



Original Article

Circ-LDLRAD3/miR-655-3p/MAPK1 axis enhances cell migration and invasion in papillary thyroid carcinoma

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Article Info

Abstract



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Papillary thyroid carcinoma (PTC) is a prevalent histological subtype of thyroid cancer, whose occurrence and development may be related to circRNA dysregulation. This research proposed to unravel circ-LDLRAD3-related mechanisms in PTC. First, circ-LDLRAD3, miR-655-3p and MAPK1 levels in PTC were quantitatively measured. Then, plasmid vectors or oligonucleotides that interfere with circ-LDLRAD3, miR-655-3p, or MAPK1 were transfected into PTC cells, followed by the analysis of proliferation, apoptosis, migration, and invasion. Finally, the targeted binding sites between miR-655-3p and circ-LDLRAD3 or MAPK1 were predicted by starBase and experimentally verified. Statistically, PTC samples expressed high circ-LDLRAD3 and MAPK1 and low miR-655-3p. Knocking down circ-LDLRAD3 or enhancing miR-655-3p hindered PTC cell proliferation, migration, and invasion, and forced apoptosis. circ-LDLRAD3 bound to miR-655-3p to affect MAPK1 expression. Elevating MAPK1 rescued circ-LDLRAD3 knockdown-allowed obstruction of PTC cell growth. In conclusion, circ-LDLRAD3 stimulates PTC development by releasing miR-655-3p-targeted MAPK1.

Keywords: Circ-LDLRAD3, miR-655-3p, Papillary thyroid carcinoma

1. Introduction

Thyroid cancer (TC) is a malignant tumor originating from thyroid follicular epithelium or parafollicular epithelial cells, and its incidence is on the rise worldwide, which is almost entirely due to papillary thyroid carcinoma (PTC) [1]. PTC is characterized by follicular cell proliferation with distinct nuclear features such as nuclear grooves, pseudo inclusions, and ground-glass appearance [2]. Surgical treatment of TC has been evolving to avoid overtreatment of patients by employing an appropriate risk-based approach as a viable alternative to immediate surgical treatment [3]. The majority of patients diagnosed with PTC have excellent long-term survival, but tumor recurrence may occur in some cases [4, 5]. To effectively treat patients with PTC, a profound insight into PTC pathogenesis is clinically required.

Research progress has been made in targeting circRNAs in the occurrence and progression of PTC [6] and circRNAs have become a novel potential strategy to treat TC [7]. For instance, artificial knockdown of oncogenic circRNAs, such as hsa_circ_0058124 [8], Hsa_circRNA_102002 [9], and circTP53 [10], has tumor-preventive effects on PTC cells. Regarding circ-LDLRAD3, it has

been previously discussed to have tumor-promoting properties in pancreatic cancer [11], non-small cell lung cancer (NSCLC) [12], and gastric cancer (GC) [13]. However, few are reported on the role of circ-LDLRAD3 in PTC.

Many circRNAs exert biological functions by acting as miRNA or protein decoys, by mediating protein function, or by self-translation [14]. Dysregulated miRNAs are closely associated with thyroid dysfunction and oncogenicity leading to PTC [15], and miRNAs are of significance as valuable markers in the diagnosis, therapy, and prognosis of PTC patients [16, 17]. In addition to that, miRNAs with anti-tumor potentials are of interest in the management of PTC progression, including but not limited to miR-144-5p [18], miR-148a [19], and miR-506-3p [20]. Previous reports have mentioned and validated the functional actions of miR-655-3p in human tumors, such as ovarian cancer [21], glioma [22], and hepatocellular carcinoma (HCC) [23].

This study confirmed the interlink between circ-LDLRAD3 and miR-655-3p, and therefore, supposed that circ-LDLRAD3 mediates PTC cell progression by miR-655-3p mediating posttranscriptional gene silencing of MAPK1, a member of MAPK signaling pathway involved

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in tumor pathogenesis [24, 25]. In a word, this work hopes to develop a fresh ceRNA network in PTC and strengthen the feasibility of the ceRNA network in cancer therapy.

2. Materials and methods

2.1. Clinical sample collection

Paired PTC tissues and adjacent normal tissues ($n = 40$) were collected in Affiliated Sinopharm Dongfeng General Hospital, Hubei University of Medicine, and all samples were stored at -80°C immediately to avoid RNA loss and confirmed by a pathologist. Patients who had received any treatment (chemotherapy, radiotherapy, or others) before surgery and those diagnosed with other cancers and serious systemic infectious diseases were excluded. This study was approved by the ethics committee of Affiliated Sinopharm Dongfeng General Hospital, Hubei University of Medicine, and all patients signed informed consent.

2.2. Cell culture

Normal thyroid cells (Nthy-ori3-1) and PTC cell lines (BCPAP, TPC-1, and SW579) were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and cultured in RPMI-1640 medium containing 10% fetal bovine serum and 1% penicillin-streptomycin solution at 37°C , 5% CO_2 .

2.3. Cell transfection

Small interfering RNA (siRNA) of circ-LDLRAD3 (si-LDLRAD3) and its negative control (si-NC), circ-LDLRAD3 overexpression vector (oe-LDLRAD3) and its negative control (oe-NC), miR-655-3p mimic, mimic NC, miR-655-3p inhibitor, inhibitor NC, si-LDLRAD3 + oe-MAPK1 and si-LDLRAD3 + oe-NC were transfected into TPC-1 cells. The above plasmid vectors or oligonucleotides were purchased from GenePharma (Shanghai, China).

2.4. CCK-8 assay

TPC-1 cells were seeded into 96-well plates (2×10^3 cells/well) for 72 h, treated with 10 μl CCK-8 reagent (Sigma) for 2 h, and analyzed on a microplate reader (Thermo Fisher, USA) to read the absorbance at 450 nm.

2.5. Colony formation assay

TPC-1 cells were seeded into 6-well plates (200 cells/well) and cultured in a 37°C incubator supplemented with 5% CO_2 for 2 weeks. Then, visible cells were fixed with

methanol and stained with 0.1% crystal violet (Sigma) to take images under a light microscope (Olympus, Tokyo, Japan).

2.6. Flow cytometry

Cells were harvested after 48 h of culture and made into a suspension with a binding buffer (1×10^6 cells/ml). Next, staining with Annexin V-FITC (Beyotime) and propidium iodide (Beyotime) was performed and data were collected on a FACS Calibur flow cytometer (BD Biosciences).

2.7. Transwell assay

Diluted Matrigel solution (Sigma) was solidified on the upper chamber to analyze cell invasion ability, and the uncoated upper chamber was applied to analyze cell migration ability. TPC-1 cells suspended in 100 μL of serum-free medium were seeded into the upper chamber, and a total of 500 μL of RPMI-1640 medium supplemented with 10% FBS was added to the lower chamber as a chemotactic agent. After 24 h, invading or migrating cells were fixed using methanol, stained using crystal violet (Sangon Biotech), and counted under a light microscope (Olympus).

2.8. RT-qPCR

Total RNA was extracted from PTC tissues and cell lines using Trizol reagent (Thermo Fisher). For circ-LDLRAD3 or MAPK1, reverse transcription was performed using the TaqMan Reverse Transcription Kit (Thermo Fisher) applied for reverse transcription, while the miRNA first-strand cDNA synthesis kit (TIANGEN, Beijing, China) was for miR-655-3p. PCR was carried out with the SYBR Premix Ex TaqIM II kit (Takara). Relative expression was analyzed using the $2^{-\Delta\Delta\text{Ct}}$ method and normalized to U6 (for miR-655-3p) or GAPDH (for circ-LDLRAD3 and MAPK1). The primer sequences are shown in Table 1.

2.9. Immunoblot analysis

Tissue and cells were lysed using RIPA lysis buffer (Beyotime) and determined for concentration using a BCA assay kit (Bio-Rad). Proteins (30 μg /lane) were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes (Bio-Rad), followed by blocking with 5% nonfat milk for 1 h and combination with primary antibodies MAPK1 (1:1000, Cell Signaling Technology) or GAPDH (1:1000, Abcam) overnight at 4°C . After that,

Table 1. Primer sequences in PCR.

Genes	Primer sequences
circ-LDLRAD3	Forward: 5'-CTTGCTGGACCAGAGAAC-3' Reverse: 5'-CATGAGGTTGTTCCGCTTC-3'
miR-655-3p	Forward: 5'-CCGCGATAATACATGGTTAACCTC-3' Reverse: 5'-GCAGGGTCCGAGGTATTC-3'
MAPK1	Forward: 5'-CGCGCTACTAACCCTCTCC-3' Reverse: 5'-TGCCAGTACGTCTGATGCTC-3'
U6	Forward: 5'-CTCGCTTCGGCAGCACA-3' Reverse: 5'-AACGCTTCACGAATTTGCGT-3'
GAPDH	Forward: 5'-CACCCACTCCTCCACCTTTG-3' Reverse: 5'-CCACCACCCTGTTGCTGTAG-3'

Note: circ-LDLRAD3, circular RNA LDLRAD3; miR-655-3p, microRNA-655-3p; MAPK1, Mitogen Activated Protein Kinase1; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase.

membranes were co-incubated with horseradish peroxidase-conjugated secondary antibody (Abcam) for 1 h before band development using an ECL kit (Beyotime).

2.10. Dual-luciferase reporter gene assay

The wild-type sequence of circ-LDLRAD3 or MAPK1 3'UTR containing the miR-655-3p seed site was cloned into pmirGLO to form the corresponding luciferase reporter vectors (circ-LDLRAD3-WT and MAPK1-WT). Mutant circ-LDLRAD3-MUT and MAPK1-MUT were obtained by mutating the seed site. The reporter gene plasmid and miR-655-3p mimic or mimic NC were co-transfected into TPC-1 cells by Lipofectamine 2000 (Thermo Fisher) and cells were assayed 48 h later for luciferase activity.

2.11. Statistical analysis

Data processing was finished by SPSS 21.0, and measurement data were reported in the form of mean \pm standard deviation. Data in normal distribution were subjected to two-group comparison by Student's t-test and multiple-group comparison by one-way ANOVA and Tukey's test. Correlation analysis in clinical samples was done with Pearson. $P < 0.05$ indicates that the difference is statistically significant.

3. Results

3.1. Circ-LDLRAD3 is upregulated in PTC

RT-qPCR detection supported that circ-LDLRAD3 levels were high in PTC tissues (Figure 1A). By analyzing patients' clinical information, it was found that circ-LDLRAD3 expression was associated with tumor size, TNM stage, and lymph node metastasis (Table 2). Circ-LDLRAD3 high expression was also examined in PTC cell lines (BCPAP, TPC-1, and SW579), with the most up-regulation in TPC-1 cells (Figure 1B), so TPC-1 cells were selected for subsequent experiments.

3.2. Circ-LDLRAD3 knockdown inhibits the malignant activities of TPC-1 cells

Plasmids targeting circ-LDLRAD3 were transfected

into TPC-1 cells, with si-LDLRAD3 suppressing while oe-LDLRAD3 elevating circ-LDLRAD3 expression levels (Figure 2A). Cell proliferation assays observed that TPC-1 cell proliferation was attenuated after the suppression of circ-LDLRAD3 (Figures 2B, C). Apoptosis analysis data obtained from flow cytometry indicated that silencing circ-LDLRAD3 forced TPC-1 cell apoptotic activities (Figure 2D). Transwell assays revealed that loss of circ-LDLRAD3 obstructed TPC-1 cells from migrating and invading (Figure 2E). However, circ-LDLRAD3 up-regulation led to the opposite results (Figures 2B-E).

3.3. miR-655-3p is a downstream molecule of circ-LDLRAD3

Through the analysis of the bioinformatics website starBase, a targeted binding site was found between circ-LDLRAD3 and miR-655-3p (Figure 3A). Then, a dual-luciferase reporter gene assay observed that the luciferase activity was weakened after co-transfection of LDLRAD3-WT with miR-655-3p mimic (Figure 3B). miR-655-3p expression was tested to be lower in PTC samples than normal samples and negatively correlated with circ-LDLRAD3 expression (Figures 3C, D). In TPC-1 cells, miR-655-3p levels were recovered after silencing circ-LDLRAD3, or further suppressed after overexpress-

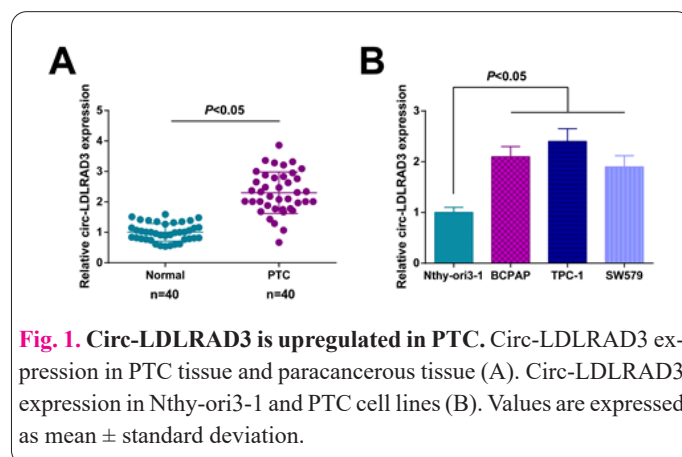


Fig. 1. Circ-LDLRAD3 is upregulated in PTC. Circ-LDLRAD3 expression in PTC tissue and paracancerous tissue (A). Circ-LDLRAD3 expression in Nthy-ori3-1 and PTC cell lines (B). Values are expressed as mean \pm standard deviation.

Table 2. Relationship between circ-LDLRAD3 expression and clinical features.

Characteristics	Number	circ-LDLRAD3		P
		High (n=20)	Low (n=20)	
Gender				0.751
Male	18	8	10	
Female	22	12	10	
Age (years)				1
<45	23	11	12	
≥ 45	17	9	8	
Tumor size (mm)				0.001
<10	28	9	19	
≥ 10	12	11	1	
TNM Stage				0.006
I+II	27	9	18	
III+IV	13	11	2	
Lymph node metastasis				0.003
No	24	7	17	
Yes	16	13	3	

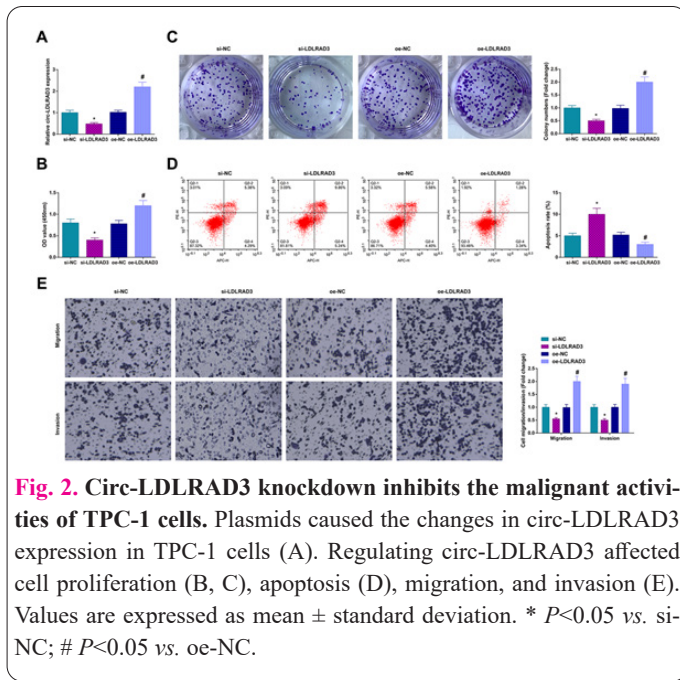


Fig. 2. Circ-LDLRAD3 knockdown inhibits the malignant activities of TPC-1 cells. Plasmids caused the changes in circ-LDLRAD3 expression in TPC-1 cells (A). Regulating circ-LDLRAD3 affected cell proliferation (B, C), apoptosis (D), migration, and invasion (E). Values are expressed as mean \pm standard deviation. * $P < 0.05$ vs. si-NC; # $P < 0.05$ vs. oe-NC.

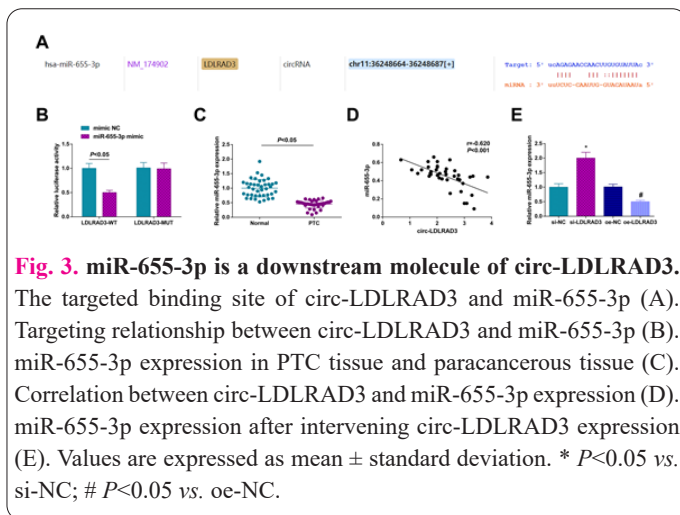


Fig. 3. miR-655-3p is a downstream molecule of circ-LDLRAD3. The targeted binding site of circ-LDLRAD3 and miR-655-3p (A). Targeting relationship between circ-LDLRAD3 and miR-655-3p (B). miR-655-3p expression in PTC tissue and paracancerous tissue (C). Correlation between circ-LDLRAD3 and miR-655-3p expression (D). miR-655-3p expression after intervening circ-LDLRAD3 expression (E). Values are expressed as mean \pm standard deviation. * $P < 0.05$ vs. si-NC; # $P < 0.05$ vs. oe-NC.

sing circ-LDLRAD3 (Figure 3E).

3.4. miR-655-3p blocks the growth of PTC cells

miR-655-3p oligonucleotides were treated in TPC-1 cells, contributing to miR-655-3p upregulation after miR-655-3p mimic treatment, and miR-655-3p downregulation after miR-655-3p inhibitor interference (Figure 4A). The cell experiments manifested that in response to miR-655-3p upregulation, TPC-1 cells were impaired in the aspects of proliferating, migrating, invading and anti-apoptotic activities. While miR-655-3p downregulation caused the opposite results (Figures 4B-E).

3.5. MAPK1 is a potential target gene of miR-655-3p

On the bioinformatics website starBase, miR-655-3p shared a targeted binding site with MAPK1 (Figure 5A). In TPC-1 cells, it could see the reduction of luciferase activity due to co-transfection of MAPK1-WT with miR-655-3p mimic (Figure 5B). PTC samples expressed high MAPK1 mRNA expression, which was negatively correlated with miR-655-3p expression (Figures 5C, D). Moreover, MAPK1 expression decreased in TPC-1 cells overexpressing miR-655-3p and increased in those lowly expressing miR-655-3p (Figure 5E).

3.6. MAPK1 overexpression makes a reversal of circ-LDLRAD3 knockdown-allowed growth inhibition of PTC cells

Finally, a co-transfection scheme was established, with oe-MAPK1 leading to MAPK1 expression recovery based on si-LDLRAD3 (Figure 6A). Then, cell activities were observed, discovering a reversal of circ-LDLRAD3 knockdown-allowed inhibitory effects on TPC-1 cell proliferation, anti-apoptosis, migration, and invasion (Figures 6B-E).

4. Discussion

PTC accounts for the majority of differentiated TC in clinics and is frequently diagnosed in TC populations. To

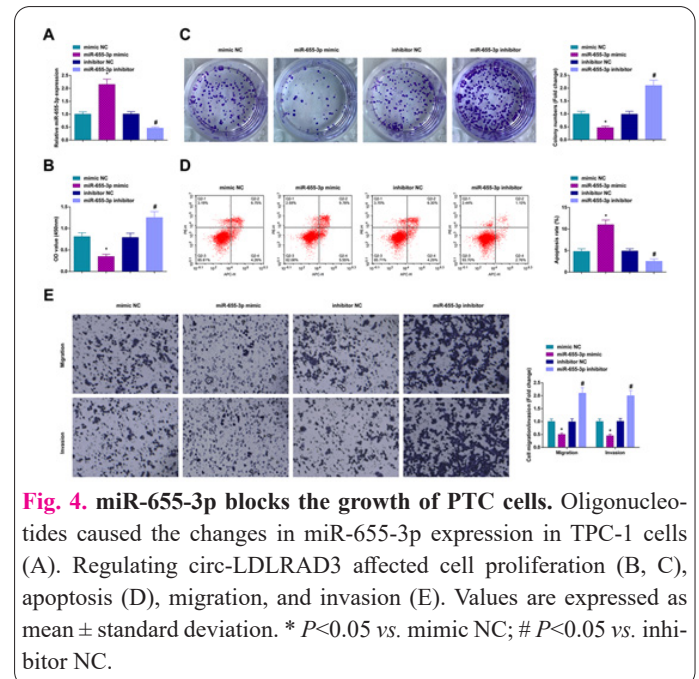


Fig. 4. miR-655-3p blocks the growth of PTC cells. Oligonucleotides caused the changes in miR-655-3p expression in TPC-1 cells (A). Regulating circ-LDLRAD3 affected cell proliferation (B, C), apoptosis (D), migration, and invasion (E). Values are expressed as mean \pm standard deviation. * $P < 0.05$ vs. mimic NC; # $P < 0.05$ vs. inhibitor NC.

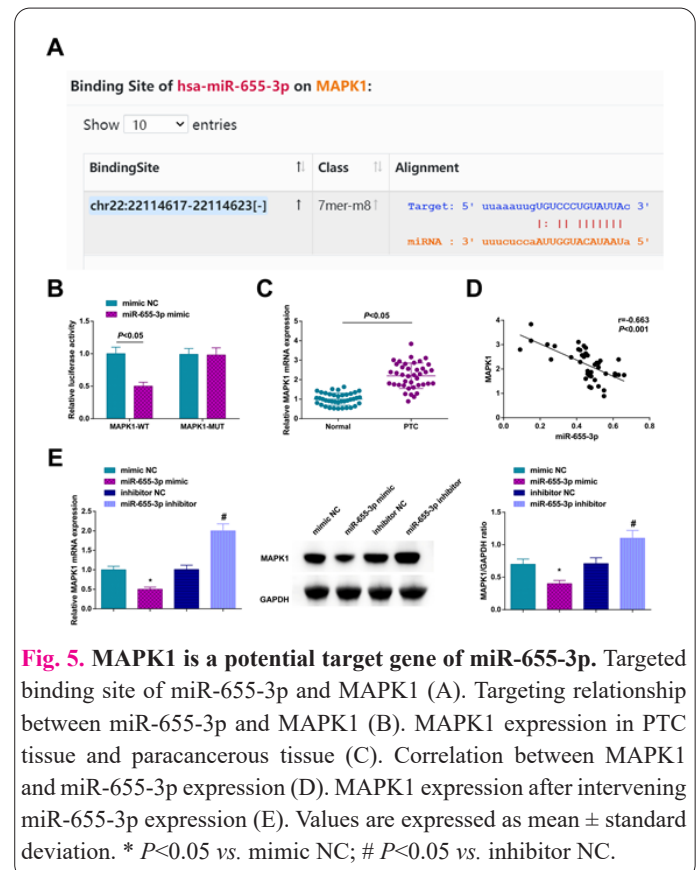
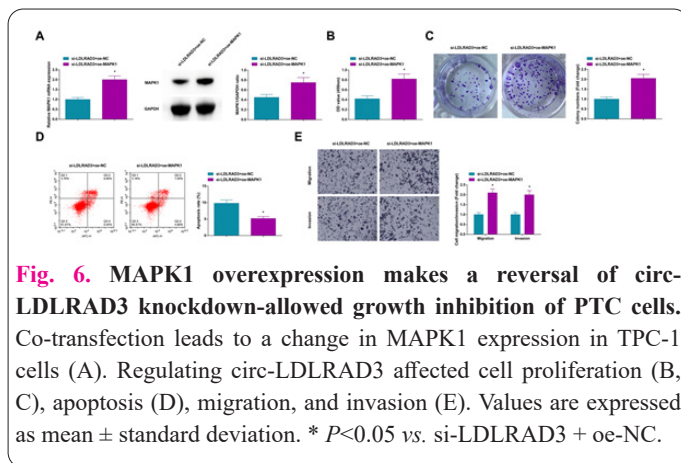


Fig. 5. MAPK1 is a potential target gene of miR-655-3p. Targeted binding site of miR-655-3p and MAPK1 (A). Targeting relationship between miR-655-3p and MAPK1 (B). MAPK1 expression in PTC tissue and paracancerous tissue (C). Correlation between MAPK1 and miR-655-3p expression (D). MAPK1 expression after intervening miR-655-3p expression (E). Values are expressed as mean \pm standard deviation. * $P < 0.05$ vs. mimic NC; # $P < 0.05$ vs. inhibitor NC.



overcome PTC malignancy, circRNA, miRNA, and mRNA formulate an interaction network at the molecular level that has been tested and expanded for decades. In a similar fashion, this study evaluated the construct mediated by circ-LDLRAD3, miR-655-3p, and MAPK1 in PTC, and concluded that circ-LDLRAD3 promoted PTC progression by reducing miR-655-3p-allowed regulation of MAPK1.

Erstwhile documents have paid great attention to circRNAs in cancer development and identified circ-LDLRAD3 as a tumor promoter. Yao *et al.* [11] have measured an increment in circ-LDLRAD3 expression in pancreatic cancer and further assessed the preventive effects of silenced circ-LDLRAD3 concerning malignant cell growth. Also, Yang *et al.* [26] have noticed a correlation between circ-LDLRAD3 expression with venous invasion and lymphatic metastasis in pancreatic cancer and further confers circ-LDLRAD3 with diagnostic values in this disease. In the course of NSCLC, circ-LDLRAD3 upregulation is correlated with TNM stage and lymphatic metastasis in patients, and circ-LDLRAD3 downregulation is effective in obstructing cell invasion and proliferation, and enhancing cell apoptotic activity [12]. In addition, circ-LDLRAD3 high expression has been observed and tested in GC, and circ-LDLRAD3 knockdown contributes to preventing GC cells from behaving aggressively [13]. Surprisingly, circ-LDLRAD3 overexpression is recognized in chemotherapy-resistant GC, and direct suppression of circ-LDLRAD3 prevents cellular survival and aggressiveness, thereby effectuating chemotherapy [27]. Here, circ-LDLRAD3 expression was maintained at a high level in PCa and indicative of tumor size, TNM stage, and lymph node metastasis. Later on, loss-of-function assays determined that suppressing circ-LDLRAD3 was of assistance to hindering PCa cell growth, while gain-of-function assays obtained the opposite results.

CircRNA-mediated silencing of miR-655-3p is inductive to HCC tumorigenesis, as manifested in proliferation and anti-apoptosis [28]. Consistently, overexpression of pro-tumor circRNA is causal for miR-655-3p deficiency by competitive absorption, thereby facilitating tumor growth and metastasis [29]. Direct elevation of miR-655-3p in ovarian cancer cells is suppressive for tumor development [30]. More extendedly, miR-655-3p is expressed lowly in HCC, and this decrease is associated with clinicopathological features and the survival of HCC patients [31]. This study ensured the crosstalk of circ-LDLRAD3 and miR-655-3p and functionally validated the tumor-suppressing

effect of overexpressed miR-655-3p, and the tumor-promoting effect of low-expressed miR-655-3p in PTC. At last, MAPK1 became the target topic of miR-655-3p in PTC, and MAPK1 overexpression led to the reversal of circ-LDLRAD3 silencing-induced effects on PTC cells. As a matter of fact, forced expression MAPK1 is noticed in NSCLC and can accelerate tumor progression. Furthermore, based on the co-regulation of circRNAs/long noncoding RNA and miRNAs, MAPK1 enhancement in TC cells aggravates tumor malignancy [32-35].

All in all, this research mechanistically illustrates that circ-LDLRAD3 enhances PTC development through enhancing MAPK1 expression by downregulating miR-655-3p. Anyway, the findings to a certain level enhance the academic understanding of molecule-contributed pathogenesis of PTC and renew a management clue for PTC patients. However, certain limitations are waiting for resolution, such as assessing the value of circ-LDLRAD3 in diagnosis and prognosis. Also, animal experiments are required to further validate and support the cell experimental results.

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Authors' contributions

ZB conducted the experiments and wrote the paper; PY analyzed and organized the data; ZL conceived, designed the study and revised the manuscript.

Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

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