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Circular RNA circZFR contributes to papillary thyroid cancer cell proliferation and invasion by sponging miR-1261 and facilitating C8orf4 expression

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ABSTRACT

In recent years, more and more circular RNAs (circRNAs) have been identified in multiple tissues and cells. Increasing evidences show circRNAs play important roles in human cancers. However, the role of circRNAs in papillary thyroid carcinoma (PTC) remains largely unknown. In this study, we identified a new circRNA circZFR that was significantly upregulated in PTC tissues compared to adjacent normal tissues. Furthermore, circZFR expression level was negatively correlated with clinical severity. We found that circZFR knockdown dramatically inhibited the proliferation, migration and invasion of PTC cells *in vitro*. Mechanistically, we found circZFR could promote C8orf4 expression via serving as a competing endogenous RNA (ceRNA) of miR-1261 in PTC cells. Rescue assays indicated that restoration of C8orf4 significantly attenuated the inhibitory effects of circZFR knockdown on PTC cell proliferation, migration and invasion. In summary, our findings demonstrated that circRNA circZFR exerted oncogenic roles via regulating miR-1261/C8orf4 axis in PTC, which suggested circZFR might be a potential therapeutic target.

1. Introduction

Thyroid cancer is one of the most frequent cancers in endocrine system and contributes to a large proportion of cancer-related death in the world [1,2]. Notably, the incidence of thyroid cancer is still increasing rapidly [3]. Among all thyroid cancer cases, papillary thyroid cancer (PTC) accounts for over 80% [4]. Although most PTC patients show a good prognosis, about 20% of PTC patients display aggressive behaviors and poor outcomes [5]. Thus, it is very critical to understand the underlying molecular mechanism of PTC progression and develop novel therapeutic targets.

More than 90% of human genomic transcripts are noncoding RNAs (ncRNA), such as long noncoding RNA (lncRNA), microRNAs (miRNAs) and circular RNAs (circRNA) [6–8]. miRNAs and lncRNAs have been widely investigated while the study on circRNA function is just emerging. circRNA is characterized with a covalently closed loop lacking 5′-3′ ends and a poly A tail [9]. More and more studies suggest that circRNAs exert very essential roles and involved in human cancers, such as glioma [8], hepatocellular carcinoma [10], lung cancer [11], breast cancer [12] and bladder cancer [13]. CircRNAs could regulate cancer cell proliferation, migration, invasion and survival to participate in tumor progression. For example, circHIPK3 promotes colorectal cancer growth and metastasis by sponging miR-7 [14]. circMAN2B2 facilitates lung cancer cell proliferation and invasion via miR-1275/FOXK1 axis [15]. Although several cancer-related circRNAs have been identified, the function of circRNAs in PTC has not been reported until now.

In the present study, we identified a new circRNA circZFR (circBase ID: hsa_circ_0072088) located at chromosome 5p13.3. circZFR was highly expressed in PTC tissues compared to adjacent normal tissues. The expression level of circZFR was positively correlated with clinical severity. We also showed circZFR promoted the proliferation, migration and invasion of PTC cells via upregulating C8orf4 by sponging miR-1261, which suggested the circZFR/miR-1261/C8orf4 axis might be a therapeutic target.

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2. Materials and methods

2.1. Human samples

Fresh samples from PTC tumor tissues (n = 41) and corresponding normal thyroid (NT) tissues adjacent to PTC tumors (n = 41) were obtained from patients with sporadic PTC undergoing surgical resection at Jinan Maternity and Child Care Hospital. Then the samples were snap-frozen in liquid nitrogen and stored at −80 °C. This study was approved by the Human Research Ethics Committee from The Second Hospital of Jinan Maternity and Child Care Hospital. Informed consent was obtained from all patients.

2.2. Cell culture and transfection

Normal human thyroid follicular epithelial cell Nthy-ori3-1 cell and human thyroid cancer K-1, TPC-1, SW579 and FTC133 purchased from American Type Culture Collection (ATCC) were cultured in RPMI-1640 medium with 10% fetal bovine serum (FBS), and 1% penicillin-streptomycin (Sigma, USA) in the humidified incubator with 5% CO_2 at 37 °C.

For cell transfection, PTC cell lines were cultured in 6-well plates with serum-free medium overnight and then transfected with a short interfering RNA targeting the junction region of circZFR (si-circZFR) plasmid (100 nM), miR-1261 mimics, or C8orf4 plasmid and their corresponding controls using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions, for at least 5 h before the medium was replaced with fresh medium. After culture at 37 °C for 72 h, the treated cells were harvested for the subsequent experiments.

2.3. Transwell assay

Cell migration and invasion were assessed using Transwell Chamber Cell Culture (10 μm pore membrane, BD Biosciences). A total of 1×10^5 cells in 100 μl of serum-free medium were added to the top chamber (Matrigel pre-coated for invasion assay) of 24-well plates. The bottom well contained growth medium with 20% FBS. Transwell chambers were placed at 37 °C for 48 h. Cells in chamber were fixed with methanol for 30min and then staining with Crystal violet for 15min. Migrated or invaded cells were finally observed under a microscope and the number was counted with randomly nine field for each experiment.

2.4. CCK-8 assay

Cells were suspended in DMEM with 10% FBS, and seeded into 96-well plates at a density of 2×10^3 cells per well. 24, 48, 72 or 96 h later, 10 μl CCK-8 solution (Dojindo, Japan) was added and incubated in dark for another 2 h. Absorbance was measured at 450 nm at indicated time points with the absorbance value measured on the first day as a control. Each measurement was performed in triplicate, and the experiments were conducted at least three times.

2.5. Colony formation assay

Cells were seeded in 6-well plates at 5×10^2 per well. And then, they were incubated for 2 weeks. After that, cells were washed with PBS, fixed with paraformaldehyde, and stained with crystal violet (Sigma, China). The number of colonies was counted under a microscope (Olympus IX81, Japan).

2.6. Luciferase assay

The fragments of circZFR and C8orf4 containing miR-1261 binding sites or the corresponding mutated binding sites were synthesized by Shanghai Sangon Biotech. These fragments were sub-cloned into the luciferase vector psiCHECK-2 (Promega, Madison, WI, USA). Cells were then seeded into 24-well plates at a density of 1×10^5 cells/well, and incubated at 37 °C overnight. Cells were transfected with circZFR-wild-type (circZFR-WT), circZFR-Mutant (circZFR-Mut), C8orf4-WT or C8orf4-Mut constructs in the presence of miR-1261 mimics or its control by using Lipofectamine 2000 (Invitrogen). Luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. Renilla luciferase activity was normalized to the activity of Firefly luciferase.

2.7. qRT-PCR

Total RNAs of PTC tissue and cell lines were extracted using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and the samples were used to produce cDNA by reverse transcription using the GoScript RT System (Promega, USA) and All-in-One mRNA Reverse Transcription Kit (GeneCopoeia, USA). The qRT-PCR analysis was performed using a FastStart Universal SYBR-Green Master (Roche, Indianapolis, IN, USA). The PCR programs were set as follows: 95 °C for 10min; and 35 cycles of 95 °C for 40s, 58 °C for 30s for primer annealing, and 72 °C for 30s. Each reaction was performed in triplicate. GAPDH or U6 were used as the internal references, and the $2^{-\Delta\Delta Ct}$ method was applied to calculate the relative expression.

2.8. Statistical analyses

All statistical data analyses were analyzed with SPSS 17.0 software (IBM, Chicago, IL). Results are shown as mean ± SD. The significance of the differences was determined by Student's t-test or one-way ANOVA followed by Tukey's post hoc test as appropriately. $P < 0.05$ was considered significant.

3. Results

3.1. circZFR was highly expressed in PTC tissues

To explore the function of circRNAs in PTC, we looked for differentially expressed circRNAs by bioinformatics analysis according to an online dataset (GSE93522) in NCBI database. We found many circRNAs were upregulated or downregulated in PTC tissues compared to adjacent normal tissues (Fig. 1A). Among these circRNAs, circZFR was the most upregulated circRNA in PTC tissues (Fig. 1A). To validate it, we analyzed circZFR expression in 41 pairs of PTC tissues and adjacent normal tissues by qRT-PCR. The results indicated that circZFR expression was significantly upregulated in PTC tissues (Fig. 1B). To examine the correlation between circZFR expression and clinical severity, we determined circZFR expression in different TNM stages of PTC samples and metastatic or non-metastatic PTC samples. As shown, the expression of circZFR was significantly higher in samples of stage III-IV (n = 25) than that in samples of stage I-II (n = 16) (Fig. 1C). Besides, metastatic samples (n = 12) have higher expression of circZFR than non-metastatic samples (n = 29) (Fig. 1D). Consistently, we found that circZFR expression was also higher in PTC cell lines compared to Nthy-ori3-1 cells (Fig. 1E). Moreover, Kaplan-Meier curve analysis indicated higher expression of circZFR in PTC patients was correlated with worse prognosis (Fig. 1F). Taken together, these results indicated circZFR was upregulated in PTC tissues and correlated with clinical severity, suggesting circZFR might be involved in PTC progression.

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