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CircHIPK3 Promotes Thyroid Cancer Tumorigenesis and Invasion through the Mirna-338-3p/RAB23 Axis

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Key words: Thyroid cancer • circHIPK3 • miR-338-3p • RAB23 • Proliferation • Invasion

Short Title: Mechanism of circHIPK3 occurrence and Invasion of Thyroid Cancer

Highlights of the Study

- circHIPK3 act as an oncogenetic circRNA in thyroid cancer. Knockdown of circHIPK3 significantly reduced the migration, invasion and proliferation of thyroid cancer
- circHIPK3 promotes tumorigenesis and invasiveness of thyroid cancer by sponging miR-338-3p and up-regulating the expression of its target gene RAB23
- circHIPK3 could be a novel biomarker for thyroid cancer.

Abstract

Objective: Thyroid cancer is a common type of endocrine malignancy, and its incidence has been steadily increasing in many regions of the world. Numerous studies have found that circRNAs are aberrantly expressed in various cancer types, and could be potential biological diagnostic markers and therapeutic targets. The purpose of this study was to investigate the role of circHIPK3 in the development and progression of thyroid cancer and its mechanism. **Subject and Methods:** qRT-PCR was used to detect the relative expression level of circHIPK3 in thyroid cancer cell lines (K1, CAL-62, TPC1), human thyroid cancer cell line (thy-ori 3-1), 10 pairs of thyroid cancer tissues and corresponding adjacent normal tissues. CCK-8 and Transwell assays were used to detect the proliferation and metastasis ability of cells. The targeted relationships between circHIPK3-miR-338-3p and miR-338-3p-RAB23 were predicted by bioinformatics analysis and verified by dual-luciferase reporter assays. **Results:** The downregulation of circHIPK3 significantly reduced the migration, invasion and proliferation of thyroid carcinoma. Then, we demonstrated that circHIPK3 up-regulated the expression of its target RAB23 by sponging miR-338-3p to promote the tumorigenesis and invasiveness of thyroid cancer. **Conclusion:** This study indicates that circHIPK3 plays the role of an oncogenic circRNA in thyroid cancer; this may provide new insights into how circRNA affects the progression of thyroid cancer. Our study also suggests that circHIPK3 could be a novel biomarker for thyroid cancer.

Introduction

Thyroid cancer (TC) is one of the most common endocrine malignancies globally and originates from follicular or parafollicular thyroid cells [1]. The most common type of thyroid cancer is thyroid papillary carcinoma (PTC), which accounts for about 80% of thyroid malignancies [2]. The overall 5-year survival rate of in patients with advanced PTC is 30%. Moreover, the median survival time of anaplastic thyroid cancer (ATC) is less than 6 months [3]. Despite good prognosis, TC could cause bad quality life and even become life-threatening due to invasiveness and metastasis. The precise mechanism regulating TC invasiveness and metastasis is still not well-known.

Non-coding RNAs (ncRNAs) are a major regulator of gene expression, affecting multiple biological pathways, such as proliferation, differentiation, apoptosis and immune response [4]. Dysregulation of ncRNAs has been shown to affect the prognosis of cancers. Circular RNA (circRNA) is a type of non-coding RNAs that are highly conserved and stable and generated by back-splicing pre-mRNA [5]. circRNAs have been shown to play important roles in different human diseases and could serve as biomarkers for prognosis and therapy. Emerging studies have revealed that circRNAs are regulators of progression of TC. For example, circRNA_102171 promotes the progression of TC through CTNNBIP1-mediated β -catenin pathway [6]. CircRAPGEF5 promotes TC by regulating miR-198/FGFR1 [7].

CircHIPK3 is a novel circRNA which highly expressed in human cells. Previous studies showed circHIPK3 regulated cancer cell growth, invasion and autophagy. Zheng et al. found that silencing of circHIPK3 inhibited the proliferation of colon cancer and cervical cancer cells [8]. In prostate cancer, circHIPK3 enhanced tumor cell growth and invasion by regulating

miRNA-338-3p [9]. However, circHIPK3 was shown to serve as a tumor suppressor to repress the progression of bladder tumor. Mechanically, circHIPK3 could serve as miRNA sponges to regulate their activities in human cancers [10]. A series of miRNAs, including miR-124 [11], miR-338-3p [9] and miR-30a-3p [12], have been shown to be direct targets of circHIPK3. However, the molecular functions of circHIPK3 in TC and whether it could play a regulatory role as miRNA sponges in TC are still unclear.

We investigated the effect of circHIPK3 on the proliferation of TC and the metastasis of TC using loss-of-function studies. We found that circHIPK3 could inhibit TC progression partially through modulating miR-338-3p and its target RAB23. Taken together, our study suggests that circHIPK3 is an oncogene related to TC tumorigenesis and invasiveness, and that it could act as a target for TC.

Materials and Methods

Clinical Specimens

A total of 10 clinical samples were collected from patients who were diagnosed with thyroid cancer at the affiliated hospital of Harbin Medical University between 2015 and 2019. None of them had received radiotherapy or chemotherapy before surgical resection. The study was approved by the Ethics Committee of Harbin Medical University. All patients provided written informed consent before enrollment. Thyroid cancer tissues and corresponding adjacent normal tissues were stored at -80°C.

Cell Culture

Cells (K1, CAL-62, TPC1, Nthy-ori 3-1) were all purchased from ATCC and cultured in DMEM (Invitrogen, CA, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen,

CA, USA) in a humidified incubator with 5% CO₂ at 37°C [13].

Cell Fractionation Assay

Cytoplasmic and nuclear RNA were extracted using the PARIS™ Kit (AM1921, Invitrogen, CA, USA). Briefly, cells were incubated with lysis solution on ice for 10 minutes after washing with PBS. After centrifugation, cytoplasmic RNA was extracted from the supernatant, and nuclear RNA from the nuclear pellet.

siRNA Synthesis

The chemically modified siRNA oligo (2'OMe) was synthesized by Shanghai GenePharma (Shanghai, People's Republic of China). The sequence of the siRNA for the circHIPK3 was 5'- CUACAGGUAUGGCCUCAC-3' (si-circHIPK3). The sequence of negative control siRNA (siNC) was 5'-U-NNN-CAACGUGUCACGUTT-3'. The oligonucleotides were transfected into TH1 cells using Lipofectamine 2000 (Invitrogen, CA, USA) according to the manufacturer's protocol.

Dual-Luciferase Reporter Assay

The full length of circHIPK3 and the 3'UTR fragment of RAB23 were cloned into the luciferase reporter vector. Each luciferase reporter plasmid DNA was co-transfected with miRNA using Lipofectamine 3000. The luciferase activity was measured using Dual-Luciferase Reporter Assay System from Promega according to the instructions.

Quantitative Reverse Transcription PCR (qRT-PCR)

TRIzol reagent (Invitrogen, CA, USA) was used to extract total RNA from cells and tissues. RNA was then reversely transcribed to cDNA, and qPCR was conducted to measure the relative expression level of miRNA, circRNA and mRNA (Takara, Otsu, Japan). The

amplification reaction was performed using the ABI StepOnePlus system (Applied Biosystems, CA) following the predetermined conditions. Data were analyzed by the $2^{-\Delta\Delta C_t}$ method.

Cell Growth Assay

Cell growth was measured using a CCK-8 kit (Dojindo Laboratories, Japan) according to manufacturer's instructions [14].

Cell Migration and Invasion Assays

Cell migration and invasion experiments were done on Transwell permeable supports (Corning, NY, USA) using the manufacturer's protocol. Cells were fixed in methanol after culturing for 72 hours, and the cell number on the lower surface was counted [15].

Statistical Analysis

Data were analyzed using GraphPad Prism software 5.0 and depicted as the mean $\pm$ SD. paired t-test or student's t-test was performed for comparisons. p value < 0.05 was considered as statistically significant. In all panels, values represent mean $\pm$ SEM from three independent experiments.

Results

Levels of circHIPK3 in TC Samples

Levels of circHIPK3 were higher in K1, TPC1 and CAL-62 cells than in normal thyroid cells, Fuy-ori 3-1 (Figure 1A). Furthermore, levels of circHIPK3 were markedly higher in TC tissues than matched adjacent normal tissues (Figure 1B).

Knockdown of circHIPK3 Inhibited TC Cell Growth

To study the biological effect of circHIPK3 in TC, we first examined its subcellular location in TPC1 and CAL-62 cells. Previous studies used U6 and GADPH as internal controls

(Figure 2A-B). The present study showed circHIPK3 had a similar expression pattern to GAPDH, which was located in both nuclear and cytoplasm (Figure 2C). Next, we designed an siRNA targeting circHIPK3 to knockdown its expression levels in TC cells (Figure 3A and C) after which CCK-8 assays were used to measure cell growth. Our results showed that knockdown of circHIPK3 significantly suppressed cell growth in both CAL-62 (Figure 3B) and TPC1 cells (Figure 3D).

Effect of Silencing circHIPK3 on Migration and Invasion of TC Cells

We tested the effects of circHIPK3 on the metastatic abilities of TC cells. Transwell assays showed that silencing circHIPK3 notably weakened the migratory capability of TPC1 cells (Figure 4A-B) and CAL-62 (Figure 4C-D). Besides, as illustrated in Figure 5, circHIPK3 knockdown also reduced cell invasion. The invaded cell numbers were decreased by 82% in TPC1 cells (Figure 5A-B) and by 81% in CAL-62 cells (Figure 5C-D) after knockdown of circHIPK3.

CircHIPK3 Serves as a Sponge of miR-338-3p to Induce RAB23

Previous studies have shown that circRNA are good sponges for miRNAs [10]; we speculated that circHIPK3 might also serve as a miRNA sponge in TC. By using TARGETSCAN and Starbase, we showed that miR-338-3p/RAB23 was regulated by circHIPK3. By using TCGA we found that miR-338 was down-regulated in TC (Supplementary Fig. 1A). Despite the lack of statistical significance, we found the TC patients with higher expression of miR-338 have a potential longer survival time through the analysis of the TCGA database (Supplementary Fig. 1B). RAB23 has been reported to be an oncogene in human cancers [16].

To further validate these findings, we first tested the expression of RAB23 after miR-338-3p overexpression or circHIPK3 knockdown in TC cells. We found that RAB23 levels were significantly decreased after knockdown of circHIPK3 (Figure 6A-B) or overexpression of miR-338-3p (Figure 6C-E) in TC cells. We also observed that miR-338-3p overexpression suppressed the expression of circHIPK3 (Figure 6F-G) in TC cells. We then performed dual-luciferase assays to ascertain whether miR-338-3p could directly regulate circHIPK3 and RAB23 (Figure 6H). The miR-338-3p, circHIPK3 and RAB23 sequences are shown in Supplementary Figure 2. The co-transfection of the report vector containing 3'-UTR regions of RAB23 or circHIPK3 with miR-338-3p mimic led to a striking decrease of luciferase activity when compared to the control vector (Figure 6I-J). Moreover, we constructed a report vector containing mutated 3'-UTR regions of RAB23 and circHIPK3 sequence. The predictive 2D interaction between miRNA sequence and 3'-UTR of mutation were predicted using RegRNA 2.0 (<http://regRNA2.mbc.nctu.edu.tw/doc/box.html>). The analysis showed that miR-338-3p could bind to the wild type circHIPK3 and RAB23 sequence. However, the mutated circHIPK3 sequence could bind to miR-18a-3p and the mutated RAB23 could bind to hsa-miR-483-3p, but not to miR-338-3p. The dual-luciferase assay demonstrated overexpression of miR-338-3p did not have a significant effect on the luciferase activity of the Report vector containing 3'-UTR-mut regions of RAB23 or circHIPK3-mut (Figure 6K-L). Our results indicated that miR-338-3p could directly bind to circHIPK3 and RAB23.

Discussion

Cancer metastasis is main reason of mortality in TC patients. However, the mechanisms underlying TC metastasis remained largely unclear. Multiple studies had demonstrated that

circRNAs were involved in regulating TC metastasis. For example, hsa_circ_0058124 [17], circRAPGEF5 [7], circ_0025033 [18] and circZFR [19] were reported to promote cell migration and invasion, while circ-ITCH was found to suppress cell metastasis in TC [20]. CircHIPK3, a highly expressed circRNA, was shown to play a crucial role in regulating the growth, migration, invasion and autophagy of cancer cells. However, the roles of circHIPK3 in TC has remained unclear. This study for the first time demonstrated that circHIPK3 may act as a metastasis promoter in TC. Knockdown of circHIPK3 significantly suppressed the proliferation, migration and invasion of TC cells.

CircHIPK3 could serve as miRNA sponges to regulate cellular activities in human cancers. Zheng reported that circHIPK3 regulated cancer cell growth by sponging multiple miRNAs, such as miR-124, miR-584, miR-152 and miR-510 [10]. Ke et al. reported that circHIPK3 could bind to miR-4288 to induce ELF3 expression in nasopharyngeal carcinoma [21]. The present study identified miR-338-3p as downstream target of circHIPK3. Overexpression of miR-338-3p suppressed RAB23 and circHIPK3 expression. Luciferase assay showed that miR-338-3p reduced the activity of miR-RB-Report vector containing 3'-UTR regions of RAB23 or circHIPK3, but not mutated plasmids. These results showed that both RAB23 and circHIPK3 could be direct targets of miR-338-3p.

MiR-338-3p was reported to suppress cancer progression in several human cancers such as lung cancer, melanoma, ovarian cancer and prostate cancer. For example, in NSCLC, miR-338-3p decreased the growth and invasion of tumor cells through targeting IRS2 [22]. In melanoma, miR-338-3p decreased cell growth and metastasis by targeting MACC1 [23]. MiR-338-3p also played a crucial role in suppressing TC progression by inhibiting AKT3.

Interestingly, the present study also revealed that miR-338 was suppressed in TC samples compared to normal tissues, which was consistent with a previous report [24]. Of note, miR-338-3p was found to be a key mediator involved in regulating the functions of lncRNAs and circRNAs. For example, lncRNA DSCAM-AS1 promotes hepatocellular carcinoma through sponging miR-338-3p to induce CyclinD1 and SMO expression [25]. LncRNA LINCO1013 increases glioma cell growth, metastasis, and glycolysis through miR-338-3p/PDK12 axis [26]. Hsa_circ_0008945 induced the progression of breast cancer by targeting miR-338-3p to induce HOXA3 expression [27]. Inhibiting hsa_circ_0001313 induced sensitivity of colon cancer cells to radiation through regulating miR-338-3p [28]. A recent study indicated that circHIPK3 promotes fibroblast-to-myofibroblast transition by acting as an endogenous miR-338-3p sponge to increase SOX4 and COL1A1 expression [29]. Our study demonstrated that circHIPK3 might sponge miR-338-3p to promote RAB23 expression.

RAB23 is a member of the RAB GTPase family, which plays a role in the process of vesicular transportation and endocytic progression to lysosomes. Previous studies showed that RAB23 acted as an oncogene in various cancers such as bladder cancer [16], esophageal cancer [30] and prostate cancer [31]. Overexpression of RAB23 promoted bladder cancer cell proliferation and invasion via NF- κ B and the integrin β 1/Rac1 pathway, and promoted resistance to cisplatin through the Shh-Gli-ABCG2 pathway [16]. However, the expression pattern and molecular functions of RAB23 in TC remained largely unclear. Here, we showed that RAB23 may act as a direct target of miR-338-3p in TC.

Several limitations should be noted in this study. First, the regulation of RAB23 and circHIPK3 by miR-338-3p were confirmed by qRT-PCR, Western blot and luciferase assay, but

the results do not directly prove that circHIPK3 has a sponge effect on miR-338-3p regulation. We intend to further validate the direct or indirect interaction between circHIPK3, miR-338-3p, and RAB23 in TC, using multiple assays such as Western Blotting, RNA immunoprecipitation, and RNA-pulldown assay. Second, the roles of circHIPK3 were explored in this study in vitro which should be further confirmed in vivo.

Conclusions

In conclusion, this study showed that circHIPK3 was overexpressed in TC and its knocking down could reduce the ability of TC cells to proliferate and metastasize, which indicated that its overexpression promoted the proliferation and metastasis of TC. Mechanistically, the relationship and interaction between circHIPK3, miR-338-3p and RAB23 were revealed. We conclude that circHIPK3 promotes TC tumorigenesis and invasiveness by sponging miR-338-3p and upregulating RAB23 expression. These findings suggest that circHIPK3 could be a promising therapeutic strategy for TC patients.

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Ethics approval and consent to participate

This study was approved by the Ethics Committee of Harbin Medical University and was conducted in accordance with the government policies and the Declaration of Helsinki. Written informed consent was obtained from all patients.

Conflict of interest disclosure statement

The authors declare that they have no competing interests.

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Authors' contributions

Conception and design: Xiaorong Zhan; Development of methodology: Taipengfei Shu;

Sample collection: Lin Yang; Analysis and interpretation of data: Lijie Sun, Jixuan

Writing, review, and/or revision of the manuscript: Xiaorong Zhan, Taipengfei S.

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Figure Legends

Figure 1: The expression level and location of circHIPK3 in TC cell lines and TC samples.

(A) The expression levels of circHIPK3 RNA was up-regulated in K1 and CAL-62 and TPC1 cell lines compared to normal thyroid cell line, Nthy-ori 3-1. (B) In eight of the ten pairs of thyroid cancer tissues and their adjacent normal tissues, circHIPK3 RNA levels in the cancer tissues were higher than normal tissues. (C) qRT-PCR assay showed circHIPK3 was mainly located in nuclear in CAL-62 and TPC1. (D) qRT-PCR assay showed circHIPK3 was mainly located in cytoplasm in CAL-62 and TPC1 cells. (E) qRT-PCR assay showed circHIPK3 was mainly located in cytoplasm of TC cells. In all panels, values represent mean $\pm$ SEM from three independent experiments. Significance was defined as $p < 0.05$ (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

Figure 2: Knockdown of circHIPK3 inhibited TC cell proliferation.

(A) The expression level of circHIPK3 RNA was decreased after transfecting with si-circHIPK3 compared to transfecting with siNC in CAL-62 cells. (B) The optical density (OD) of CAL-62 cells with down-regulated circHIPK3 at 450 nm was significantly lower than that of the control group. (C) The expression level of circHIPK3 RNA was decreased in the TPC1 cells transfected with si-circHIPK3, compared with the TPC1 cells transfected with siNC. (D) After downregulation of circHIPK3 in TPC1 cells, the optical density (OD) at 450 nm was significantly reduced. In all panels, values represent mean $\pm$ SEM from three independent experiments. Significance was defined as $p < 0.05$ (* $p < 0.05$; ** $p < 0.01$).

Figure 3: Knockdown of circHIPK3 suppressed cell migration and invasion in TPC1 and CAL-62 cells.

(A) Knockdown of circHIPK3 reduced cell migration in TPC1 cells (left), quantification of the results (right), data is presented as the mean $\pm$ SEM. (B) Knockdown of circHIPK3 reduced cell migration in CAL-62 cells (left), quantification of the results (right), data is presented as the mean $\pm$ SEM. (C) Knockdown of circHIPK3 reduced cell invasion in TPC1 cells (left), quantification of the results (right), data is presented as the mean $\pm$ SEM. (D) Knockdown of circHIPK3 reduced cell invasion in CAL-62 cells (left), quantification of the results (right), data is presented as the mean $\pm$ SEM. In all panels, values represent mean $\pm$ SEM from three independent experiments. Significance was defined as $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***).

Figure 4: CircHIPK3 promoted RAB23 by miR-338-3p.

(A-B) RAB23 was down-regulated after knockdown of circHIPK3 in TPC1 (A) and CAL-62 (B) cells. (C-D) RAB23 was down-regulated after overexpressing miR-338-3p in TPC1 (C) and CAL-62 (D) cells. (E) RAB23 protein was down-regulated after knockdown of circHIPK3 or overexpression of miR-338-3p. (F-G) CircHIPK3 RNA was down-regulated after overexpressing miR-338-3p in TPC1 (F) and CAL-62 (G) cells. (H) Bioinformatics analysis revealed the predicted binding sites between RAB23 or circHIPK3 and miR-338-3p. (I-J) Luciferase reporter assay demonstrated miR-338-3p mimics significantly decreased the luciferase activity of circHIPK3-wt and RAB23-wt in TC cells. (K-L) Luciferase reporter assay demonstrated miR-338-3p mimics did not affect the luciferase activity of circHIPK3-mut and RAB23-mut in TC cells. All tests were performed at least three times. Data were expressed as mean $\pm$ SD. * $p < 0.05$.

Supplementary Figure 1: The expression and prognosis of MiR-338 in TC and the binding of miR-338-3p to different circHIPK3 and RAB23 sequence.

(A) MiR-338 was down-regulated in TC tissues compared with normal tissues by analyzing TCGA. (B) The TC patients with higher expression of miR-338 have a potential longer survival time by using TCGA database. (C, E) Wild-type circHIPK3 and RAB23 sequences that can bind to miR-338-3p. (D, F) The designed mutant circHIPK3 and RAB23 sequences are not binding to miR-338-3p.

Fig. 1

A

TPC1-migration

B

CAL-62-migration

C

TPC1-invasion

D

CAL-62-invasion

Fig. 3

Fig. 4

Supplementary Fig. 1