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Has_circ_0008274 promotes cell proliferation and invasion involving AMPK/mTOR signaling pathway in papillary thyroid carcinoma

G.-K. ZHOU[1], G.-Y. ZHANG[2], Z.-N. YUAN[1], R. PEI[1], D.-M. LIU[1]

[1]Department of Head and Neck Surgery, Harbin Medical University Cancer Hospital, Harbin, Heilongjiang, China
[2]Department of Pharmacy, The First Hospital of Harbin, Harbin, Heilongjiang, China

Abstract. – OBJECTIVE: Circular RNAs (circRNAs) have been known as important regulators in tumorigenesis. Whether circRNAs are involved in papillary thyroid carcinoma (PTC) requires to be determined. In the present study, we aimed to investigate the expression and function of has_circ_0008274 in PTC.

PATIENTS AND METHODS: Tissue expression of has_circ_0008274 was evaluated in Gene Expression Omnibus datasets (GSE93522). Real-time PCR assays were used to detect the expression of has_circ_0008274 in human PTC tissues and cell lines. The correlation of has_circ_0008274 expression with clinicopathological factors was statistically analyzed. The MTT assay, colony formation assay, transwell assays were performed to analyze and compare cell viability and invasion. Western blot analysis was used to quantify the expression of AMPK/mTOR signaling pathway proteins.

RESULTS: We found that has_circ_0008274 was significantly upregulated in PTC tissues, and the level of has_circ_0008274 was negatively associated with TNM stage and lymph node metastasis. Loss-of-function assay indicated that knockdown of has_circ_0008274 suppressed PTC cells proliferation and invasion *in vitro*. Mechanistically, has_circ_0008274 could inhibit the activation of AMPK/mTOR signaling pathway, which was demonstrated by measuring the expression levels of p-AMPK and p-mTOR.

CONCLUSIONS: These results demonstrate that increased has_circ_0008274 expression modulates has_circ_0008274 to enhance PTC cells proliferation and invasion. Has_circ_0008274/ AMPK/mTOR axis may be a novel therapeutic candidate target in PTC treatment.

Key Words:
Has_circ_0008274, Papillary thyroid carcinoma, Proliferation, Invasion, AMPK/mTOR signaling pathway.

Introduction

Thyroid cancer is the most common endocrine malignancy, and has had a steadily increasing incidence over the last several decades[1,2]. Of the various histologic subtypes, papillary thyroid carcinoma (PTC) accounts for 85 to 90% of all thyroid carcinoma cases[3]. Several factors[4] are implicated in the development of PTC including genetic alterations, growth factors and radiation. Currently, most PTC can be managed successfully with a combination of radioiodine and levothyroxine after complete thyroidectomy[5]. However, in the case of aggressive PTC, around 10% of the patients manifest recurrences or distant metastases within 10 years[6]. Up to date, the precise molecular mechanisms underlying the pathogenesis of thyroid carcinogenesis remain unclear. Therefore, further research to understand the underlying mechanisms of tumor progression is needed before more targeted therapies can be realized. Circular RNAs (circRNAs) are closed-loop RNAs produced through end-to-end joining of RNA transcription fragments during transcription[7]. The recent development of high-throughput RNA sequencing (RNA-Seq) has led to the direct detection of more circular RNAs in tumor celles[8,9]. Compared with traditional linear RNA, such as miRNA, circRNAs are structured as a covalently closed loop without 5' to 3' polarity or polyadenylated tail[10]. Evidence indicate that circNRAs are important players in normal cellular differentiation and tissue homeostasis as well as in disease development[11,12]. Researchers have indicated that circRNAs are involved in carcinogenesis of several types of cancers, such

Corresponding Authors: Da-Ming Liu, MD; e-mail: lii77814999@163.com
Rong Pei, MD; e-mail: ring88163@yeah.net

as lung cancer[13], osteosarcoma[14], cervical cancer[15] and esophageal squamous cell carcinoma[16]. In PTC, only a few circRNAs have been identified. The expression pattern and biological function of circRNAs in PTC remains largely unclear. Has_circ_0008274, a newly identified circRNA, has been reported to be upregulated in PTC by previous microarray and bioinformatics analysis[17]. The gene of has_circ_0008274 is located at chr11: 96485180-96489456, and its associated-gene symbol are UGGT2. The expression pattern and biological of circ_0008274 were rarely reported. In this study, we chose has_circ_0008274 as our target circRNA and explored its expression and biological function in PTC. We confirmed that has_circ_0008274 expression was significantly upregulated in both PTC tissues and cell lines and associated with advanced clinical progression. Loss of function experiments indicated that knockdown of has_circ_0008274 could suppressed PTC cells proliferation and invasion by modulating the AMPK/mTOR signaling pathway which was an important signaling pathway involved in progression and development of PTC[18].

Patients and Methods

Human PTC Tissue Samples

Primary PTC tissues and corresponding non-cancerous normal tissues were collected from the surgical specimen archives of Harbin Medical University Cancer Hospital, between April 2012 and March 2014. The clinical characteristics of the patients were summarized in Table I. None of the enrolled patients received radiotherapy or chemotherapy prior to surgery. These tissues were immediately frozen in liquid nitrogen after surgical resection and stored in liquid nitrogen until use. All the patients enrolled in this study had written the informed consent before surgery. The study was approved by the Ethics Committee of the Harbin Medical University Cancer Hospital.

Cell Lines and Cell Culture

The human thyroid follicular epithelial cell line Nthy-ori3-1 and human thyroid cancer cell lines (TPC-1, 8505C and SW1736) were purchased from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Xuhui, Shanghai, China). The cell lines were all cultured in Dulbecco's Modified Eagle's Medium (DMEM, Meilun Biotechnology, Dalian, China) containing 10% fetal bovine serum (FBS, EverGreen, Hangzhou, Zhejiang, China), 100 U/ml penicillin (Yeasen, Pudong, Shanghai, China), and 100 μg/ml streptomycin (Yeasen, Pudong, Shanghai, China). All cells were maintained in a humidified incubator with 5% CO_2 at 37°C.

Cell Transfection

Small interfering RNAs against has_circ_0008274 (si-circ-0008274-1 and si-circ-0008274-2) and negative control siRNAs were synthesized by GenePharma (Pudong, Shanghai, China). The siRNAs transfection was carried out using RNAiMax (Invitrogen, Carlsbad, CA, USA), following the manufacturer's protocols. In brief, TPC-1 or SW1736 cells (2×10^5 cells per well) were seeded into 6-well plates (NEST, Wuxi, Jiangsu, China) to obtain 60-70% confluence at the time of transfection. Subsequently, the mixture of 5 μl siRNA duplex (20 μM) and 7.5 μl RNAiMax regents were added into each well. After 24 h incubation, the medium was changed with fresh medium and the cells were used for further analyzed.

RNA Extraction and Real-Time Quantitative RT-PCR (qRT-PCR)

Total RNAs from PTC tissues and cell lines were isolated using TRIzol reagents (Leagene Biotechnology, Beijing, China) according to manufacturer's instructions. Then, 1 μg of total RNA was reverse transcribed to cDNA using ABScript II cDNA First Strand Synthesis Kit (ABclonal, Wuhan, China). Real-time PCR analyses were conducted by TransStart Tip Green qPCR SuperMix Kit (Transgen Biotech, Haidian, Beijing, China) and performed using the ABI PRISM 7000 Fluorescent Quantitative PCR System (Applied Biosystems, Foster City, CA, USA). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an endogenous control. The mRNA expression levels were measured as relative fold changes based on $2^{-\Delta\Delta Ct}$ method. The PCR primers for has_circ_0008274 or GAPDH were as follows: has_circ_0008274 sense, 5'-TGGGTGGAGTATGATGCTGA-3' and reverse, 5'-CACACCAGGTTTCACACCAC-3'; GAPDH sense, 5'-GTCAACGGATTTGGTCTGTATT-3' and reverse, 5'-AGTCTTCTGGGTGGCAGTGAT-3'.

Western Blot Analysis

Cells were lysed in Radio Immunoprecipitation Assay (RIPA) protein extraction reagents (Solarbio, Pudong, Beijing, China) supplemented

with protease inhibitor cocktail (Beyotime, Pudong, Shanghai, China). Cell extracts were boiled in loading buffer (Beyotime, Pudong, Shanghai, China) and equal amount of cell extracts were separated on 8-15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels. The subjects were then transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad, Hercules, CA, USA) followed by 3% BSA (Beyotime, Pudong, Shanghai, China) blocking. The primary antibodies-anti-AMPK (5832, Abcam, Cambridge, MA, USA), anti-p-AMPK (BM4718, BOSTER, Wuhan, Hubei, China), anti-mTOR (20657-1-AP, Proteintech, Wuhan, Hubei, China), anti-p-mTOR (5536, Cell Signaling Technology, Pudong, Shanghai, China) and anti-GAPDH (ab153802, Abcam, Cambridge, MA, USA) were diluted at a ratio of 1:1000 according to the protocols and incubated overnight at 4°C. The membranes were then incubated with secondary antibodies for 1 h at room temperature. Subsequently, enhanced chemiluminescence (Beyotime, Pudong, Shanghai, China) was used for visualization and Bio-Rad Quantity One system (Hercules, CA, USA) was applied for further analysis. Then, quantitative analysis was carried out by ImageJ software (NIH, Bethesda, MD, USA).

Cell Proliferation Assay

PTC cells' changes of proliferative capacity were measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay kit (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions. In short, the equal number of cells (at a density of 1×10^3 cells per well) were plated in triplicate in 96-well culture dish (NEST, Wuxi, China) and cultured in 10% fetal bovine serum (FBS) growth medium for 24 h. Afterwards, 20 µl MTT solution (0.5 mg/ml) were added into each well and the cells were continued to culture for another 4 h. Subsequently, the reaction was stopped by lysing the cells with 200 µl of dimethylsulfoxide (DMSO; Beyotime, Pudong, Shanghai, China) for 15 min. The plates were then measured at a wavelength of 490 nm by a microplate Reader (Biotek, Winooski, VT, USA).

Colony Formation Assay

Cells (500 cells/wells) were exposed to the indicated treatments accordingly and were seeded in 6-well plates (NEST, Wuxi, Jiangsu, China) supplemented with medium containing 10% FBS and antibiotics. After incubating for 14 days, cell colonies were fixed with 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO, USA) and stained with violet 0.1% crystal violet (Qianchen Biotech, Pudong, Shanghai, China) in phosphate-buffered saline for 15 min. Visible colonies were manually counted. Experiments were performed in triplicate.

Transwell Assay

For transwell invasion assays, 200 µl (2.5×10^5 cells) PTC cells were seeded onto the top chamber with Matrigel-coated membrane (Millipore, Billerica, MA, USA), and incubated in fetal bovine serum (FBS)-free medium with 5% CO_2 at 37°C. Afterwards, the lower chambers were filled with medium plus 10% FBS (EverGreen, Hangzhou, Zhejiang, China) which served as a chemo-attractant. After 24 h incubation at 37°C, the non-invasive cells on upper side of the membrane were removed by cotton wool. The cells that attached to the lower surface were stained with fixed in 4% formaldehyde (Sigma-Aldrich, St. Louis, MO, USA) and stained with violet 0.1% crystal violet (Qianchen Biotech, Pudong, Shanghai, China). The number of invaded cells was counted using an inverted microscope (Olympus, Tokyo, Japan).

Statistical Analysis

Statistical analysis was performed using SPSS 19.0 software (IBM Corp., IBM SPSS Statistics for Windows, Armonk, NY, USA) and GraphPad Prism 5.02 (GraphPad, La Jolla, CA, USA). Data are expressed as mean ± standard error of the mean (SEM). Comparisons between two groups were completed by Student's *t*-test, and comparisons among multi-groups were performed by One-way ANOVA. Tukey's post-hoc test was used to validate the ANOVA for comparing measurement data between groups. Association between has_circ_0008274 levels and clinicopathological factors was also analyzed by x^2-test. In all cases, $p < 0.05$ was considered statistically significant.

Results

Has_circ_0008274 was Highly Expressed in PTC Tissues and Cell Lines

To identify circRNAs that might play a role in the development and progression of PTC, we firstly downloaded microarray dataset GSE93522 from Gene Expression Omnibus (GEO) database containing six PTC tissues and six corre-

sponding normal tissues. Subsequently, bioinformatics analysis was carried out to evaluate the differentially expressed circRNAs in PTC. As the data presented in Figure 1A and B, among these dysregulated circRNAs, we found that a circRNAs termed has_circ_0008274 was significantly up-regulated in PTC tissues compared with matched normal tissues in the microarray datasets. To confirm the bioinformatics analysis results, we further conducted qRT-PCR assays to assess the expression levels of has_circ_0008274 in 142 paired PTC tissues. The data demonstrated has_circ_0008274 expression was indeed increased in PTC tissues compared with the corresponding normal tissues (Figure 1C). In addition, qRT-PCR analysis also found that has_circ_0008274 expression levels were also clearly upregulated in the four PTC cell lines (Figure 1D). Furthermore, in order to explore the clinical significance of has_circ_0008274 in PTC patient, using the median has_circ_0008274 expression in all PTC patients as a cutoff, the patients were divided into a high- has_circ_0008274 group and a has_circ_0008274-expression group. As shown in Table I, we observed that high circ_0008274 expression was significantly associated with

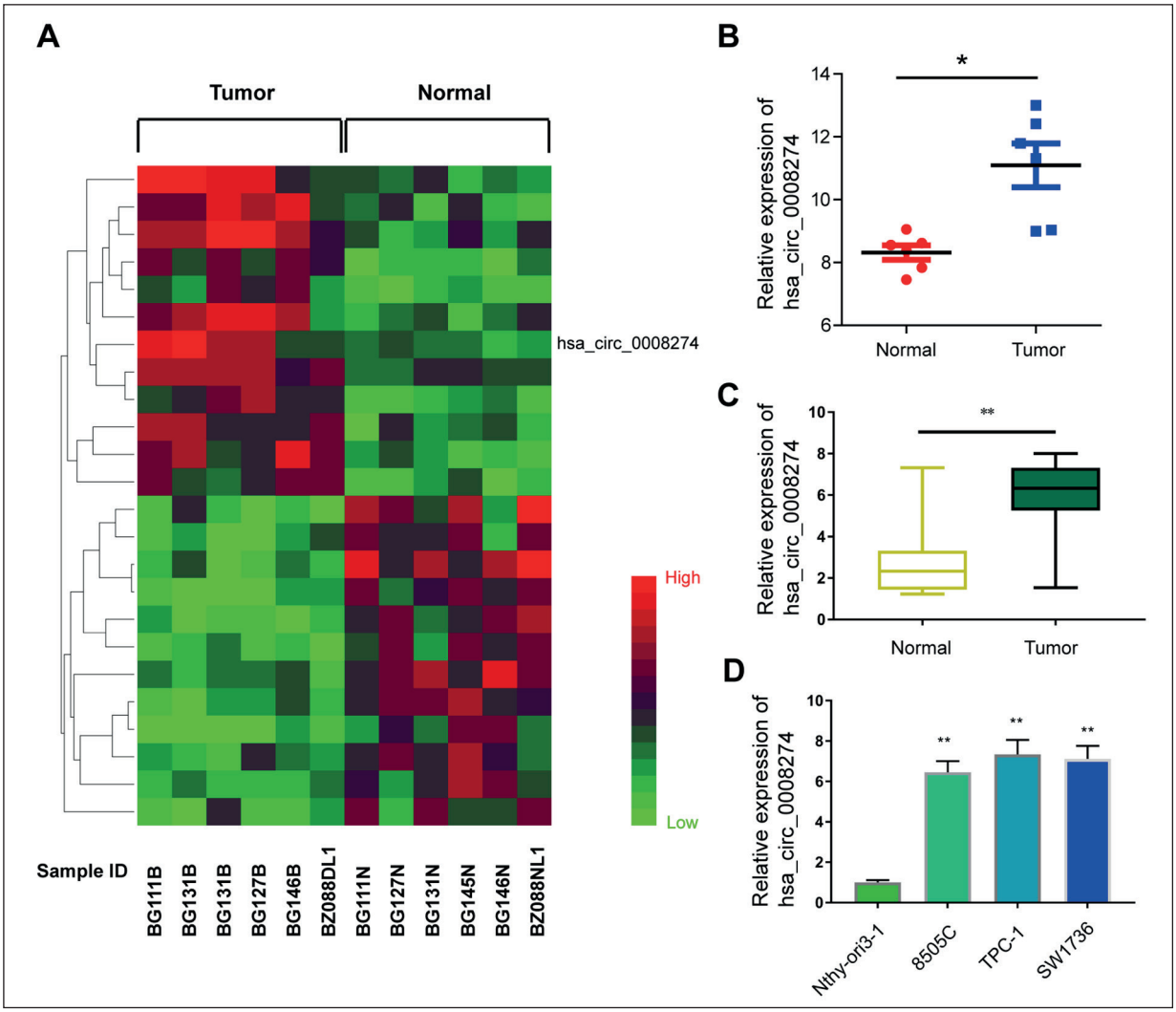

Figure 1. The has_circ_0008274 profiling and expressing levels in thyroid carcinoma tissues and cell lines. ***(A)*** Heat map of the dysregulated circRNAs expression in thyroid carcinoma and normal tissue samples was analyzed using the GEO datasets. ***(B)*** The relative expression levels of has_circ_0008274 were analyzed using microarray dataset GSE93522. ***(C)*** The relative expression levels of has_circ_0008274 were detected by qRT-PCR assays in 142-paired thyroid carcinoma and normal tissue samples. ***(D)*** The relative expression levels of has_circ_0008274 in Nthy-ori3-1, 8505C, TPC-1 and SW1736 via qRT-PCR assays. *$p < 0.05$, **$p < 0.01$.

TNM stage (p = 0.015) and lymph node metastasis (p = 0.004). However, the expression levels of has_circ_0008274 were not significantly correlated with age, gender, extra thyroidal extension, primary tumor and tumor size (p > 0.05). Taken together, these results revealed that has_circ_0008274 was highly expressed in PTC and involved in the progression.

Knockdown of has_circ_0008274 Inhibited PTC Cell Proliferation and Invasion

Given that has_circ_0008274 was significantly overexpressed in PTC tissues and cell lines and associated with advanced clinical stages in PTC patients, it is necessary to further investigate whether has_circ_0008274 depletion could affect the biological functions in PTC cells. To achieve this, specific siRNAs against has_circ_0008274 (si-circ-0008274-1 and si-circ-0008274-2) were utilized to silence the expression of has_circ_0008274 in TPC-1 and SW1736 PTC cells. As shown in Figure 2A, the siRNAs significantly impaired the expression of has_circ_0008274 in both the TPC-1 and SW1736 cells by qRT-PCR assay. Cell proliferation was then evaluated using MTT assays. The results showed that has_circ_0008274 knockdown markedly reduced the proliferation of both the TPC-1 and SW1736 cells (Figure 2B and C). Consistent with these data, colony-formation assays revealed that suppression of has_circ_0008274 significantly deceased the colony forming capabilities of both TPC-1 and SW1736 cells compared with the controls (Figure 2D). Furthermore, the effects of has_circ_0008274 on cell invasive abilities were also explored in TPC-1 and SW1736 cells. We found that in TPC-1 and SW1736 cells, has_circ_0008274 knockdown significantly reduced the invasive cell number compared to negative control siRNA transfected cells (Figure 2E). To sum up, these results suggested that has_circ_0008274 knockdown could efficiently repress proliferation and invasion of PTC cells *in vitro*, which implied that has_circ_0008274 played critical roles in regulating the development and progression of PTC.

Depression of has_circ_0008274 Suppressed the Activation of the AMPK/mTOR Signaling Pathway

To further ascertain the molecular mechanisms underlying the involvement of has_circ_0008274 in PTC, the AMPK/mTOR signaling pathway was investigated using Western blot assays. As presented in Figure 3A, depletion of has_circ_0008274 impaired AMPK signaling as revealed by the decreased phosphorylation of AMPK (p-AMPK), and promoted mTOR pathway as shown by the increase of mTOR phosphorylation (p-mTOR) in

Table I. Correlation between clinicopathological features and circ_0008274 expression in the PTC cases.

Variables	No. of cases	Circ_0008274 expression		*p*-value
		High	Low	
Gender				0.179
Male	71	31	40	
Female	71	39	32	
Age (years)				0.491
< 45	65	30	35	
≥ 45	77	40	37	
Extrathyroidal extension				0.467
Yes	55	25	30	
No	87	45	42	
Primary tumor				0.141
T1–2	91	41	50	
T3–4	50	29	21	
Tumor size (cm)				0.065
< 3	84	36	48	
≥ 3	58	34	24	
TNM stage				0.015
I-II	95	40	55	
III-IV	47	30	17	
Lymph node metastasis				0.004
Yes	41	28	13	
No	101	42	59	

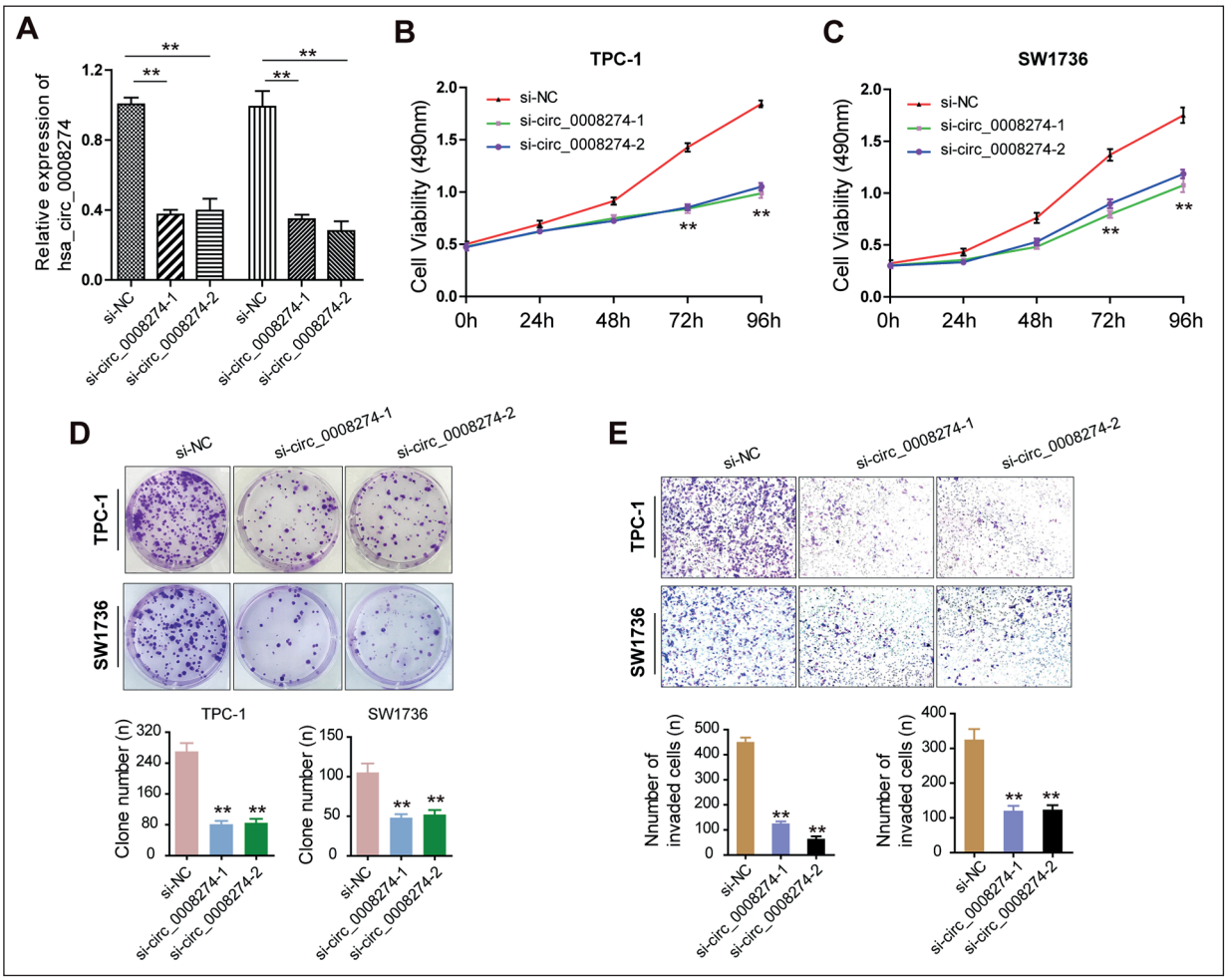

Figure 2. The effects of has_circ_0008274 knockdown on thyroid carcinoma cell proliferation and invasion. ***(A)*** Expression of has_circ_0008274 was knocked down by transfection of has_circ_0008274 siRNAs and verification by qRT-PCR. ***(B*** and ***C)*** Cell proliferation of thyroid carcinoma cells (TPC-1 and SW1736) after has_circ_0008274 siRNAs or negative control siRNAs transfection was evaluated by MTT assay. ***(D)*** Representative images of colony formation assay using TPC-1 and SW1736 cells, and quantification analysis of colony numbers. ***(E)*** Transwell invasion assays were conducted to assess the cell invasive abilities of thyroid carcinoma cells (TPC-1 and SW1736) after has_circ_0008274 siRNAs or negative control siRNAs transfection. *$p < 0.05$, **$p < 0.01$.

TPC-1 cells. Consistent with the results observed in TPC-1 cells, knockdown of has_circ_0008274 also markedly decreased the protein levels of both p-AMPK and p-mTOR in SW1736 cells (Figure 3B). Therefore, our data suggested that attenuation of has_circ_0008274 suppressed AMPK/mTOR signaling pathway in PTC.

Discussion

PTC is the most common endocrine tumor whose biological features and clinical outcomes vary considerably[19]. Up to date, the majority of the PTC patients have a good prognosis. However, cancer related mortality and cancer recurrence are noted in a portion of patients with PTC[20]. The potential mechanism underlying PTC and metastasis remains largely unclear. And there is an urgent requirement to look for novel diagnostic biomarkers and therapeutic targets for PTC treatment. Many studies have shown that the expression profile of ncRNAs, such as miRNAs, long noncoding RNAs and circRNAs, is different in cancer from normal, and also suggest that miRNA could contribute to the development of cancer[21-23]. Many miRNAs and long noncoding RNAs have been well investigated[24,25]. However, the potential function of circRNAs remains largely unknown. In addition, increasing evidence

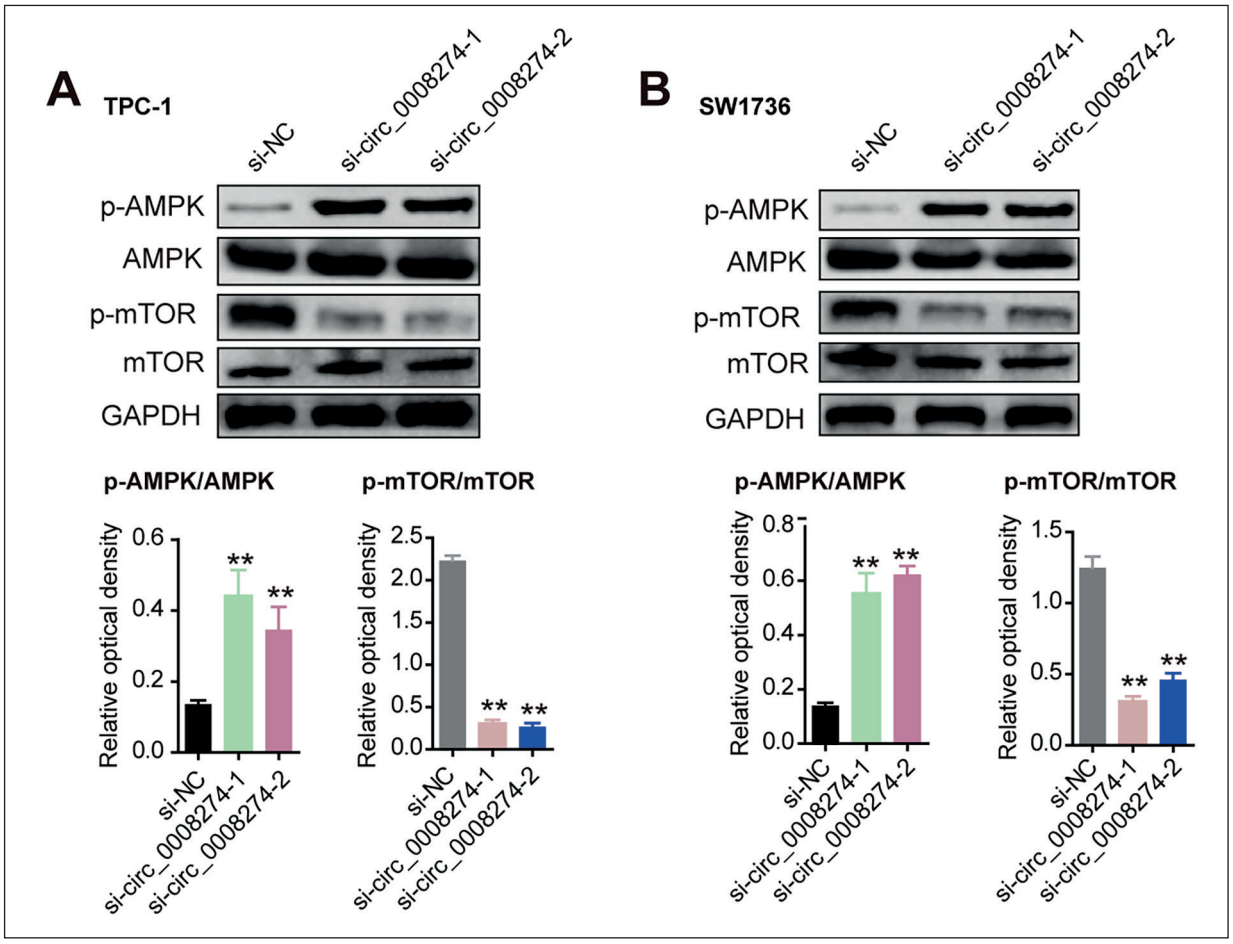

Figure 3. The effects of has_circ_0008274 inhibition on AMPK/mTOR signaling pathway in TPC-1 and SW1736. ***(A*** and ***B)*** The protein levels of AMPK, p-AMPK, p-mTOR and mTOR were assessed in TPC-1 or SW1736 cells following transfection with has_circ_0008274 siRNAs or negative control siRNAs using Western blot assays. *$p < 0.05$, **$p < 0.01$.

indicated that circRNAs might serve as biomarkers for tumor diagnosis or prognosis. In this study, our attention focused on a newly identified cicRNA has_circ_0008274. Previously, several studies have reported that circRNAs were strongly associated with the invasion and metastasis of various cancer cells. Zheng et al[26] reported that circ-TTBK2 was highly expressed in glioma and its forced expression could promoted cell proliferation, migration, and invasion, while inhibited apoptosis by regulating miR-217/HNF1β/Derlin-1 pathway. Xia et al[27] found that has_circ_0067934 expression was up-regulated in both esophageal squamous cell carcinoma tissues and cells. In their loss-of function assay, it was observed that knockdown of has_circ_0067934 could suppressed esophageal squamous cell carcinoma cells proliferation and invasion. Xie et al[28] reported that circRNA_001569 was significantly up-regulated in colorectal cancer and associated with distant metastasis and differentiation. *In vitro* assay showed that circ_001569 promotes the proliferation and invasion of colorectal cancer cells by regulating miR-145. However, the relationship between circRNA and PTC progression remains largely unknown. In this study, by analyzing the data from microarray analysis, we found that has_circ_0008274 expression was significantly upregulated in PTC. Next, we performed RT-PCR to demonstrate the results, finding that the expression levels of has_circ_0008274 were also significantly higher in PTC tissues and cell lines. Then, we found that has_circ_0008274 expression was associated with TNM stage and lymph node metastasis, suggesting that has_circ_0008274 might be involved in the carcinogenesis of PTC. Functionally, we knockdown has_circ_0008274 and explore its role on tumor behavior, find-

ing that knockdown of has_circ_0008274 significantly decreased PTC cell growth, induced apoptosis, and inhibited invasion, indicating that has_circ_0008274 could be a potential therapeutic target for PTC. Adenosine monophosphate-activated protein kinase (AMPK) is a serine/threonine protein kinase that has emerged as an important intracellular signaling pathway, and is a major tumor suppressor kinase, through a p53-dependent regulation of cell cycle[29,30]. Mammalian target of rapamycin (mTOR) is an atypical serine/threonine protein kinase that acts as an important regulator in the regulation of cellular growth, survival and motility[31]. Moreover, deregulations of the mTOR signaling pathway is one of the most commonly observed pathological alterations in human cancers[32,33]. Activation of this pathway inhibites of autophagy and enhances cell growth and proliferation in both normal and tumor cells[34]. Micic et al[35] indicate that AMPK acts as a negative regulator in mTOR signaling pathway. In order to explore the potential mechanism by which has_circ_0008274 promotes PTC cells proliferation and invasion, our attention focused on the effects of has_circ_0008274 on AMPK/mTOR signaling pathway. As expectedly, has_circ_0008274 knockdown can promote the expression of p-AMPK and suppress the expression of p-mTOR, indicating that has_circ_0008274 served as a tumor promoter in PTC by modulating AMPK/mTOR signaling pathway.

Conclusions

We demonstrated that has_circ_0008274 was upregulated in PTC cells and clinical samples. Furthermore, we also revealed a new tumor promoter role of the has_circ_0008274 by modulating AMPK/mTOR signaling pathway in PTC. Thus, has_circ_0008274 may serve as a potential therapeutic candidate in the treatment of PTC.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Acknowledgements

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