European Review for Medical and Pharmacological Sciences 2019; 23: 232-240

MiR-299-3p functions as a tumor suppressor in thyroid cancer by regulating SHOC2

X. CHEN, M. QI, Q. YANG, J.-Y. LI

Department of Breast and Thyroid Surgery, Shandong Provincial Hospital Affiliated to Shandong University, Jinan, China

Abstract. – OBJECTIVE: Thyroid cancer (TC) is one of the most common malignancies in the world. The prognosis of TC patients with advanced stage or recurrence is still poor. However, the biological role of miR-299-3p in TC remains unknown. The aim of our current research was to investigate the role of miR-299-3p in TC progression.

PATIENTS AND METHODS: MiR-299-3p expression level in both TC tissues and cell lines was determined by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). Cell proliferation ability was examined by Cell Count Kit-8 (CCK-8) assay and 5-ethynyl-2'-deoxyuridine (EdU) incorporation assay. Cell cycle progression and cell apoptosis were determined by flow cytometric analysis. Dual-Luciferase reporter assay was recruited to validate whether SHOC2 was a downstream target of miR-299-3p. In addition, the protein expression SHOC2 in transfected cells was examined by Western blotting.

RESULTS: We found that miR-299-3p was significantly downregulated in TC tissues and cell lines. To verify the role of miR-299-3p in TC, we transfected mimics and inhibitor in selected cell lines for over-expressing or down-expressing miR-299-3p, respectively. After transfection, cell functional experiments were subsequently employed. The results indicated that miR-299-3p could inhibit cell proliferation and cell cycle progression, whereas remarkably promote cell apoptosis in TC cell lines. Bioinformatics predicted that SHOC2 might be a potential target of miR-299-3p. Subsequent Dual-Luciferase reporter analysis validated our hypothesis. Rescue assay showed that miR-299-3p functioned as a tumor suppressor by targeting SHOC2 in TC.

CONCLUSIONS: MiR-299-3p functioned as a tumor suppressor in TC by targeting SHOC2. Our research provided novel insights into the molecular mechanism underlying TC progression, which might afford some new understanding in biomarkers and therapeutic strategies in TC development.

Key Words

MiR-299-3p, Proliferation, Cell cycle, Apoptosis, SHOC2.

Introduction

Thyroid cancer (TC) is one of the most common malignancies in the world[1]. Over the past decades, numerous researches have been performed on TC. However, the incidence rate of TC is still growing[2]. Due to early detection and efficient treatment, the mortality rate of TC is less than 10%[3]. Nevertheless, the prognosis of TC patients with advanced stage or recurrence is still poor[3]. Hence, it is vital to give efforts in searching novel biomarkers and systematic treatment, as well as understanding the underlying mechanism of TC progression.

MicroRNAs (miRNAs) are a class of small non-coding RNAs. They can modulate post-transcriptional gene expression by translational repression or degradation[4]. MiRNAs have been well studied by numerous researchers, and the physiological roles of miRNAs have been reported. The biological functions of miRNAs are various, including cell differential, proliferation, apoptosis, metastasis, invasion and senescence[5-7]. Moreover, it has been reported that aberrant expression of miRNAs can lead to multiple different malignant tumors including GC[8].

In this study, we examined the expression level of miR-299-3p in TC. The results showed that miR-299-3p was significantly downregulated in TC tissues and cell lines. After transfection, cell physiological experiments were performed in transfected cell lines. All the results indicated that miR-299-3p markedly inhibited cell proliferation in TC. Through bioinformatics analysis and Dual-Luciferase reporter assay, we validated that SHOC2 was a downstream target of miR-299-3p. Besides, tumor xenograft model showed that up-regulated miR-299-3p remarkably suppressed tumor formation *in vivo*. Taken together, we suggested that miR-299-3p functioned as a tumor suppressor in TC by regulating SHOC2 expression.

Corresponding Author: Jiyu Li, MD; e-mail: bigli2004@163.com

Patients and Methods

Tissue Specimens

In the current study, all TC tissues and paired adjacent tissues underwent surgical resection in our hospital from April 2015 to September 2017. This study was approved by the Ethics Committee of Shandong Provincial Hospital Affiliated to Shandong University. Signed informed consents were obtained from all participants before the study. All tissue specimens were stored at liquid nitrogen after resection. Tissue specimens were deposited at -80°C until use.

Cell Culture

Three thyroid cancer cell lines (8505C, BCPAP, and TPC-1) and one normal human thyroid cell line Nthy-ori3-1 were obtained from Cell Bank of Chinese Academy of Sciences (Shanghai, China). All cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) (HyClone, South Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA), 100 U/mL penicillin and 100 μg/mL streptomycin in a 37°C, 5% CO_2 incubator.

Cell Transfection

Over-expressing or down-expressing miR-299-3p (mimics or inhibitor) oligonucleotides and their negative controls were purchased from GenePharma (Shanghai, China). The plasmid pcDNA-3.1 (GenePharma Biotechnology, Shanghai, China) was used to up-regulate the expression of SHOC2, and the empty pcDNA-3.1 plasmid was taken as a control. Transfection efficiency was examined by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR).

RNA Extraction and qRT-PCR

Total RNA in all cell lines and tissues were extracted *via* TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the standard protocol. Complementary deoxyribose nucleic acids (cDNAs) were synthesized according to the instructions of Reverse Transcription Kit (TaKaRa, Dalian, China). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) or U6 was taken as normalization. Primer sequences used in this study were as follows: SHOC2, F: 5'-CTTACTCCAGGAACCCCCTC-3', R: 5'-AAGGACTAGGGATGTGTCCG-3'; miR-299-3p, F: 5'-TTCAGTGTAAACATCCTCGACTG-3', R: 5'-TGGCAATGTCGTGGAGTCG-3'; U6: F: 5'-GCTTCGGCAGCACATATACTAAAAT-3', R: 5'-CGCTTCAGAATTTGCGTGTCAT-3'; GAPDH: F: 5'-CGCTCTCTGCTCCTCCTGTTC-3', R: 5'-ATCCGTTGACTCCGACCTTCAC-3'.

Cell Counting Kit-8 Assay

Cell Counting Kit-8 (CCK-8) assay was recruited to examine the proliferation ability of TC cells. The transfected cells were plated on 96-wells plates (7×10^3/well), and 10 μL CCK-8 solution (Beyotime, Shanghai, China) was added, followed by incubation for 2 h at 37°C in the dark. Optical density (OD) value at 450 nm was evaluated by a spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

5-Ethynyl-2'-Deoxyuridine (EdU) Incorporation Assay

The EdU proliferation assay (RiboBio, Nanjing, China) was used to determine cell proliferation ability following the manufacturer's protocol. After transfection, cells were incubated with EdU for 2 h. EdU positive cells were observed under a fluorescence microscope after Apollo staining and 4',6-diamidino-2-phenylindole (DAPI) staining (Thermo Fisher Scientific, Waltham, MA, USA).

Flow Cytometric Analysis

To study cell apoptosis, the Annexin V-FITC/PI Apoptosis Detection Kit (Vazyme, Nanjing, China) was performed following the standard protocol. To detect cell cycle, transfected cells were immersed in cold 70% ethanol overnight and stained with propidium iodide (PI) (Vazyme, Nanjing, China). Flow cytometric analysis was performed by BD FACS Canto II (BD Biosciences, Franklin Lakes, NJ, USA) flow cytometry.

Bioinformatics Analysis

To seek for the potential target genes of miR-299-3p, we searched 4 public available databases, including RNA22, TargetScan, miRWalk, and MiRanda. The results predicted that 3'-UTR of SHOC2 had potential binding sites with miR-299-3p. Thus, we considered that SHOC2 might serve as a target of miR-299-3p.

Dual-Luciferase Reporter Assay

Wild SHOC2 3'-UTR sequence or mutant SHOC2 3'-UTR sequence was combined with top-GL3 promoter vector (GenScript, Nanjing, China). Luciferase activity was determined in Victor 1420 Multilabel Counter (Wallac, Finland) by Luciferase Assay System (Promega, Madison, WI, USA) following the manufacturer's instructions.

Western Blot

Total protein was extracted by radioimmunoprecipitation assay (RIPA) buffer (Thermo Fisher Scientific, Waltham, MA, USA) containing phenylmethanesulfonyl fluoride (PMSF). Extracted protein lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Then the membrane was incubated the primary antibody of rabbit anti-SHOC2 (1:1000, CST, Danvers, MA, USA) at 4°C overnight. Rabbit anti-GAPDH (1:5000, CST, Danvers, MA, USA) was taken as a loading control. The relative expression level of protein was evaluated by Image J software.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 19.0 software (SPSS, Chicago, IL, USA) was used for all statistical analyses. All experiments in this study were performed at least three times independently. All data were expressed as mean ± standard deviation (SD). Pearson correlation was performed for correlation analysis between miR-299-3p and SHOC2. The Student's unpaired *t*-test was used to compared the difference between the two groups. $p<0.05$ was considered statistically significant.

Results

MiR-299-3p Was Downregulated in TC

The relative expression of miR-299-3p in TC was examined by qRT-PCR. The results showed that the expression level of miR-299-3p in TC tissues was significantly lower than that of paired adjacent tissues (Figure 1A). Consistently, as shown in Figure 1B, the expression level of miR-299-3p in TC cell lines was markedly lower than normal hu-

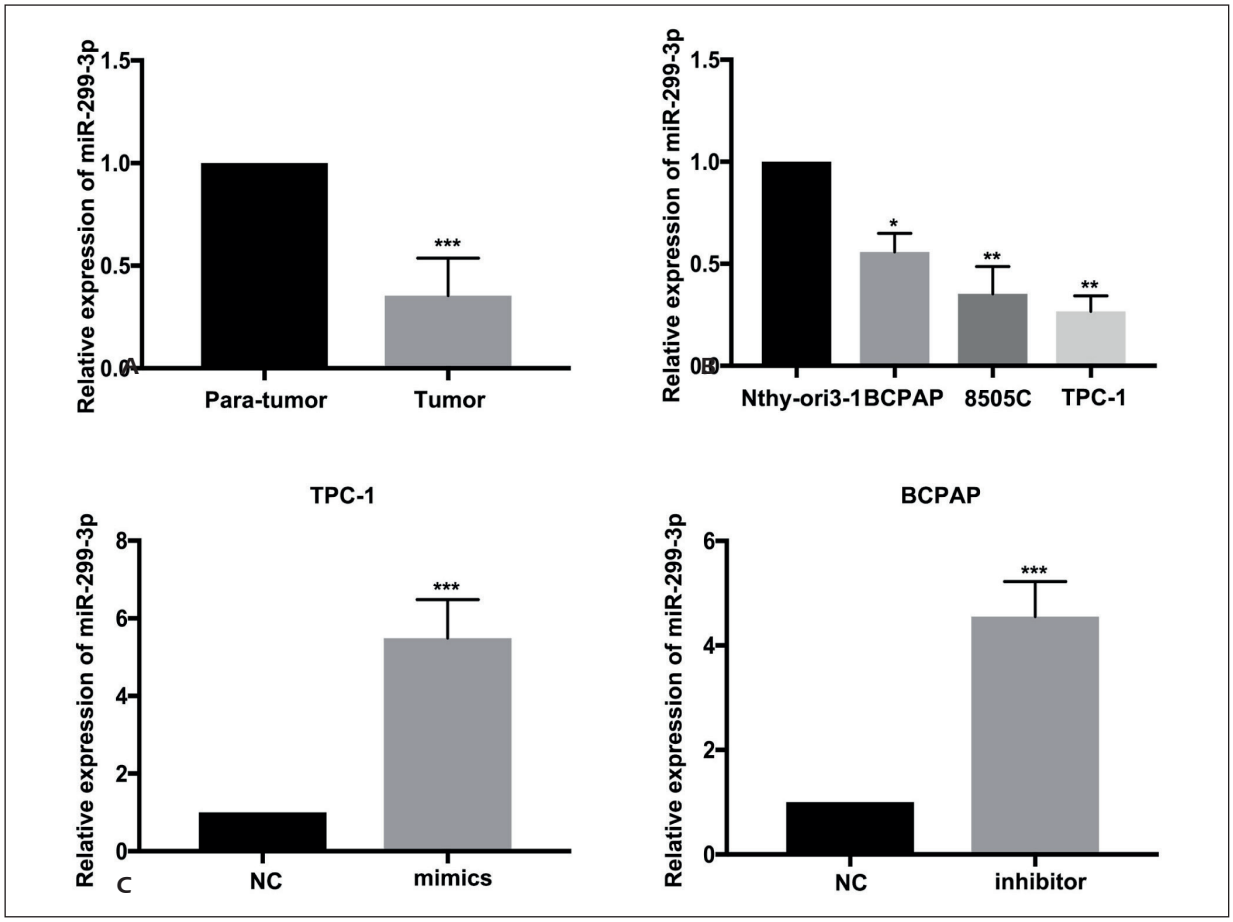

Figure 1. Expression level of miR-299-3p in thyroid cancer and transfected cells. **A**, The relative expression level of miR-299-3p was detected in 30 paired thyroid cancer and adjacent tissues. **B**, QRT-PCR was used to verify the expression of miR-299-3p in thyroid cancer cell lines. **C**, Transfection efficiency was determined by qRT-PCR. *$p<0.05$, **$p<0.01$, ***$p<0.001$, compared with the control group. Data were expressed as mean ± SD.

man thyroid cell line. Subsequently, we transfected mimics or inhibitor in selected cells to up-regulate or downregulate miR-299-3p expression. Transfection efficiency was determined by qRT-PCR (Figure 1C). Our data validated that the expression of miR-299-3p was down-regulated in TC.

Overexpressing MiR-299-3p Suppressed the Proliferation of TC Cells

After transfection, CCK-8 assay and EdU proliferation assay were performed to elucidate the proliferation of TC cells. As shown in Figure 2A, up-regulated miR-299-3p cells had a relative lower OD value when compared with control cells. The opposite result was observed in the inhibitor group. Subsequently, EdU proliferation assay was used to examine the influence of miR-299-3p in cell proliferation. Up-regulated miR-299-3p group had a relative lower EdU positive rate in comparison with the control group. However, the reverse influence was emerged in the inhibitor group (Figure 2B). Taken together, all results suggested that miR-299-3p could inhibit the proliferation of TC cells.

Up-regulated MiR-299-3p Induced Cell Cycle Arrest and Promoted Cell Apoptosis in TC

To elucidate the role of miR-299-3p in cell cycle and cell apoptosis, we recruited flow cytometric analysis. As shown in Figure 3A, in the miR-299-3p over-expressing group, cell distribution was significantly arrested in G1/0 fraction. However, in the miR-299-3p downexpression group, cell cycle progression was promoted. Flow cytometric analysis confirmed that miR-299-3p could inhibit cell cycle progression and promote cell apoptosis (Figure 3B).

SHOC2 Was a Downstream Target of MiR-299-3p in TC

To search for the downstream targets of miR-299-3p, several public available databases were recruited for prediction. As shown in Figure 4A, SHOC2 had a potential binding site with miR-299-3p its 3'-UTR region. Subsequently, Dual-Luciferase reporter assay was performed for validation. The relative expression level of SHOC2 in both TC tissues and adjacent normal tissues were examined by qRT-PCR (Figure 4B). The correlation between miR-299-3p expression and SHOC2 expression in TC was determined by Pearson correlation analysis. The result indicated that miR-299-3p expression level was negatively correlated with SHOC2 expression level in TC (Figure 4C). To further study the correlation between miR-299-3p and SHOC2, the protein expression of SHOC2 in transfected cells was detected by Western blotting. The results demonstrated that the protein expression of SHOC2 was significantly downregulated in the mimics group, whereas was markedly up-regulated in the inhibitor group (Figure 4D). Hence, all data indicated that SHOC2 was a direct downstream target of miR-299-3p in TC.

MiR-299-3p Functioned as a Tumor Suppressor in TC by Regulating SHOC2

To further determine the role of miR-299-3p and SHOC2 in TC, we recruited rescue assay. As shown in Figure 5A, when co-transfected with SHOC2 overexpression plasmid, the expression level of SHOC2 was significantly up-regulated. The up-regulated SHOC2 reversed the influence of miR-299-3p in cell proliferation (Figure 5B). Similarly, SHOC2 overexpression promoted cell cycle progression and suppressed the cell cycle arrest effect of miR-299-3p (Figure 5C). As shown in Figure 5D, up-regulated SHOC2 abolished the influence of miR-299-3p in cell apoptosis in TC. In sum, we validated that miR-299-3p functioned as a tumor suppressor in TC by targeting SHOC2.

Discussion

Currently, the incidence of TC is still increasing in the world[9]. Although early diagnosis and treatment has greatly improved, the mortality rate of TC has not declined[10]. Hence, it is of vital importance to investigate the molecular mechanism in TC progression. Mounting evidence has shown that miRNAs play an important role in tumor progression[11]. Herein, in this study, we validated that miR-299-3p functioned as a tumor suppressor in TC by regulating SHOC2, which might bring novel insights into the search for new biomarkers and treatment strategies in TC.

MiRNA has been reported for years, and its biological functions have been well studied. Previous studies have shown that miRNAs can take part in many cell progression and cancer types. As reported, inhibition of miR-16 leads to the progression of oral squamous cell carcinoma by targeting Tousled-like Kinase 1[12]. MiR-199b-5p has been proved to suppress cell proliferation, migration and invasion by targeting DDR1 in triple negative breast cancer[13]. In another study, microRNA-663b has been reported to promote the

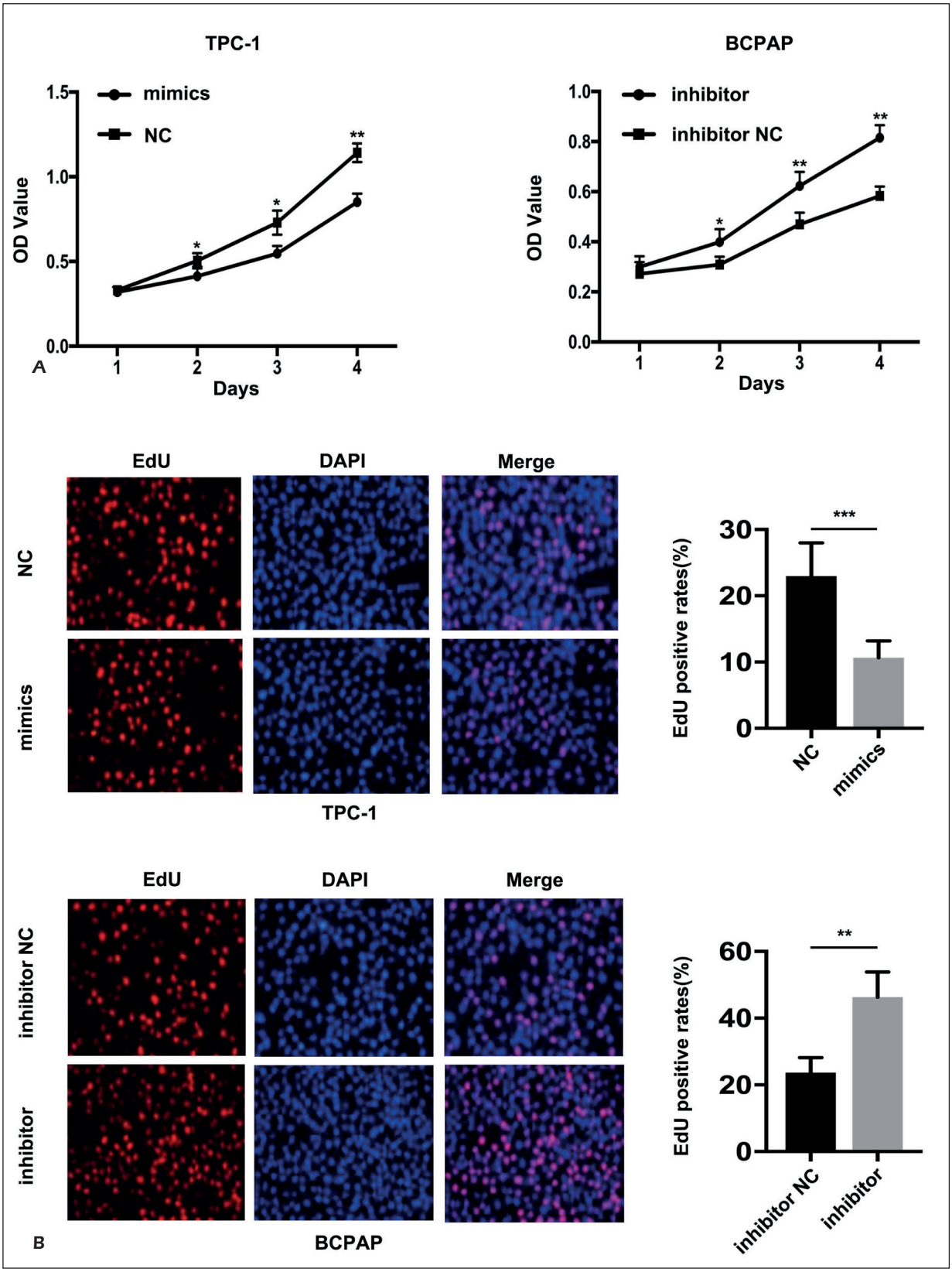

Figure 2. MiR-299-3p over-expression suppressed the proliferation of TC cell lines. **A**, CCK-8 was used to detect cell proliferation ability, and higher OD values represented more cell proliferation. **B**, Cell proliferation ability was expressed as EdU positive ratio. *p<0.05, **p<0.01, ***p<0.001, compared with the control group. Data were expressed as mean ± SD.

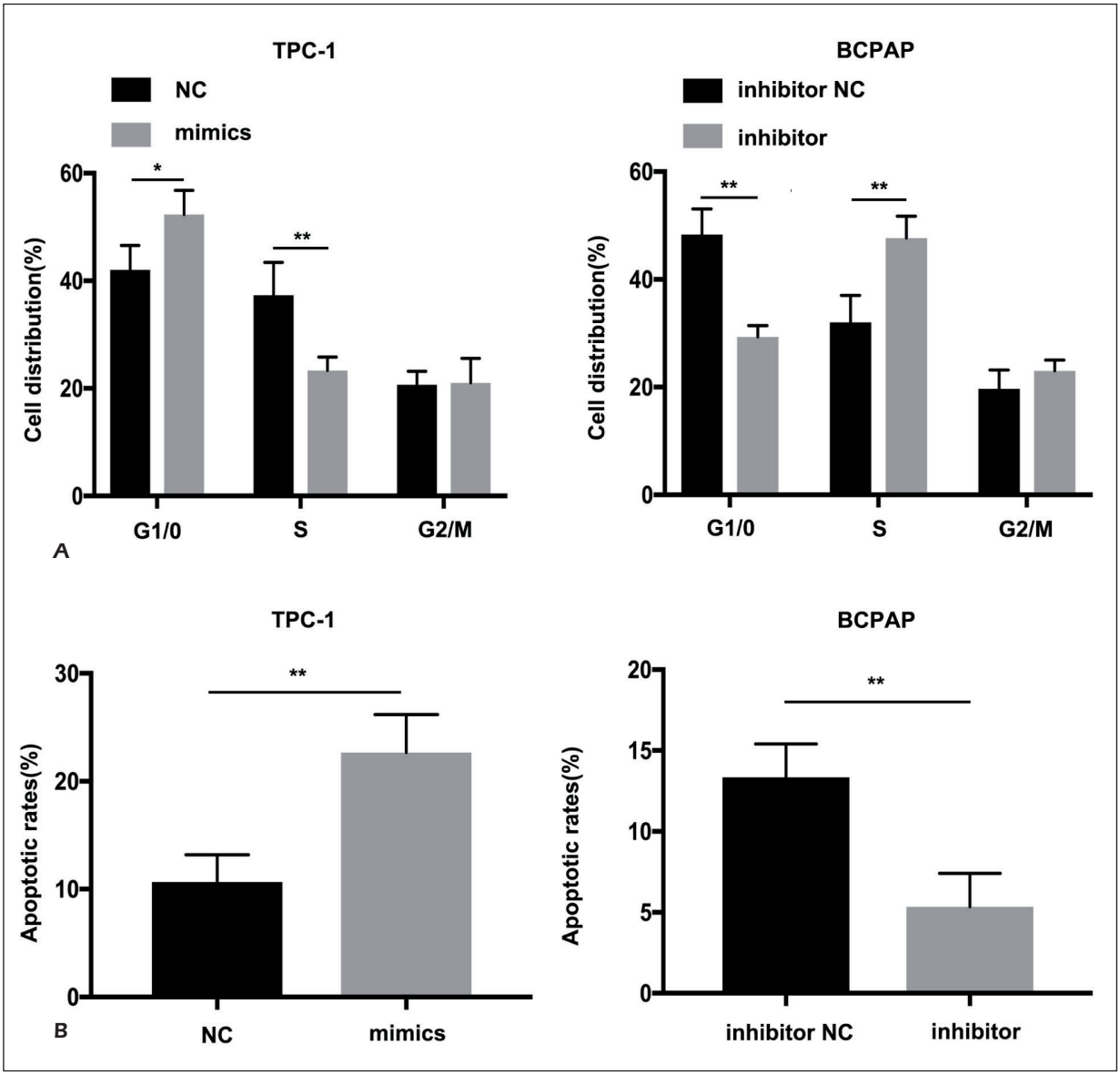

Figure 3. MiR-299-3p suppressed cell cycle progression and promoted cell apoptosis. **A**, Cell cycle was detected by flow cytometry. **B**, The rate of living cells was examined by flow cytometry. *$p<0.05$, **$p<0.01$, compared with the control group. Data were expressed as mean ± SD.

proliferation and epithelial-mesenchymal transition of nasopharyngeal carcinoma by regulating SMAD7[14]. Meanwhile, miRNAs are confirmed to play a vital role in diverse cell biological functions, such as cell proliferation, cell metastasis and invasion, cell cycle, cell apoptosis and cell senescence[15-17]. Thus, efforts on the research of miRNAs are essential. In recent years, miR-299-3p has been reported in several cancer types[18,19]. Nevertheless, the functional role of miR-299-3p in TC is still unknown. Hence, in the current study, we examined the expression level of miR-299-3p in TC. The results indicated that miR-299-3p was significantly down-regulated in TC. Subsequently, a series of cell functional experiments were used to prove that miR-299-3p over-expression could suppress cell proliferation and cell cycle progression, as well as promote cell apoptosis.

Scaffold protein SHOC2 (a leucine-rich repeat scaffold protein, also known as SUR-8) has been reported to positively regulate Ras-mediated signal transduction[20]. SHOC2 is a conserved leucine-repeat rich protein involved in fibroblast growth factor receptor signaling[21]. Aberrant expression

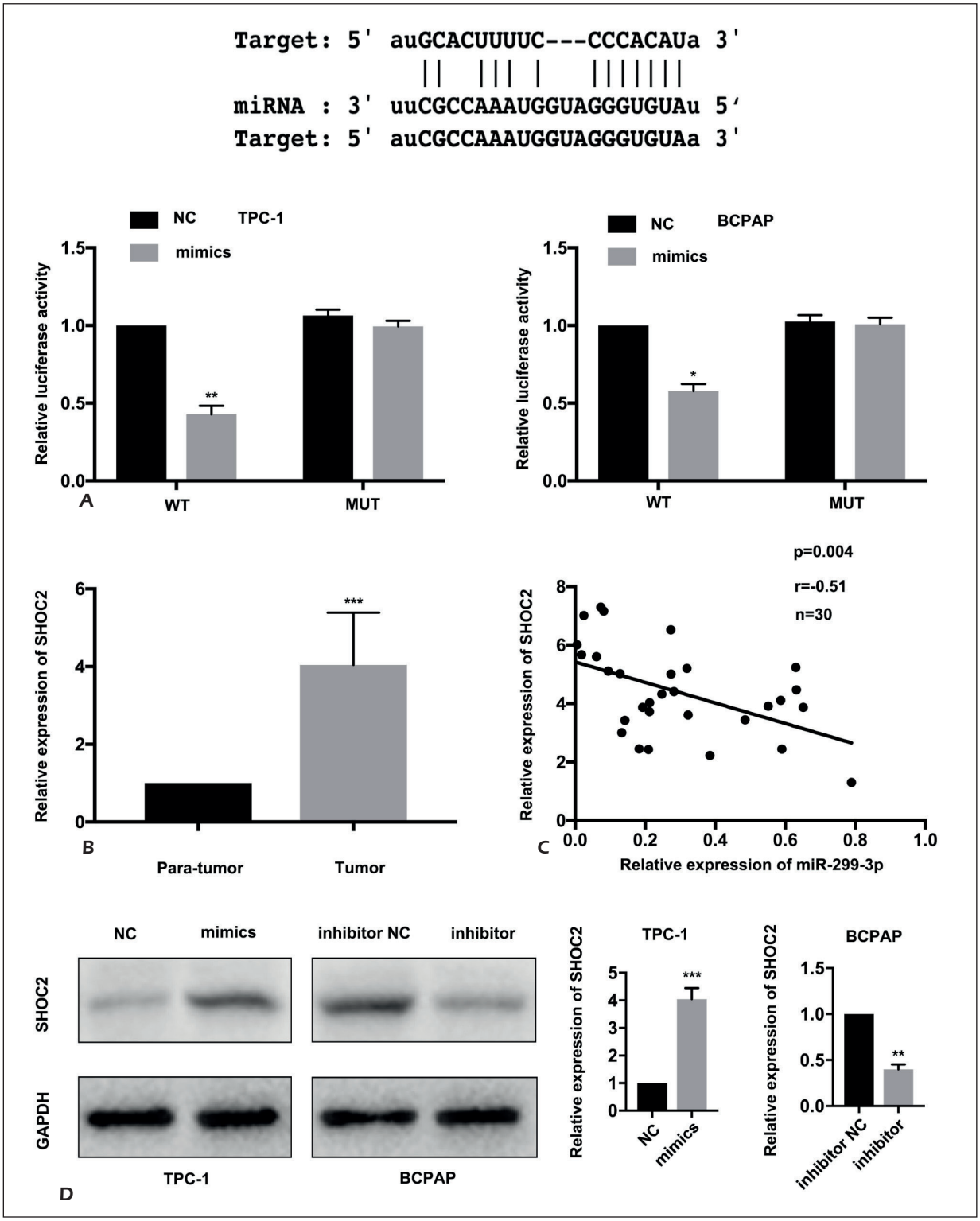

Figure 4. SHOC2 was proved to be the target gene of miR-299-3p. **A**, Dual Luciferase reporter assay proved that miR-299-3p directly bound to the 3'-UTR region of SHOC2. The sequence of wild-type and mutant versions in SHOC2 3'-UTR was shown. After co-transfection with wild-type or mutant 3'-UTR reporter plasmids, the relative Luciferase activity of SHOC2 was calculated and recorded by histogram. **B**, The expression of SHOC2 in thyroid cancer and adjacent normal tissues. **C**, SPSS was used to analyze the correlation be-tween the mRNA level of SHOC2 and miR-299-3p (p=0.004). **D**, Western blotting was used to measure the protein level of SHOC2 in transfected cell lines. *p<0.05, **p<0.01, ***p<0.001, compared with the control group. Data were expressed as mean ± SD.

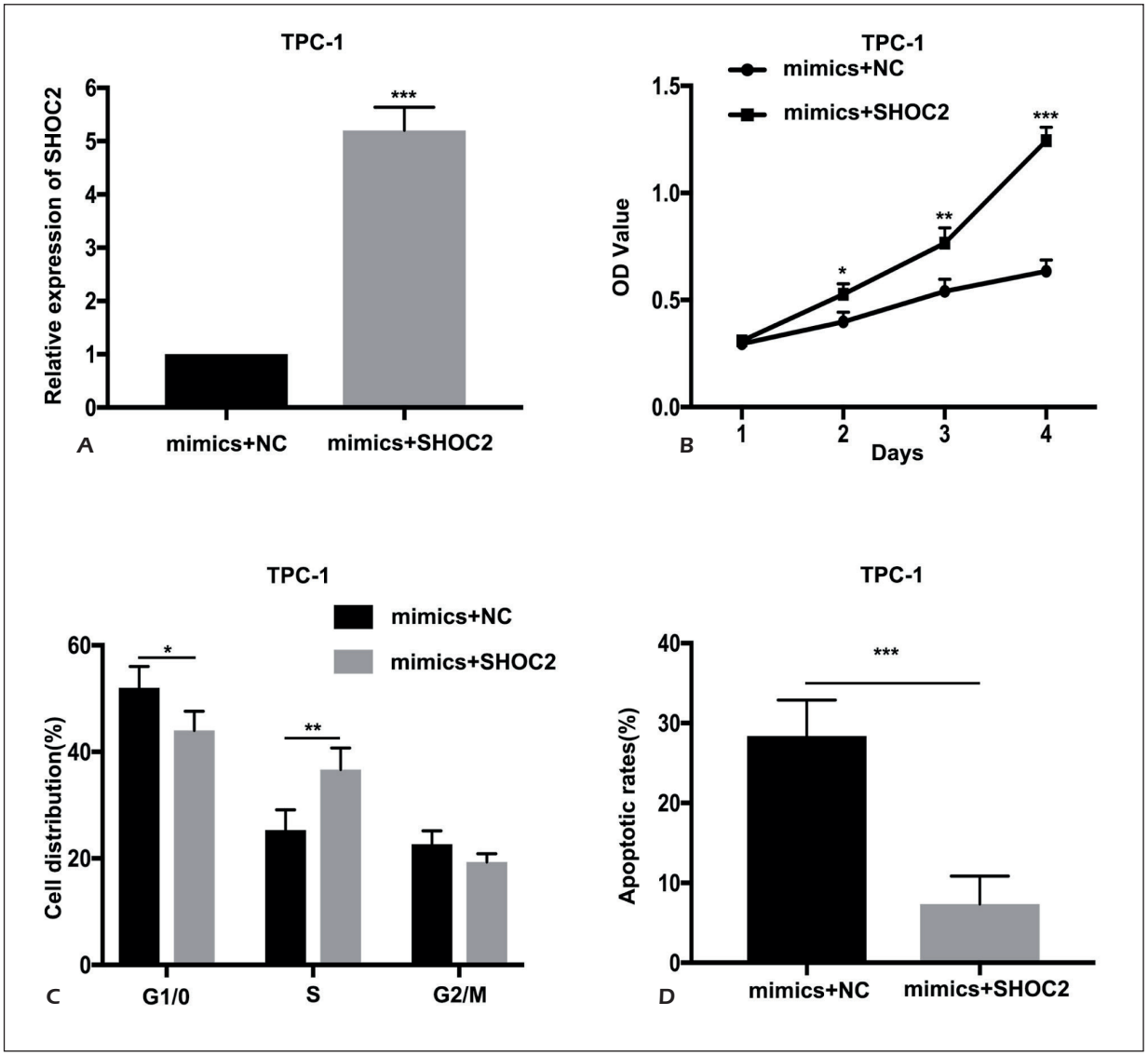

Figure 5. MiR-299-3p functioned as a tumor suppressor by directly targeting SHOC2. **A**, QRT-PCR was used to examine the mRNA level of SHOC2 in cells co-transfected with plasmids and mimics. **B**, CCK-8 assay was performed to elucidate the effect of miR-299-3p on cell proliferation in co-transfected cells. **C**, Flow cytometry was used to examine cell cy-cle. **D**, Flow cytometry was used to detect the apoptotic rates. *p<0.05, **p<0.01, ***p<0.001, compared with the control group. Data were expressed as mean ± SD.

of SHOC2 has been confirmed to play an important role in diverse cancer types[22,23]. However, the biological function of SHOC2 in TC remains unknown. Thus, in our study, we validated that SHOC2 was a direct downstream target of miR-299-3p by binding to its 3'-UTR region. Meanwhile, qRT-PCR results verified that SHOC2 was significantly up-regulated in TC. Subsequent Pearson correlation analysis showed that the expression of miR-299-3p was negatively correlated with SHOC2 in TC. In addition, rescue assay demonstrated that miR-299-3p functioned as a tumor suppressor in TC by regulating SHOC2.

Conclusions

We showed that miR-299-3p was significantly downregulated in TC tissues and cell lines. To validate of the role of miR-299-3p in TC, we transfected mimics and inhibitor in selected cell lines for over-expressing or down-expressing miR-299-3p, respectively. After transfection, cell functional experiments were performed. The results indicated that miR-299-3p could inhibit cell proliferation and cell cycle progression, and promote cell apoptosis in TC cell lines. Bioinformatics predicted that SHOC2 might be a potential

target of miR-299-3p. Subsequent Dual-Luciferase reporter analysis validated this hypothesis. Rescue assay showed that miR-299-3p functioned as a tumor suppressor by targeting SHOC2 in TC. In sum, our research provided novel insights into the molecular mechanism underlying TC progression. Moreover, our findings might afford some new understanding of biomarkers and therapeutic strategies in the TC development.

Acknowledgements

Shandong key research and development program (2015GSF118023).

Conflict of Interests

The authors declare that they have no conflict of interest.

References

1) Siegel RL, Miller KD, Jemal A. Cancer Statistics, 2017. CA Cancer J Clin 2017; 67: 7-30.
2) Pellegriti G, Frasca F, Regalbuto C, Squatrito S, Vigneri R. Worldwide increasing incidence of thyroid cancer: update on epidemiology and risk factors. J Cancer Epidemiol 2013; 2013: 965212.
3) McLeod DS, Sawka AM, Cooper DS. Controversies in primary treatment of low-risk papillary thyroid cancer. Lancet 2013; 381: 1046-1057.
4) Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 2004; 116: 281-297.
5) Xu J, Wang F, Wang X, He Z, Zhu X. miRNA-543 promotes cell migration and invasion by targeting SPOP in gastric cancer. Onco Targets Ther 2018; 11: 5075-5082.
6) Jiang Y, Zhang M, Guo T, Yang C, Zhang C, Hao J. MicroRNA-21-5p promotes proliferation of gastric cancer cells through targeting SMAD7. Onco Targets Ther 2018; 11: 4901-4911.
7) Li Q, Li Z, Wei S, Wang W, Chen Z, Zhang L, Chen L, Li B, Sun G, Xu J, Li Q, Wang L, Xu Z, Xia Y, Zhang D, Xu H, Xu Z. Overexpression of miR-584-5p inhibits proliferation and induces apoptosis by targeting WW domain-containing E3 ubiquitin protein ligase 1 in gastric cancer. J Exp Clin Cancer Res 2017; 36: 59.
8) Ouyang Y, Yuan W, Qiu S. MicroRNA-153 functions as a tumor suppressor in gastric cancer via targeting Kruppel-like factor 5. Exp Ther Med 2018; 16: 473-482.
9) Enewold L, Zhu K, Ron E, Marrogi AJ, Stojadinovic A, Peoples GE, Devesa SS. Rising thyroid cancer incidence in the United States by demographic and tumor characteristics, 1980-2005. Cancer Epidemiol Biomarkers Prev 2009; 18: 784-791.
10) La Vecchia C, Malvezzi M, Bosetti C, Garavello W, Bertuccio P, Levi F, Negri E. Thyroid cancer mortality and incidence: a global overview. Int J Cancer 2015; 136: 2187-2195.
11) Chen G, Zhou H. MiRNA-708/CUL4B axis contributes into cell proliferation and apoptosis of osteosarcoma. Eur Rev Med Pharmacol Sci 2018; 22: 5452-5459.
12) Hu S, Wang H, Yan D, Lu W, Gao P, Lou W, Kong X. Loss of miR-16 contributes to tumor progression by activation of tousled-like kinase 1 in oral squamous cell carcinoma. Cell Cycle 2018: 1-12.
13) Wu A, Chen Y, Liu Y, Lai Y, Liu D. miR-199b-5p inhibits triple negative breast cancer cell proliferation, migration and invasion by targeting DDR1. Oncol Lett 2018; 16: 4889-4896.
14) Wang M, Jia M, Yuan K. MicroRNA-663b promotes cell proliferation and epithelial mesenchymal transition by directly targeting SMAD7 in nasopharyngeal carcinoma. Exp Ther Med 2018; 16: 3129-3134.
15) Chen X, Zeng K, Xu M, Hu X, Liu X, Xu T, He B, Pan Y, Sun H, Wang S. SP1-induced lncRNA-ZFAS1 contributes to colorectal cancer progression via the miR-150-5p/VEGFA axis. Cell Death Dis 2018; 9: 982.
16) Liao XH, Xie Z, Guan CN. MiRNA-500a-3p inhibits cell proliferation and invasion by targeting lymphocyte antigen 6 complex locus K (LY6K) in human non-small cell lung cancer. Neoplasma 2018; 65: 673-682.
17) Taguchi YH. Inference of target gene regulation via mirnas during cell senescence by using the miRaGE server. Aging Dis 2012; 3: 301-306.
18) Li X, Zhang X, Yang C, Cui S, Shen Q, Xu S. The lncRNA RHPN1-AS1 downregulation promotes gefitinib resistance by targeting miR-299-3p/TNFSF12 pathway in NSCLC. Cell Cycle 2018; 17: 1772-1783.
19) Dang S, Zhou J, Wang Z, Wang K, Dai S, He S. MiR-299-3p functions as a tumor suppressor via targeting Sirtuin 5 in hepatocellular carcinoma. Biomed Pharmacother 2018; 106: 966-975.
20) Sieburth DS, Sun Q, Han M. SUR-8, a conserved Ras-binding protein with leucine-rich repeats, positively regulates Ras-mediated signaling in C. elegans. Cell 1998; 94: 119-130.
21) Selfors LM, Schutzman JL, Borland CZ, Stern MJ. soc-2 encodes a leucine-rich repeat protein implicated in fibroblast growth factor receptor signaling. Proc Natl Acad Sci U S A 1998; 95: 6903-6908.
22) Kaduwal S, Jeong WJ, Park JC, Lee KH, Lee YM, Jeon SH, Lim YB, Min DS, Choi KY. Sur8/Shoc2 promotes cell motility and metastasis through activation of Ras-PI3K signaling. Oncotarget 2015; 6: 33091-33105.
23) Lee YM, Kaduwal S, Lee KH, Park JC, Jeong WJ, Choi KY. Sur8 mediates tumorigenesis and metastasis in colorectal cancer. Exp Mol Med 2016; 48: e249.