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Upregulated hsa_circ_0004458 Contributes to Progression of Papillary Thyroid Carcinoma by Inhibition of miR-885-5p and Activation of RAC1

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Background: Circular RNAs (circRNAs), a class of noncoding RNAs, may act as biomarkers and therapeutic targets of various cancers. However, the effects of hsa_circ_0004458 in papillary thyroid carcinoma (PTC) are still very much unclear. We aimed to demonstrate the potential roles of hsa_circ_0004458 in the progression of PTC.

Material/Methods: In our study, qRT-PCR assay was performed to assess hsa_circ_0004458, miR-885-5p and RAC1 expressions. Dual-luciferase reporter assay was used to detect the regulatory effects of hsa_circ_0004458 on miR-885-5p, and miR-885-5p on RAC1. MTT and flow cytometry assays were used to measure the cell proliferation, cycle, and apoptosis abilities. Tumor formation assay in nude mice was performed to measure the tumor growth *in vivo*.

Results: Our results indicated that hsa_circ_0004458 was upregulated in PTC tissues and cells, while silencing of hsa_circ_0004458 suppressed PTC cell proliferation and promoted PTC cell cycle arrest and apoptosis *in vitro*. Tumor formation assay in nude mice showed that knockdown of hsa_circ_0004458 by siRNAs inhibited the growth of PTC tumor *in vivo*. In addition, we found that miR-885-5p was a direct target of hsa_circ_0004458, and silencing of hsa_circ_0004458 inhibited PTC cell proliferation by miR-885-5p. We also demonstrated that RAC1 was a direct target of miR-885-5p and silencing of RAC1 suppressed PTC cell proliferation.

Conclusions: We found that hsa_circ_0004458 promoted the progression of PTC by inhibition of miR-885-5p and activation of RAC1, and hsa_circ_0004458 may serve as a potential therapeutic target and biomarker for PTC.

MeSH Keywords: Carcinoma, Papillary • MicroRNAs • rac1 GTP-Binding Protein • RNA, Untranslated

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Background

Papillary thyroid carcinoma (PTC) is a differentiated malignant tumor originating from thyroid follicular epithelial cells, and is also the most common pathological type of thyroid carcinoma [1]. The incidence of thyroid cancer increases year by year, and among them, PTC grows faster than others and with a younger average age of onset [2]. The clinical symptoms of PTC are atypical and are often characterized by slow growth of thyroid nodules, which are easy to misdiagnose, and make patients miss the best treatment period [3]. Although PTC has a relatively good prognosis, some PTC patients have tumor invasion and lymph node metastasis in the early stage and are prone to repeated recurrence and metastasis after surgery [4]. Therefore, it is crucial to explore effective new markers in diagnosis, treatment, and prognosis of PTC.

Circular RNAs (circRNAs), a type of noncoding RNA (ncRNA), possess a covalently closed continuous loop structures without 5' or 3' tails, which make them more stable and resistant to RNase R [5–7]. Research increasingly suggests that circRNAs are differentially expressed in many types of cancers and are closely related to the development of many cancers, including gastric cancer, colorectal cancer, breast cancer, and hepatocellular carcinoma [8–12]. Therefore, circRNAs may be potential biomarkers and therapeutic targets for cancer therapy. Furthermore, many studies have shown that circRNAs can participate in the occurrence and development of many kinds of cancers by acting as microRNA (miRNA) sponges [13,14]. Thus, circRNAs can protect target genes from miRNA-mediated mRNA degradation. The circRNA-miRNA-mRNA networks are involved in many critical cellular processes, such as cell proliferation, cycle regulation, differentiation, apoptosis, invasion, and metastasis [15–18]. Hsa_circ_0004458 (chr8: 18656804-18662408) is a product of PSD3 mRNA splicing and is 448 nucleotides in length. Hsa_circ_0004458 was first discovered in gastric cancer and PTC [19,20], but the function and mechanism of hsa_circ_0004458 have not been defined.

MiRNAs are small noncoding RNAs that negatively regulate gene expression at the posttranscriptional level [21]. Emerging evidence suggests that miRNAs play important roles in regulation of tumor biology and inflammation [22]. Altered expression of miRNA has been reported in a variety of cancers, and their expression profiles can be used as hallmarks for diagnosis, classification, and prognosis of human malignancies [23–26]. Their target genes are involved in processes such as cell proliferation, apoptosis, differentiation, and metastasis [27]. However, the biological function and mechanism of action of miR-885-5p in PTC are not well understood and need further investigation.

In this study, we verified the carcinogenesis of hsa_circ_0004458 as a ceRNA in PTC to modulate RAC1 expression

and identified miR-885-5p as the specific miRNA inhibited by hsa_circ_0004458. Furthermore, we showed that hsa_circ_0004458 promoted proliferation and inhibited cell cycle arrest and apoptosis of PTC by inhibition of miR-885-5p and activation of RAC1.

Material and Methods

Tissue specimens

We obtained 48 pair of PTC tissue samples (PTC tissues and the matched para-cancerous thyroid tissues) from Zhejiang Taizhou Municipal Hospital from February 2013 to June 2017. Written informed consent was obtained from all patients, and the study was approved by the Ethics Committee of Zhejiang Taizhou Municipal Hospital. All these tissues were immediately stored at 70°C.

Cell lines and cell culture

Human thyroid follicular epithelial cells (Nthy-ori 3-1) cells and PTC cells lines (BCPAP, TPC, K1, and IHH4) were purchased from ATCC (Manassas, VA, USA). Nthy-ori 3-1 cells were cultured in RPMI1640 medium (Gibco, New York, USA); BCPAP, TPC, K1, and IHH4 cells were grown in Dulbecco's modified Eagle's medium (DMEM, cat#: 11960-044, Invitrogen, USA). Both mediums included 10% fetal bovine serum (FBS, cat. 10082-147, Gibco), 100 U/mL penicillin, and 100 U/mL streptomycin (cat. no. 15140-12, Invitrogen, Carlsbad, CA). All cells were maintained at 37°C in a 5% CO₂ incubator.

Cell treatment

Hsa_circ_0004458 siRNA (si-circ_0004458), negative control siRNA (si-control), and RAC1 siRNA (si-RAC1) were obtained from RiboBio (Guangzhou, China). The scrambled control and miR-885-5p inhibitors (anti-miR-885-5p) were purchased from GenePharma (Shanghai, China). Briefly, TPC-1 and K1 cells were grown in 6-well plates and were transiently transfected with si-circ_0004458 (50 nM) or si-control (50 nM), si-control (50 nM) or si-RAC1 (50 nM), scramble (50 nM) or anti-miR-885-5p (50 nM) for 48 h by using Lipofectamine™ 2000 (Invitrogen) according to the instructions provided by the manufacturer.

RNA extraction and quantitative real-time PCR (qRT-PCR)

Total RNA was isolated from cells and tissue samples by using Trizol reagent (#9109, Takara, China). RNA concentrations were quantified using a NanoDrop ND-2000 spectrophotometer (ThermoScientific, Waltham, MA), and then RNA was transcribed to cDNA by using a cDNA synthesis kit (#6130, Takara, China). miR-885-5p expression was quantified by TaqMan

Table 1. Primer sequences for qRT-PCR analysis.

Gene	Primer sequences
GAPDH	Forward: 5'-TATGATGATATCAAGAGGGTAGT-3' Reverse: 5'-TGTATCCAAACTCATTGTCATAC-3'
U6	Forward, 5'-CTCGCTTCGGCAGCACATA-3' Reverse 5'-AACGATTCACGAATTGCGT-3'
Hsa_circ_0004458	Forward: 5'-GTCCATTGCCTTACCTGTGC-3' Reverse: 5'-CTGTCCAGCTGATAAAGGCG-3'
MiR-885-5p	Forward: 5'-TCCATTACACTACCCTGCCTAT-3' Reverse: 5'-CGCTACGTAACGGCATGACAGT-3'
RAC1	Forward: 5'-ATGCAGGCCATCAAGTATGTGGTG-3' Reverse: 5'-TTACAACAGCAGG CATTCTCTTCC-3'

microRNA assay (Applied Biosystems, San Diego, CA), and U6 was used as an endogenous control. Hsa_circ_0004458 and RAC1 expressions were measured by using SYBR Green (Applied Biosystems, cat #4368577) according to manufacturer's instructions, and GAPDH was used as the reference gene. All reactions were performed in triplicate on the ABI 7500 System (Applied Biosystems, Foster City, CA). Relative expressions were determined based on $2^{-\Delta\Delta C_t}$ method [28]. All primers were purchased from Invitrogen Co., Ltd., and the sequences are shown in Table 1.

MTT assay

The TPC-1 and K1 cells were seeded onto 96-well plates and transfected with the corresponding siRNAs or anti-miR-885-5p for 48 h. At 0, 12, 24, 48, and 72 h after transfection, we added 20 μ l of MTT reagent (0.5 mg/ml, Sigma Aldrich, St. Louis, Missouri, USA) to each well. Cells were then incubated at 37°C for 4 h, and 150 μ l dimethyl sulfoxide (DMSO; Sigma Chemical Co., St. Louis, MO) was added into each well. Finally, the absorbance was measured by using a microplate reader at 570 nm (Sunrise™; Tecan Group Ltd., Männedorf, Switzerland).

Colony formation assay

The transfected TPC-1 and K1 cells (5×10^3 cells/well) were seeded onto 6-well plates and incubated at 37°C in a 5% CO₂ incubator for 14 days. The colonies were fixed with 10% formaldehyde (cat. no. BDH0500-1LP) and stained with 1% crystal violet (BD Biosciences) for 5 min. After washing with PBS, the visible colonies were photographed and counted.

Flow cytometry

For cell cycle assay, the transfected TPC-1 and K1 cells were collected and fixed in 70% ethanol. According to the manufacturer's protocol, the cell cycle was measured by using a Cycletest Plus DNA Kit (BD Biosciences, Franklin Lakes, NJ).

Cells were treated with RNase A and dyed with propidium iodide (cat. # P4170, Sigma) and the results were detected using a FACS Calibur flow cytometer (BD Biosciences).

For cell apoptosis assay, the transfected TPC-1 and K1 cells were washed with phosphate-buffered saline (PBS; cat #14190-250; Invitrogen) and resuspended in Annexin-binding buffer, after which cells were stained with Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) (cat. no. ab14085; Abcam, Cambridge, UK) for 15 min. The results were analyzed using a FACS Calibur flow cytometer (BD Biosciences).

Dual-luciferase activity assay

The fragments of the wild or mutant 3'-UTR of hsa_circ_0004458 and RAC1 containing the miR-885-5p binding site were amplified, then the fragments were cloned into a luciferase vector psi-CHECK (Promega, Madison, USA). K1 and TPC-1 cells were co-transfected with wild (circ_0004458-3'-UTR-WT) or mutant (circ_0004458-3'-UTR-Mut) circ_0004458 3'-UTRs, miR-control, or miR-885-5p, respectively. TPC-1 cells were transfected with scramble or miR-885-5ps and RAC1-3'-UTR-WT or RAC1-3'-UTR-Mut, respectively. Luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega) according to manufacturer's instructions.

Tumor formation in nude mice

The transfected TPC-1 cells (2×10^6 cells) were subcutaneously injected into the 6-week-old BALBc nude mice for 7, 14, 21, 28, and 35 days. The height, width, and depth of tumors were measured, tumor volumes were measured weekly, and the tumor weight was measured.

Statistical analysis

All data are presented as mean $\pm$ SD. The data were analyzed with SPSS 17.0 and GraphPad Prism software (Ver. Prism 7, La Jolla, CA, USA). The differences between the groups were analyzed by one-way analysis of variance (ANOVA) with the Tukey post hoc test. All experiments were performed 3 times.

Results

Hsa_circ_0004458 was upregulated in PTC

To identify potential circRNAs that may be expressed in PTC, previous research has analyzed the circRNAs expression profile in 6 pairs of PTC tissue samples (6 PTC tissues and 6 normal thyroid tissues) by using circRNA microarray [20]. According to the expression intensity sorting, we screened out the top 10 up- or down-regulated circRNAs (Figure 1A). We then analyzed

Figure 1. Hsa_circ_0004458 was upregulated in PTC. **(A)** Heat map of the top 10 up- or down-regulated circRNAs in 6 paired PTC tissues and normal thyroid tissues. Each column indicates the expression profile of a tissue sample; red is high expression, green is low expression. **(B)** Hsa_circ_0004458 expression was analyzed by qRT-PCR assay in 48 pairs of PTC tissues and matched para-cancerous thyroid tissues. **(C)** Hsa_circ_0004458 expression was detected by qRT-PCR assay in Nthy-ori 3-1 cells and PTC cells lines (BCPAP, TPC, K1, and IHH4) (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). **(D)** Hsa_circ_0004458 comes from the exon 5 and exon 8 of PSD3 gene.

Table 2. Correlations between hsa_circ_0004458 expression and clinicopathologic characteristics in PTC (dCt).

Characteristics	No. of patients		Mean ± SD	P value
Total no. of patients	48			
Age (year)				
>50	21	(43.8%)	10.02±1.88	0.7314
≤50	27	(56.2%)	9.84±1.72	
Gender				
Male	19	(39.6%)	10.31±2.43	0.5317
Female	29	(60.4%)	9.97±1.95	
Tumor size (cm)				
<3	26	(54.2%)	11.38±2.02	0.016*
≥3	22	(45.8%)	10.14±1.25	
Invasion				
T0–T2	31	(64.6%)	11.96±1.71	<0.001***
T3–T4	17	(35.4%)	9.18±1.89	
Lymphatic metastasis				
N0	34	(70.8%)	11.94±1.03	<0.001***
N1–N3	14	(29.2%)	9.13±1.24	
Distal metastasis				
M0	40	(83.3%)	11.87±1.94	0.0019**
M1	8	(16.7%)	9.47±1.49	
TNM stage				
0 & I & II	32	(66.7%)	11.34±1.74	<0.001***
III & IV	16	(33.3%)	9.23±1.24	

* $P<0.05$; ** $P<0.01$; *** $P<0.001$.

hsa_circ_0004458 expression in PTC tissues and cells, and the results indicated that hsa_circ_0004458 expression was significantly upregulated in PTC tissues (n=48) compared with matched para-cancerous thyroid tissues (n=48) (Figure 1B). We also analyzed the correlations between hsa_circ_0004458 expression and clinicopathologic characteristics in PTC and found that hsa_circ_0004458 expression was related to tumor size (cm) ($P=0.016$), invasion ($P<0.001$), lymphatic metastasis ($P<0.001$), distal metastasis ($P=0.0019$), and TNM stage ($P<0.001$) (Table 2). Hsa_circ_0004458 expression was also higher in PTC cells lines (BCPAP, TPC-1, K1, and IHH4) than in Nthy-ori 3-1 cells. The expression level of hsa_circ_0004458 was higher in TPC and K1 cells than in other PTC cell lines, so we selected TPC-1 and K1 cells as the research cell lines ($P<0.05$, $P<0.01$, $P<0.001$, Figure 1C). According to the database accessed at <http://www.circbase.org/>, we found that hsa_circ_0004458 is located in chr8: 18656804-18662408 and is formed from the exon 5 and exon 8 of the PSD3 gene, the best

transcript is NM_015310, and the spliced length is 448 bp. Hsa_circ_0004458 was amplified by qRT-PCR assay with the divergent primers, and the sequence of the circular junction was also validated by Sanger sequencing (Figure 1D).

Silencing of hsa_circ_0004458 suppresses the growth of PTC

To further explore whether hsa_circ_0004458 affects PTC progression, we knocked down hsa_circ_0004458 expression in TPC-1 and K1 cells by using small interfering RNA. The results showed that hsa_circ_0004458 expression was effectively inhibited in TPC-1 and K1 cells ($P<0.05$, Figure 2A). The MTT assay showed the proliferation abilities were impaired in the si-circ_0004458 group compared to the si-control group ($P<0.05$, Figure 2B, 2C). The clone formation assay indicated that silencing of hsa_circ_0004458 significantly inhibited the colony-forming abilities of TPC-1 and K1 cells ($P<0.05$, Figure 2D, 2E). In addition,

Figure 2. Silencing hsa_circ_0004458 suppressed the growth of PTC. K1 and TPC-1 cells were transfected with circ_0004458 siRNAs (si-circ_0004458) and si-control, respectively. (A) qRT-PCR assay was used to evaluate the effect of RNA interference (* $P<0.05$). (B, C) MTT assay was performed to measure PTC cell proliferation (* $P<0.05$). (D, E) Clone formation assay was used to assess PTC cell clonogenesis ability (* $P<0.05$). (F) The transfected TPC-1 cells (2×10^6 cells) were injected into the nude mice for 7, 14, 21, 28, and 35 days. The height, width, and depth of tumors were measured and the tumor volumes were assessed (* $P<0.05$). (G) The dissected tumors were obtained. (H) The tumor weight was measured (* $P<0.05$).

we also demonstrated that silencing hsa_circ_0004458 inhibited tumor growth *in vivo* by injecting TPC-1 cells subcutaneously into nude mice. The dissected tumors were obtained, then we measured the height, width, and depth of tumors every 7 days, as well as the tumor volumes and weights. The results revealed that tumor volume and weight were smaller in the si-circ_0004458 group than in the si-control group ($P<0.05$, Figure 2F–2H). Therefore, we suggest that silencing hsa_circ_0004458 suppressed the growth of PTC *in vitro* and *in vivo*.

Silencing hsa_circ_0004458 induces PTC cell cycle arrest and apoptosis

We further proved the effects of hsa_circ_0004458 on PTC-1 and K1 cell cycle arrest and apoptosis. Our results verified that the cell cycle distributions represented significant S phase reduction in the si-circ_0004458 group compared with

the si-control group ($P<0.05$, Figure 3A–3C). We also found that the cell apoptosis rate was significantly increased in the si-circ_0004458 group compared with the si-control group ($P<0.05$, Figure 3D). Therefore, we suggest that silencing of hsa_circ_0004458 promotes PTC cell cycle arrest and apoptosis.

Silencing of hsa_circ_0004458 inhibits PTC cell proliferation by regulating miR-885-5p

Previous studies demonstrated that circRNAs might act as ceRNA to miRNA [29,30], while the exact regulatory mechanism of hsa_circ_0004458 remains unclear. According to bioinformatics analysis using StarBase 2.0, the miRNAs predicted targeted hsa_circ_0004458 by TargetScan are shown in Table 3. We then found and selected 10 miRNAs that have binding sites with the hsa_circ_0004458 sequence. Dual-luciferase reporter assay showed that hsa_circ_0004458 could reduce miR-885-5p

Figure 3. Silencing hsa_circ_0004458 induces PTC cell cycle arrest and apoptosis. K1 and TPC-1 cells were transfected with si-circ_0004458 and si-control, respectively. (A–C) Cell cycle distribution was analyzed by flow cytometry (* $P < 0.05$). (D) Cell apoptosis was measured by flow cytometry (* $P < 0.05$).

expression (Figure 4A). Therefore, we further analyzed the regulation of hsa_circ_0004458 on miR-885-5p. We constructed wild (circ_0004458-3'-UTR-WT) or mutant (circ_0004458-3'-UTR-Mut) circ_0004458 3'-UTRs (Figure 4B). K1 and TPC-1 cells were co-transfected with circ_0004458-3'-UTR-WT or circ_0004458-3'-UTR-Mut, and miR-control or miR-885-5p, respectively. The results indicated that the luciferase intensity of wild-type hsa_circ_0004458 was significantly inhibited by miR-885-5p, while miR-885-5p had no effect on mutated hsa_circ_0004458 ($P < 0.05$, Figure 4C). We also found that silencing of hsa_circ_0004458 significantly upregulated miR-885-5p expression ($P < 0.05$, Figure 4D). In addition, we demonstrated that silencing of hsa_circ_0004458 significantly inhibited the proliferation abilities of K1 and TPC-1 cells, and miR-885-5p inhibitor (anti-miR-885-5p) then attenuated the inhibition effects mediated by hsa_circ_0004458 knockdown (Figure 4E, 4F).

RAC1 is a direct target of miR-885-5p

Previous research has also shown miRNAs can serve as negative regulators of gene expression by targeting mRNAs [31,32]. We further analyzed the putative complementary site of miR-885-5p and RAC1 based on TargetScan prediction. Similarly, we constructed wild-type RAC1 (RAC1-3'-UTR-WT) and mutant-type RAC1 (RAC1-3'-UTR-Mut) (Figure 5A, 5B). TPC-1 cells were transfected with scramble or miR-885-5ps, RAC1-3'-UTR-WT, or RAC1-3'-UTR-Mut, respectively. The results showed that the luciferase intensity of wild-type RAC1 was significantly inhibited by miR-885-5p, while miR-885-5p had no effect on mutated RAC1 ($P < 0.05$, Figure 5C).

Table 3. miRNAs predicted targeted has_circ_0004458 by Targetscan.

CircRNA Mirbase ID	CircRNA (Top) miRNA (Bottom) pairing	Context+ score percentile
hsa_circ_0004458 (5'...3')	GAAAAUCUCAGUAAU-----GGUACCAG	96
hsa-miR-1263 (3'...5')	 UGAGUCAUACGGUCCCAUGGUA	
hsa_circ_0004458 (5'... 3')	CAGUCACUCAGGUAAUUCUUUAA	92
hsa-miR-186 (3'... 5')	 UCGGGUUUUCCUCUUAAGAAAC	
hsa_circ_0004458 (5'... 3')	UAAAGCAUUCUCUCUUGUGGGAG	93
hsa-miR-188-3p (3'... 5')	 ACGUUUGGGACGUACACCCUC	
hsa_circ_0004458 (5'... 3')	AAACGCCUUUAUCAGCUGGACAG	88
hsa-miR-198 (3'... 5')	 CUUGGAUAGAGGGGAGACCUGG	
hsa_circ_0004458 (5'... 3')	NNNNNNNNNNNNNNNCAAUGCUGGG	NA
hsa-miR-338-3p (3'... 5')	 GUUGUUUUAGUGACUACGACCU	
hsa_circ_0004458 (5'... 3')	UCCAAUAGAUUUUUUAUUGUAA	92
hsa-miR-338-5p (3'... 5')	 GUGAGUCGUGGUCCUAUAACAA	
hsa_circ_0004458 (5'... 3')	CAAAGAUCAGAUGUUGCAAAC	82
hsa-miR-507 (3'... 5')	 AAGUGAGGUUUUCCACGUUUU	
hsa_circ_0004458 (5'... 3')	CAAAGAUCAGAUGUUGCAAAC	82
hsa-miR-557 (3'... 5')	 UCUGUUCGGGUGGGCACGUUUG	
hsa_circ_0004458 (5'... 3')	GAAAUUGGGAGCACUGAAAUUUU	94
hsa-miR-513a-3p (3'... 5')	 GGAAGAGUCUUUCCACUUUAAAU	
hsa_circ_0004458 (5'... 3')	CUCUUGUGGGAGAAACUCAAGAA	99
hsa-miR-526b (3'... 5')	 UGUCUUUCACGAAGG--GAGUUCUC	
hsa_circ_0004458 (5'... 3')	AAAGCAUUCUCUCUUGUGGGAGA	99
hsa-miR-532-3p (3'... 5')	 ACGUUCGGAACCCACACCCUCC	

Table 3 continued. miRNAs predicted targeted has_circ_0004458 by TargetsCan.

hsa_circ_0004458 (5'... 3')	ACUUCUCCAUAUAGAUUUUUUUAU 	94
hsa-miR-548c-3p (3'... 5')	CGUUUUCAUUAACUCUAAAAAC	
hsa_circ_0004458 (5'... 3')	AACAACACGGCUAGAAGCUCAUU 	94
hsa-miR-556-5p (3'... 5')	GAGUAUAAUGUUACUCGAGUAG	
hsa_circ_0004458 (5'... 3')	CAGAUACCAUUGCUUCACAAGAU 	86
hsa-miR-581 (3'... 5')	UGACUAGAUCUCUUGUGUUCU	
hsa_circ_0004458 (5'... 3')	AGCAGCAAUGUGGAAGCAGCCAA 	91
hsa-miR-604 (3'... 5')	CAGGACUUAAGGCGUCGGA	
hsa_circ_0004458 (5'... 3')	AAUUUUGGAAAAGGAGACCCAG 	82
hsa-miR-615-5p (3'... 5')	CUAGGCUCGUGGCCCCUGGGGG	
hsa_circ_0004458 (5'... 3')	GAGUUUUAAUACACUUCUCCAAU 	93
hsa-miR-1270 (3'... 5')	UGUGUCGAGAAGGUUAUAGAGGUC	
hsa_circ_0004458 (5'... 3')	GAGUUUUAAUACACUUCUCCAAU 	94
hsa-miR-620 (3'... 5')	UAAAGAUUAGAUAGAGGUA	
hsa_circ_0004458 (5'... 3')	UUUUGGAAAAGGAGACCCAGAA 	89
hsa-miR-637 (3'... 5')	UGCGUCUCGGGCUUCGGGGGUA	
hsa_circ_0004458 (5'... 3')	UUACAGGAUAGCGUGGAUCAG 	87
hsa-miR-640 (3'... 5')	UCUCCGUCCAAGG----ACCUAGUA	
hsa_circ_0004458 (5'... 3')	AGCAGCAAUGUGGAAGCAGCCAA 	99
hsa-miR-647 (3'... 5')	CUUCCUUCACUCACGUCGGUG	
hsa_circ_0004458 (5'... 3')	CCCCAGAAAUCUCAGUAAUGGU 	95
hsa-miR-885-5p (3'... 5')	UCUCCGUCCCAUCACAUUACCU	
hsa_circ_0004458 (5'... 3')	CGAAUUUAGCAAACUAGUUGCAG 	86
hsa-miR-891b (3'... 5')	AGUUACUGAGUCCAUAACGU

Figure 4. Silencing hsa_circ_0004458 inhibited PTC cell proliferation by regulating miR-885-5p. **(A)** Dual-luciferase reporter assay was performed to measure the relative luciferase intensities of 10 miRNAs. **(B)** miR-885-5p binding site in circ_0004458 3'-UTR predicted with software. **(C)** K1 and TPC-1 cells were co-transfected with wild (circ_0004458-3'-UTR-WT) or mutant (circ_0004458-3'-UTR-Mut) circ_0004458 3'-UTRs, miR-control, or miR-885-5p, respectively. The luciferase intensity was measured by dual-luciferase reporter assay (* $P<0.05$). **(D)** The relative expression level of miR-885-5p was analyzed by qRT-PCR assay in K1 and TPC-1 cells transfected with si-circ_0004458 or si-control (* $P<0.05$). **(E, F)** K1 and TPC-1 cells were transfected with si-circ_0004458 or anti-miR-885-5p, respectively. Cell viability was measured by MTT assay (* $P<0.05$ vs. si-control+scramble group; # $P<0.05$ vs. si-circ_0004458+scramble group).

Silencing of RAC1 suppressed PTC cell proliferation

In our study, we found that silencing of hsa_circ_0004458 significantly downregulated RAC1 expression, and anti-miR-885-5p then prevented downregulation of RAC1 expression mediated

by hsa_circ_0004458 knockdown ($P<0.05$, Figure 6A). Moreover, we further explored the role of RAC1 on PTC cell proliferation. K1 and TPC-1 cells were transfected with si-control or si-RAC1, respectively. The results proved that silence of RAC1 significantly inhibited K1 and TPC-1 cell proliferation ($P<0.05$, Figure 6B,

Figure 5. RAC1 is a direct target of miR-885-5p. **(A)** The putative complementary site of miR-885-5p and RAC1 was obtained based on TargetScan prediction. **(B)** The sequences of wild-type RAC1 (RAC1-3'-UTR-WT), mutant-type RAC1 (RAC1-3'-UTR-Mut), and miR-885-5p are shown. **(C)** TPC-1 cells were transfected with scramble or miR-885-5ps, RAC1-3'-UTR-WT or RAC1-3'-UTR-Mut, respectively. The luciferase intensity was measured by dual-luciferase reporter assay (* $P < 0.05$).

6C); silence of RAC1 also significantly inhibited K1 and TPC-1 cell colony-forming ability ($P < 0.05$, Figure 6D). Therefore, our results demonstrated that hsa_circ_0004458 from the PSD3 gene promoted TPC tumorigenesis by inhibition of miR-885-5p and activation of RAC1.

Discussion

Although an increasing number of circRNAs have recently been discovered and identified, the biological effects of circRNAs are not well known. Research indicates that various circRNAs exert essential functions in the pathogenesis of cancer by acting as tumor suppressors or oncogenes [33]. In our study, we have gradually demonstrated the regulatory role of hsa_circ_0004458 and its effect on miRNAs sponges in PTC by functional and molecular experiments. The results agree with our hypothesis that hsa_circ_0004458 upregulates RAC1 and promotes PTC progression by sponging miRNAs. This is the first report showing the expression, function, and mechanism of hsa_circ_0004458 in PTC.

A large number of studies have shown that circRNA serves as miRNA sponge and leads to a loss of miRNA function which further promotes the expression of its target genes [29,34]. For example, ciRS-7 eliminates the antitumor effect of miR-7 on gastric cancer through the PTEN/PI3K/AKT signaling pathway [35]; circ-ABC10 accelerates breast cancer progression

by sponging miR-1271 [36]; circ-LARP4 inhibits gastric cancer progression by sponging miR-424-5p and upregulating LATS1 level [37]; and hsa_circ_0012673 facilitates lung adenocarcinoma proliferation by serving as a sponge of miR-22 [38]. In our study, we screened 10 miRNAs which have binding sites with hsa_circ_0004458 sequence by bioinformatics analysis, and finally confirmed that miR-885-5p was able to bind with hsa_circ_0004458 by dual-luciferase reporter assay. In addition, we have also demonstrated that silencing hsa_circ_0004458 inhibited PTC cell proliferation by targeting miR-885-5p.

RAC1 protein, an important intracellular signaling molecule, belongs to the Rho family small G protein (Rho GTPase), which plays key roles in cell growth, skeleton formation, migration, invasion, and activation of various protein kinases [39,40]. The present study confirmed that Rho, especially RAC1 protein, plays important roles in malignant biological properties such as tumor migration and invasion [41]. We also demonstrated that RAC1 was a direct target of miR-885-5p and that silencing hsa_circ_0004458 significantly downregulated RAC1 expression by miR-885-5p, while silencing of RAC1 suppressed PTC cell proliferation.

Conclusions

Our data indicate hsa_circ_0004458 was significantly upregulated in PTC tissues and cell lines. Silencing of hsa_circ_0004458

Figure 6. Silencing RAC1 suppressed PTC cell proliferation. **(A)** The relative expression level of RAC1 was analyzed by qRT-PCR assay in K1 and TPC-1 cells transfected with si-circ_0004458 or anti-miR-885-5p, respectively (* $P<0.05$). **(B, C)** Cell viability was detected by MTT assay in K1 and TPC-1 cells transfected with si-control or si-RAC1, respectively (* $P<0.05$). **(D)** The cell clonogenesis ability was accessed by clone formation assay in K1 and TPC-1 cells transfected with si-control or si-RAC1, respectively (* $P<0.05$). **(E)** The schematic representation of circ_0004458/miR-885-5p/RAC1 in PTC cells.

suppressed the growth of PTC and induced PTC cell cycle arrest and apoptosis. Additionally, mechanistic analysis showed that hsa_circ_0004458 regulated RAC1 by specifically sponging miR-885-5p. Our study discloses a novel

hsa_circ_0004458-miR-885-5p-RAC1 signaling pathway that is involved in PTC cell progression. Therefore, hsa_circ_0004458 may serve as a therapeutic target for PTC treatment.

Conflict of interest

None.

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