Hsa_circ_0000839 Inhibits Papillary Thyroid Carcinoma Proliferation by Targeting CDC27

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Abstract

Background: Circular RNA (circRNA) has been reported to play multiple roles in a variety of cancers. However, the role of circRNA in papillary thyroid carcinoma (PTC) remains mostly unknown.

Methods: The expression, function and potential molecular mechanisms of hsa_circ_0000839 in PTC in vitro were evaluated by quantitative RT-PCR, western blot, flow cytometry, CCK8, Edu, RNA-sequencing, luciferase reporter, and RNA immunoprecipitation assay. The function of hsa_circ_0000839 in PTC in vivo was evaluated by xenograft tumors assay.

Results: Hsa_circ_0000839 was significantly downregulated in PTC tissues and plasma from patients with PTC, and its downregulation was correlated with larger tumor size in patients with PTC. The role of hsa_circ_0000839 in the proliferation of PTC cell lines was evaluated in both vitro and in vivo. Mechanistically, hsa_circ_0000839 regulated the level of CDC27 via sponging miR-149-5p in PTC.

Conclusions: Hsa_circ_0000839 might act as a tumor suppressor of PTC through the hsa_circ_0000839/miR-149-5p/CDC27 axis. Hsa_circ_0000839 could serve as a potential biomarker and therapeutic target for patients with PTC.

Background

Over the past several decades, thyroid cancer has been the most common endocrinerelated malignancy, accounting for more than 90% of endocrine cancers [1]. 80% of thyroid cancers are papillary thyroid carcinomas (PTCs), named for their papillary histological architecture [2]. PTC less than or equal to 1 cm is also called papillary thyroid microcarcinoma (PTMC), which has the highest rate of increase among PTC with different diameters in recent years [3]. However, the increasing incidence of thyroid cancer is not only the result of greater diagnostic scrutiny, but also of other explanations, including environmental influences and molecular pathways, which should be investigated [4]. Thus, it is necessary to explore new biomarkers and their molecular mechanisms to better understand the progression of PTC.

Circular RNAs (circRNAs) is a recently discovered type of endogenous non-coding RNA characterized by covalently closed loops without 5' to 3' polarity or polyadenylation tails [5]. Previous studies have revealed the evolutionary conservation, high abundance, and structural stability of circRNA in various tissues and cell lines [6, 7]. The circular structure is the reason for the stability of circRNA, which makes it an ideal diagnostic and prognostic biomarker for multiple diseases. Moreover, circRNA has been reported to have many important functions, including acting as microRNA (miRNA) sponges and combining with RNA-associated proteins to form RNA-protein complexes that regulate gene transcription [8]. In recent years, more and more reports have shown that circRNA plays an important role as miRNA sponges in the biological functions of competing endogenous RNA (ceRNA) networks [9]. CircRNA can competitively bind to miRNA, thus, regulating the expression of miRNA target genes to further regulate tumor development.

In the present study, we aimed to identify the circRNA associated with the progression of PTC, and further investigated the molecular mechanism of the identified circRNA. We found hsa_circ_0000839, which derived from exons 2, 3, and 4 of the TTC39C gene, was significantly downregulated in PTC tissues, as well as plasma samples from patients with PTC. We further confirmed that hsa_circ_0000839 sponges miR-149-5p to regulate the expression level of CDC27, ultimately acting as a tumor suppressor in PTC. Our findings provide insight into the future treatment of PTC and reveal a novel potential biomarker of PTC.

Methods

Clinical specimens

With the approval of the Ethics Committee of the First Hospital of China Medical University (Shenyang, China), all samples were collected from patients with PTC and healthy volunteers in the First Hospital of China Medical University. Paired PTC tissues and adjacent non-cancerous tissues were collected from 118 patients with PTC who underwent surgery at the First Hospital of China Medical University from May 2018 to May 2019. Each sample was confirmed by two independent professional pathologists. After resection, all samples immediately snap-frozen in liquid nitrogen and stored at -80°C until further investigation. Plasma samples were collected from patients with PTMC preoperatively and healthy volunteers at the First Hospital of China Medical University from May 2018 to August 2020. All blood samples were collected in Vacutainer tubes (Becton Dickinson, USA) and centrifuged for 10 min at 3800 rpm. Plasma samples were stored at -80°C until further investigation.

Cell culture

Human normal thyroid cell line Nthy-ori 3-1 and human PTC cell line K1 were obtained from the European Collection of Cell Culture (Porton Down, UK). Human PTC cell line TPC1 was a gift from Professor Meiping Shen (Department of General Surgery, The First Affiliated Hospital of Nanjing Medical University, Nanjing, Jiangsu). HEK-293T and Human PTC cell line BCPAP were bought from the Stem Cell Bank, Chinese Academy of Sciences (Shanghai, China). The Nthy-ori 3-1 and BCPAP cells were maintained in RPMI 1640 supplemented with 10 % fetal bovine serum (FBS). TPC1 and HEK-293T cells were maintained in DMEM with 10 % FBS. K1 cells were cultured in DMEM mixed with MCDB105 and Ham's F12 medium (2:1:1), supplemented with 10% FBS. All cell lines were cultured at 37°C in a humidified chamber with 5% CO_2 .

RNA extraction, isolation of nuclear and cytoplasmic fractions, and quantitative RT-PCR

Total RNAs were extracted from cell lines, tissues, and plasma using RNAiso (Takara, Japan) according to the manufacturer's instructions. Cytoplasmic and nuclear RNA were extracted from cells using a Cytoplasmic and Nuclear RNA Purification Kit (Norgen Biotek, Canada). For circRNA, mRNA, and cytoplasmic RNA, cDNAs were synthesized using a PrimeScript™ RT Reagent Kit (Takara, Japan). For miRNA and nuclear RNA, cDNAs were synthesized using a miRcute Reverse Transcription Kit (Tiangen, China). Quantitative RT-PCR (qRT-PCR) of mRNAs and circRNAs were performed using a TB Green Premix Ex Taq™ II (Takara), and qRT-PCR of miRNAs were performed using a SYBR Green qPCR Master Mix (Tiangen). The expression levels of RNAs were calculated using the $2^{-\Delta\Delta CT}$ method, and GAPDH, 18S rRNA, and small nuclear U6 levels were used as internal controls, respectively. The specific primers used in qRT-PCR are listed in Table S1.

Rnase R treatment and Actinomycin D assay

Total RNAs from TPC1 and K1 cells were either untreated (control) or treated with 3U/mg RNase R (Epicenter, USA) in the presence of 1 × Reaction Buffer, and incubated for 30 min at 37 °C. TPC1 and K1 cells were exposed to 2 µg/ml actinomycin D (MCE, China) for 0h, 4h, 8h, 12h, 24h. The stability of hsa_circ_0000839 and mRNA of TTC39C was further analyzed by qRT-PCR.

Western blotting

Cells were lysed in Total Protein Extraction Kit (KeyGEN, China). The protein was quantified using BCA Kit (Beyotime, China). The same amounts of protein were subjected to 10% SDS-PAGE and the separated proteins were transferred onto a PVDF membrane (Millipore, USA). The proteins on the membrane were blocked using 5% skim milk and then incubated with primary antibodies at 4 °C overnight. The antibodies recognizing CDC27, GAPDH, and cyclin B1 used in this study are listed in Table S2. Then, the membranes were incubated with secondary antibodies (1:5000) for 2 h. Finally, the immunoreactive proteins on the membranes were detected using an Enhanced Chemiluminescence Kit (Thermo Fisher Scientific, USA) and then quantified by Image J software and normalized to internal control GAPDH.

Xenografts in mice

The animal assay was approved by the animal management committee of the First Hospital of China Medical University, and all experimental procedures and animal care were in accordance with the institutional ethics guidelines for animal experiments. To create the xenograft tumor model, twelve 6-week-old female nude mice were separated randomly into hsa_circ_0000839 overexpressing group and control group (n = 6 for each group). Hsa_circ_0000839 overexpressing or control K1 cells (1×10^7) were injected subcutaneously into the right axilla of the nude mice, respectively. The volume of the tumors of all injected nude mice was measured every 3 days using calipers. After 21 days, all injected nude mice were sacrificed, and the volume of the excised tumor was measured.

Cell transfection

Small interfering RNAs (siRNAs) targeting hsa_circ_0000839 and CDC27 were synthesized by GenePharma (China) and the sequences were listed in Table S3. MiR-149-5p mimics were also synthesized by GenePharma. The overexpression lentivirus of hsa_circ_0000839 was synthesized by Genechem (China). PTC cell lines were transfected with siRNAs or lentivirus according to the manufacturer's instructions.

Luciferase reporter assay

The wild-type and mutant fragments of hsa_circ_0000839 and CDC27 containing miRNA-149-5p binding sites were designed, synthesized, and inserted into the firefly luciferase expression vector pMIRREPORT (Obio Technology, China) (Table S4), separately. Then, the luciferase reporter vectors and miR-149-5p mimics or negative control (NC) were co-transfected to HEK-293T cells, separately. After 48 h, according to the manufacturer's instructions, the luciferase activity in co-transfected cells were detected using the dual-luciferase reporter assay system (Promega, USA).

RNA immunoprecipitation (RIP)

RNA immunoprecipitation assays were performed using a Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore, USA) according to the manufacturer's instructions. The argonaute 2 (AGO2) antibodies used in this study are listed in Table S2.

CCK8 assay

Cell proliferation was tested using a Cell Counting 8 Kit (MCE). Cells were seeded in 96-well plates with approximate 2500 cells/ well in 100 µl of medium in quintuplicate. CCK8 was added into wells every 24 h and incubated for 2.5 h. The absorbance was measured at a wavelength of 450 nm.

EdU incorporation assay

Transfected PTC cells were seeded in 96-well plate and incubated for 24 h. Then, BeyoClick™ EdU Cell Proliferation Kit (TMB) (Beyotime) was used to detect DNA synthesis and cell proliferation according to the manufacturer's protocol. The assay was conducted in quintuplicate and the absorbance was detected at a wavelength of 450 nm.

RNA-sequencing analysis

The RNA-sequencing analysis was performed at Novogene Corporation (China). Total RNAs from hsa_circ_0000839 silenced (n=3) and NC (n=3) TPC1 cells were extracted and purified. After library preparation and cluster generation, the library preparations were sequenced on an Illumina HiSeq platform and 125 bp/150 bp paired-end reads were generated. Fastp, Hisat2 and FeatureCounts software were used respectively for process of quality control, read comparison and quantification. Differential expression analysis of two groups was performed by the DESeq2 R package. Genes with $\text{padj} < 0.05$ found by DESeq2 were assigned as differentially expressed. The GEO Accession number is GSE 160796.

Statistical analysis

The analyses were mainly performed using SPSS 22.0 (IBM Corp., Armonk, NY, USA) and a $p\text{-value} < 0.05$ was determined as statistically significant. Correlation was measured by Spearman correlation analysis. The receiver operating characteristic (ROC) curve was performed to evaluate the diagnostic value. The nonparametric test was used to assess continuous data with a skewed distribution, whereas categorical data were analyzed using the chi-squared test. A two-tailed Student's t-test was used for comparisons between two groups.

Results

Identification of the circular structure of hsa_circ_0000839

Hsa_circ_0000839 is located on chromosome 18 (NM_001135993) and is derived from exons 2, 3 and 4 of the TTC39C gene, with a length of 293 nt (position 21644103–21649235) as reported in circBase. Sanger sequencing confirmed that the qRT-PCR products amplified using divergent primers contained the head-to-tail splicing site of hsa_circ_0000839 and the sequence was consistent with the circBase database annotation (Figure. 1A). The expression ratio of hsa_circ_0000839 in cytoplasmic and nuclear fractions of cells was measured using qRT-PCR after nucleocytoplasmic separation. Most of the hsa_circ_0000839 were found in the cytoplasm (Figure. 1B).

Furthermore, we found that expression of the hsa_circ_0000839 amplicon decreased significantly when oligo-dT primers were used for reverse transcription compared with the use of random primers, indicating the absence of a poly-A tail in circRNA and verifying the circular structure of the circRNA (Figure. 1C). Given that circRNAs are more stable than linear RNAs, we further analyzed the stability of hsa_circ_0000839. After treatment with RNase R, an exonuclease, the levels of the linear forms of TTC39C decreased sharply, whereas the levels of hsa_circ_0000839 were stable (Figure. 1D). After treatment with actinomycin D, a transcription inhibitor, the half-life of the hsa_circ_0000839 was significantly longer than that of linear TTC39C in PTC cells (Figure. 1E). Both results demonstrated the stable circular structure of the hsa_circ_0000839.

Hsa_circ_0000839 is significantly downregulated in PTC, and correlated with clinical characteristics of PTC

We assessed the expression of hsa_circ_0000839 in 118 paired PTC and non-cancerous tissues, and in 50 pairs of plasma sample from patients with PTC and healthy controls using qRT-PCR. Figure 2A demonstrates the relative abundance of hsa_circ_0000839 in PTC compared with non-cancerous tissues. Furthermore, when we assessed the clinical data on the aforementioned patients, the expression level of hsa_circ_0000839 decreased in patients with a larger tumor size (Table 1). We further analysed the diagnosis value of hsa_circ_0000839 in plasma samples from patients with PTMC. The clinical characteristics of patients with PTMC are listed in Table S5. Consistent with the results of the PTC and non-cancerous tissues, the expression levels of hsa_circ_0000839 were lower in the plasma of patients with PTMC than that of the healthy controls (Figure. 2B). Furthermore, the area under the ROC curve (AUC) of hsa_circ_0000839 for distinguishing plasma samples from PTMC patients and healthy controls was 0.615 (Figure. 2C).

Next, we investigated the expression levels of hsa_circ_0000839 in different PTC cell lines (BCPAP, TPC1, and K1) and a normal thyroid cell line (Nthy-ori 3-1). The expression of hsa_circ_0000839 was significantly downregulated in PTC cell lines compared with that in the normal thyroid cell line (Figure. 2D). TPC1 and K1 cells exhibited the lowest levels of hsa_circ_0000839 among the PTC cell lines. Therefore, we conducted further experiments in these two cell lines.

Hsa_circ_0000839 inhibits cell proliferation in vitro and tumor growth in vivo

The hsa_circ_0000839 siRNA was transfected into TPC1 and K1 cells, which successfully silenced the hsa_circ_0000839, but not TTC39C mRNA, as confirmed by qRT-PCR (Figure. 3A). CCK8 assays indicated that inhibition of hsa_circ_0000839 significantly increased cell proliferation (Figure. 3B). Consistently, EdU assays indicated that silencing hsa_circ_0000839 increased DNA synthesis (Figure. 3C). These results demonstrated that hsa_circ_0000839 silencing markedly enhanced the proliferation of PTC cells.

In order to further confirm the function of hsa_circ_0000839, the lentiviruses overexpressing hsa_circ_0000839 were successfully transfected into TPC1 and K1 cells and the results were confirmed by qRT-PCR (Figure. 3D). According to the results of CCK8 assays, overexpression of hsa_circ_0000839 significantly inhibited the proliferation of PTC cells (Figure. 3E). Meanwhile, compared with the control group, overexpression of hsa_circ_0000839 also significantly decreased the DNA synthesis (Figure. 3F).

To determine whether hsa_circ_0000839 contributed to tumor growth of PTC in vivo, a xenograft mouse model was constructed. We first confirmed that K1 cells had been stably transfected with lentiviruses overexpressing hsa_circ_0000839 or NC. Subsequently, the transfected K1 cells were injected subcutaneously into the right axilla of the nude mice. Lower tumor volumes were observed in the

hsa_circ_0000839 overexpression group compared with those in the control group (Figure. 3G, Figure. S1). Taken together, these findings suggested that hsa_circ_0000839 functions as a tumor suppressor gene in the proliferation and progression of PTC cells in vitro and vivo.

Hsa_circ_0000839 inhibits cell proliferation by targeting CDC27

To explore the underlying mechanism by which hsa_circ_0000839 regulates cell progression in PTC cells, we performed RNA sequencing (RNA-Seq) analysis to identify changes in gene expression after silencing hsa_circ_0000839 in TPC1 cells. The results of RNA-Seq showed that 103 putative mRNAs were significantly downregulated in hsa_circ_0000839-silencing TPC1 cells compared with the NC. The genes that were significantly differentially expressed were presented as a volcano map (Figure. 4A).

CDC27, which is known to target cell cycle regulator cyclin B1, was selected as downstream gene of hsa_circ_0000839 for further investigation. However, other APC genes were not changed according to the results of the RNA-seq. The expression of CDC27 was positively correlated with the expression of hsa_circ_0000839 in PTC cells silencing or overexpressing hsa_circ_0000839 by qRT-PCR, respectively (Figure. 4B-C). Meanwhile, we detected the expression of CDC27 and the correlation of hsa_circ_0000839 and CDC27 expression in the 118 paired PTC and non-cancerous tissues. The results showed that the expression of CDC27 was downregulated in PTC tissues (Figure. 4D) and was positively correlated with the expression of hsa_circ_0000839 (Figure. 4E). The expression levels of CDC27 were also significantly downregulated in PTC and negatively associated with tumor stages in The Cancer Genome Atlas (TCGA) database (Figure. S2A-B) [10]. Western blotting revealed that silencing hsa_circ_0000839 significantly reduced the levels of CDC27 and upregulated the downstream cyclin B1, and the opposite results were observed when hsa_circ_0000839 was overexpressed (Figure. 4F-G, Figure. S3A-B). Therefore, CDC27 was considered as a major candidate target of hsa_circ_0000839. To explore the function of CDC27, cell viability after silencing of CDC27 was examined. The mRNA and protein levels of CDC27 were significantly reduced by the CDC27 siRNA, and the downstream cyclin B1 levels increased (Figure. 4H-I, Figure. S4). According to the results of CCK8 and EdU assays, silencing CDC27 promoted the proliferation of PTC cells (Figure. 4J-K). These results indicated that hsa_circ_0000839 might be capable of modulating the proliferation of PTC cells by targeting CDC27.

Hsa_circ_0000839 regulates CDC27 expression by targeting miR-149-5p

CircRNAs located in the cytoplasm are usually associated with miRNA sponging [11]. Therefore, we further explored whether the relationship between hsa_circ_0000839 and CDC27 was mediated by miRNAs. According to the prediction of miRNAs associated with both hsa_circ_0000839 and CDC27, miR-149-5p was selected as a candidate miRNA for subsequent experiments (Figure. 5A). MiR-149-5p levels

negatively correlated with hsa_circ_0000839 silencing or overexpression in TPC1 and K1 cells, as shown using qRT-PCR (Figure. 5B-C). CDC27 levels correlated negatively with transfection with the miR-149-5p mimics in TPC1 and K1 cells, according to the results of qRT-PCR and western blotting (Figure. 5D-E, Figure. S5A-B). Furthermore, luciferase reporters using either a wild-type or mutated miR-149-5p binding sites of hsa_circ_0000839 sequence, which were inserted into the 3' untranslated region of Renilla luciferase, showed that overexpressing miR-149-5p significantly reduced the luciferase activities of the wild-type reporter but not the mutant sequence expressed reporter (Figure. 5F). The luciferase reporter system also showed that CDC27 interacted with miR-149-5p (Figure. 5G). Meanwhile, RIP assays indicated miR-149-5p could bind either hsa_circ_0000839 or CDC27 (Figure. 5H). These findings indicated that hsa_circ_0000839 could acts as a sponge for miR-149-5p to regulate CDC27 expression.

Co-transfection of hsa_circ_0000839 and miR-149-5p could counteract the upregulation of CDC27 induced by the overexpression of CDC27 in TPC1 and K1 cells (Figure. 5I, Figure. S6). CCK8 and EdU analyses indicated that overexpression of hsa_circ_0000839 inhibited cell proliferation, but this effect could be significantly abolished by co-transfection with hsa_circ_0000839 and the miR-149-5p mimics (Figure. 5J-K).

Discussion

In the past 30 years, the incidence of PTC has increased 3 fold and the highest rate of increase was PTMC [12]. PTC is usually curable with 5-year survival of over 95% [13]. However, due to the complex pathogenesis of PTC, it is important to explore new biomarkers to detect and treat more aggressive and lethal PTC. Growing evidence indicates that circRNAs participate in the carcinogenesis of PTC [14]. Meanwhile, the stable structure of circRNAs make them ideal biomarkers for the diagnosis of diseases. In the present study, we showed that hsa_circ_0000839 was significantly downregulated in the tissues and plasma of patients with PTC compared with those in non-cancerous thyroid tissues or the healthy volunteers. Furthermore, the expression of hsa_circ_0000839 was negatively correlated with tumor size. Our experiment demonstrated that hsa_circ_0000839 inhibited the proliferation of PTC through the hsa_circ_0000839/miR-149-5p/CDC27 axis.

Hsa_circ_0000839 is a novel circular RNA, located in chromosome 18 with a length of 293 nt. However, no previous studies have showed that hsa_circ_0000839 can contribute to diseases, including PTC. We proved that hsa_circ_0000839 had a circular structure through RNase R treatment, actinomycin D treatment, and sanger sequence. We proposed for the first time that hsa_circ_0000839 was a potential biomarker of PTC. Our results revealed hsa_circ_0000839 is downregulated in the tissues and plasma samples of patients with PTC. Furthermore, the expression of hsa_circ_0000839 correlated negatively with tumor size. Although the diagnostic value of hsa_circ_0000839 was not high, we might combine other known biomarkers to predict PTC in further study. In our study, we also found that hsa_circ_0000839 acted as a tumor suppressor in PTC. Hsa_circ_0000839 inhibited the proliferation of PTC cells in vivo and vitro.

We further studied the mechanism of hsa_circ_0000839 and screened out that hsa_circ_0000839 regulated the expression level of CDC27. Our findings confirmed that CDC27 was downregulated in PTC tissues and negatively correlated with the clinical stages. In addition, our study also proved that CDC27 could inhibit the proliferation of PTC cells in vitro. Previous studies have shown that CDC27, which is one of the subunits of the anaphase promoting complex/cyclosome, is a tumor suppressor gene by targeting cyclin B1 [15]. CDC27 has been reported to act as a biomarker and tumor suppressor to predict disease progression in breast cancer [16]. Meanwhile, the downstream cyclin B1 is overexpressed in multiple types of cancer and function as an oncogene by promoting the G₂/M transition of the cell cycle [17]. High levels of cyclin B1 have been shown to be important indicators of poor prognosis in several malignant diseases [18–22], and their downregulation might be a promising antitumor mechanism [23, 24]. In the present study, we revealed that hsa_circ_0000839 could inhibit the progression of PTC by regulating the expression level of CDC27 and further influence the downstream cyclin B1.

In 2009, Seitz first proposed the competitive endogenous RNA (ceRNA) mechanism [25]. CircRNA acts as a miRNA sponge thereby increasing the expression levels of miRNA target genes via the ceRNA mechanism [26]. Previous studies have indicated that a variety of differentially expressed circRNAs between PTC and non-cancerous thyroid tissues, including circRNA_102171, circRAPGEF5, circBACH2, which can influence the progression of PTC through the ceRNA networks [27–29]. Nonetheless, the potential function as miRNA sponges of hsa_circ_0000839 in PTC are still unclear. Our results indicated that miR-149-5p could bind with hsa_circ_0000839 and CDC27, respectively. Our study further confirmed that hsa_circ_0000839 acted as a sponge to inhibit miR-149-5p in PTC, and affected the expression level of CDC27 through ceRNA mechanism. Subsequently, we validated that miR-149-5p could reverse the influence of hsa_circ_0000839 on PTC, according to the recovery experiments. It was further demonstrated that hsa_circ_0000839 functioned as ceRNA to regulate the expression of CDC27, thereby affecting the proliferation of PTC.

Conclusions

In conclusion, our study showed that hsa_circ_0000839 was downregulated in PTC. Hsa_circ_0000839 inhibited the proliferation of PTC cells in vivo and vitro. Further analysis showed that CDC27 was downregulated in PTC and the expression of hsa_circ_0000839 was positively correlated with CDC27 in PTC tissues. Moreover, hsa_circ_0000839 could efficiently sponge miR-149-5p to regulate the expression of CDC27 and the proliferation of PTC cells. Our results proved that hsa_circ_0000839 could be a biomarker in patients with PTC and the ceRNA network might be a potential therapeutic target for the treatment of PTC.

Abbreviations

PTC papillary thyroid carcinoma

PTMC papillary thyroid microcarcinoma

circRNA circular RNA

miRNA microRNA

RIP RNA Immunoprecipitation

ceRNA competitive endogenous RNA

siRNA small interfering RNA

Declarations

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Author contributions

JHG and TTL performed the experiments, data acquisition, and data analysis.; ZYS and WPT provided technical and material support. All authors have participated in drafting the paper and approved the final version to be published.

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Availability of data and materials

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

Ethics approval and consent to participate

This study was approved by the Ethical Committee of The First Hospital of China Medical University. The written informed consents were obtained from all the patients.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Siegel RL, Miller KD, Jemal A. Cancer Statistics. 2017. *CA Cancer J Clin.* 2017; **67**: 7–30. doi:10.3322/caac.21387.
2. Cancer Genome Atlas Research Network. Integrated genomic characterization of papillary thyroid carcinoma. *Cell.* 2014;159:676–90. 10.1016/j.cell.2014.09.050. doi.
3. Davies L, Welch HG. Increasing incidence of thyroid cancer in the United States, 1973–2002. *JAMA.* 2006;295:2164–7. 10.1001/jama.295.18.2164. doi.
4. Chen AY, Jemal A, Ward EM. Increasing incidence of differentiated thyroid cancer in the United States, 1988–2005. *Cancer.* 2009;115:3801–7. 10.1002/cncr.24416. doi.
5. Wilusz JE, Sharp PA. A circuitous route to noncoding RNA. *Science.* 2013;340:440–1. 10.1126/science.1238522. doi.
6. Jeck WR, Sorrentino JA, Wang K, Slevin MK, Burd CE, Liu J, et al. Circular RNAs are abundant, conserved, and associated with ALU repeats. *RNA.* 2013;19:141–57. 10.1261/rna.035667.112. doi.
7. Salzman J, Chen RE, Olsen MN, Wang PL, Brown PO. Cell-type specific features of circular RNA expression. *PLoS Genet.* 2013;9:e1003777. 10.1371/journal.pgen.1003777. doi.
8. Han B, Chao J, Yao H. Circular RNA and its mechanisms in disease: From the bench to the clinic. *Pharmacol Ther.* 2018;187:31–44. 10.1016/j.pharmthera.2018.01.010. doi.
9. Liu Y, Chen S, Peng G, Liao Y, Fan X, Zhang Z, Shen C. CircRNA NALCN acts as an miR-493-3p sponge to regulate PTEN expression and inhibit glioma progression. *Cancer Cell Int.* 2021; 21:307. doi: 10.1186/s12935-021-02001-y.
10. Chandrashekar DS, Bashel B, Balasubramanya SAH, Creighton CJ, Ponce-Rodriguez I, Chakravarthi BVSK, Varambally S. UALCAN: A Portal for Facilitating Tumor Subgroup Gene Expression and Survival Analyses. *Neoplasia.* 2017;19:649–58. 10.1016/j.neo.2017.05.002. doi.
11. Shen P, Yang Y, Liu G, Chen W, Chen J, Wang Q, Gao H, Fan S, Shen S, Zhao X. CircCDK14 protects against Osteoarthritis by sponging miR-125a-5p and promoting the expression of Smad2. *Theranostics.* 2020;10:9113–31. 10.7150/thno.45993. doi.
12. Chen AY, Jemal A, Ward EM. Increasing incidence of differentiated thyroid cancer in the United States, 1988–2005. *Cancer.* 2009; 115:3801–7. doi: 10.1002/cncr.24416.
13. Hay ID, Thompson GB, Grant CS, Bergstralh EJ, Dvorak CE, Gorman CA, et al. Papillary thyroid carcinoma managed at the Mayo Clinic during six decades (1940–1999): temporal trends in initial therapy and long-term outcome in 2444 consecutively treated patients. *World journal of surgery.* 2002; 26:879–85. doi: 10.1007/s00268-002-6612-1.

14. Cao J, Zhang M, Zhang L, Lou J, Zhou F, Fang M. Non-coding RNA in thyroid cancer - Functions and mechanisms. *Cancer Lett.* 2021;496:117–26. 10.1016/j.canlet.2020.08.021. *doi*.
15. King RW, Peters JM, Tugendreich S, Rolfe M, Hieter P, Kirschner MW. A 20S complex containing CDC27 and CDC16 catalyzes the mitosis-specific conjugation of ubiquitin to cyclin B. *Cell.* 1995;81:279–88. 10.1016/0092-8674(95)90338-0. *doi*.
16. Talvinen K, Karra H, Pitkänen R, Ahonen I, Nykänen M, Lintunen M, et al. Low cdc27 and high securin expression predict short survival for breast cancer patients. *APMIS.* 2013;121:945–53. 10.1111/apm.12110. *doi*.
17. Peters JM. The anaphase promoting complex/cyclosome: a machine designed to destroy. *Nat Rev Mol Cell Biol.* 2006;7:644–56. 10.1038/nrm1988. *doi*.
18. Santala S, Talvensaaari-Mattila A, Soini Y, Santala M. Prognostic value of cyclin B in endometrial endometrioid adenocarcinoma. *Tumour Biol.* 2015;36:953–7. 10.1007/s13277-014-2676-4. *doi*.
19. Aaltonen K, Amini RM, Heikkilä P, Aittomäki K, Tamminen A, Nevanlinna H, et al. High cyclin B1 expression is associated with poor survival in breast cancer. *Br J Cancer.* 2009;100:1055–60. 10.1038/sj.bjc.6604874. *doi*.
20. Sun X, Zhangyuan G, Shi L, Wang Y, Sun B, Ding Q. Prognostic and clinicopathological significance of cyclin B expression in patients with breast cancer: A meta-analysis. *Medicine.* 2017;96:e6860. 10.1097/MD.0000000000006860. *doi*.
21. Koliadi A, Nilsson C, Holmqvist M, Holmberg L, *de La Torre* M, Wärnberg F, et al Cyclin B is an immunohistochemical proliferation marker which can predict for breast cancer death in low-risk node negative breast cancer. *Acta Oncol.* 2010; 49:816–20. *doi*:10.3109/02841861003691937.
22. Winters ZE, Hunt NC, Bradburn MJ, Royds JA, Turley H, Harris AL, *et al*. Subcellular localisation of cyclin B, Cdc2 and p21(WAF1/CIP1) in breast cancer. association with prognosis. *Eur J Cancer.* 2001; 37:2405–12. *doi*:10.1016/s0959-8049(01)00327-6.
23. Tsaor I, Makarević J, Hudak L, Juengel E, Kurosch M, Wiesner C, *et al*. The cdk1-cyclin B complex is involved in everolimus triggered resistance in the PC3 prostate cancer cell line. *Cancer Lett.* 2011; 313:84–90. *doi*:10.1016/j.canlet.2011.08.026.
24. Baak JP, Gudlaugsson E, Skaland I, Guo LH, Klos J, Lende TH, *et al*. Proliferation is the strongest prognosticator in node-negative breast cancer: significance, error sources, alternatives and comparison with molecular prognostic markers. *Breast Cancer Res Treat.* 2009; 115:241–54. *doi*:10.1007/s10549-008-0126-y.
25. Seitz H. Redefining microRNA targets. *Curr Biol.* 2009;19:870-3. 10.1016/j.cub.2009.03.059. *doi*.
26. Fu Y, Sun H. Biogenesis, cellular effects, and biomarker value of circHIPK3. *Cancer Cell Int.* 2021; 21:256. *doi*: 10.1186/s12935-021-01956-2.
27. Bi W, Huang J, Nie C, Liu B, He G, Han J, *et al*. CircRNA circRNA_102171 promotes papillary thyroid cancer progression through modulating CTNNBIP1-dependent activation of β -catenin pathway. *J Exp Clin Cancer Res.* 2018; 37: 275. *doi*: 10.1186/s13046-018-0936-7.

28. Liu W. Zhao J, Jin M. Zhou M. circRAPGEF5 Contributes to Papillary Thyroid Proliferation and Metastasis by Regulation miR-198/FGFR1. *Mol Ther Nucleic Acids*. 2019; 14: 609–16. *doi*: 10.1016/j.omtn.2019.01.003.

29. Cai X. Zhao Z, Dong J. Lv Q, Yun B. Liu J, *et al*. Circular RNA circBACH2 plays a role in papillary thyroid carcinoma by sponging miR-139-5p and regulating LMO4 expression. *Cell Death Dis*. 2019; 10: 184. *doi*: 10.1038/s41419-019-1439-y.

Table

Due to technical limitations, table 1 is only available as a download in the Supplemental Files section.

Figures

Figure 1

Characterization of hsa_circ_0000839 in PTC. (A) Genomic locus of hsa_circ_0000839. Hsa_circ_0000839 is produced at the TTC39C gene (NM_001135993) locus, which contains exons 2–4. The back-splice junction of hsa_circ_0000839 was identified by Sanger sequencing. (B) The expression levels of hsa_circ_0000839 in the nuclear and cytoplasmic fractions of TPC1 and K1 cells. (C) qRT-PCR analysis of hsa_circ_0000839 expression using random primers or oligo-dT primers. (D) qRT-PCR analysis of hsa_circ_0000839 and TTC39C expression in PTC cells treated with or without RNase R. (E) qRT-PCR assay for the abundance of hsa_circ_0000839 and TTC39C in TPC1 and K1 cells treated with actinomycin D at the indicated time points. Data are presented as means $\pm$ SD. * $P < 0.05$

Figure 2

Hsa_circ_0000839 is downregulated in PTC tissues, patients' plasma, and PTC cell lines. (A) qRT-PCR analysis of hsa_circ_0000839 expression in PTC and non cancerous tissues (n = 118). (B) qRT-PCR analysis of hsa_circ_0000839 expression in plasma from patients with PTMC and paired healthy controls (n = 50). (C) ROC curves of hsa_circ_0000839 to distinguish PTMC plasma from normal plasma. (D) qRT-PCR analysis of hsa_circ_0000839 in PTC and normal thyroid cell lines. Data are presented as medians with range or means $\pm$ SD. *P < 0.05

Figure 3

Hsa_circ_0000839 inhibits cell proliferation and tumor growth in vitro and in vivo. (A) Expression levels of hsa_circ_0000839 and TTC39C in TPC1 and K1 cells treated with hsa_circ_0000839 siRNA. (B) CCK8 assay of PTC cells silencing hsa_circ_0000839. (C) EdU assay of PTC cells silencing hsa_circ_0000839. (D) Expression levels of hsa_circ_0000839 in TPC1 and K1 cells after transduction with hsa_circ_0000839 lentivirus. (E) CCK8 assay of PTC cells overexpressing hsa_circ_0000839. (F) EdU assay of PTC cells overexpressing hsa_circ_0000839. (G) The volume of subcutaneous xenograft tumors of K1 cells isolated from nude mice. Data are presented as means $\pm$ SD. *P < 0.05

Figure 4

Hsa_circ_0000839 inhibits cell proliferation by targeting CDC27. (A) Volcano map of differentially expressed genes between hsa_circ_0000839-silencing and NC TPC1 cells in RNA-seq analysis. (B, C) The mRNA levels of CDC27 in the PTC cells silencing or overexpressing hsa_circ_0000839. (D) qRT-PCR analysis of CDC27 expression in PTC and non cancerous tissues (n = 118). (E) Correlation analysis revealed a positive correlation between the levels of hsa_circ_0000839 and CDC27 mRNA in the tumorous tissues of the 118 patients with PTC. (F, G) The protein levels of CDC27 and downstream cyclin B1 in PTC cells silencing or overexpressing hsa_circ_0000839. (H) Expression levels of CDC27 in TPC1 and K1 cells treated with CDC27 siRNA. (I) The protein levels of CDC27 and downstream cyclin B1 in the PTC cells silencing CDC27. (J, K) CCK8 assay and EdU assay of PTC cells silencing CDC27. Data are presented as medians with range or means $\pm$ SD. *P < 0.05

Figure 5

Hsa_circ_0000839 regulates CDC27 expression by targeting miR-149-5p. (A) The predicted binding sites of miR-149-4p on hsa_circ_0000839 and CDC27. (B, C) MiR 149 5p expression in PTC cells silencing or overexpressing hsa_circ_0000839. (D) CDC27 expression in PTC cells transfected with miR-149-5p mimics. (E) The protein levels of CDC27 and downstream cyclin B1 in PTC cells transfected with miR-149-5p mimics. (F) MiR-149-5p mimics or NC were co-transfected with a luciferase reporter construct

containing circ_0000839(WT) or circ_0000839(MUT) into HEK-293T. The luciferase reporter activities are shown. (G) MiR-149-5p mimics or NC were co-transfected with a luciferase reporter construct containing CDC27(WT) or CDC27(MUT) into HEK-293T. The luciferase reporter activities are shown. (H) RIP was performed using anti-AGO2 antibodies in TPC1 cells. (I) The protein levels of CDC27 in PTC cells transfected with hsa_circ_0000839 alone or co-transfected with miR-149-5p mimics. (J, K) CCK8 and EdU assay of PTC cells transfected with hsa_circ_0000839 alone or co-transfected with miR-149-5p mimics. Data are presented as means $\pm$ SD. *P < 0.05

Supplementary Files

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