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The interaction between circular RNA hsa_circ_0000285 and miR-599 in thyroid cancer

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Abstract. – OBJECTIVE: The vital role of circular RNAs in malignant tumors has been well-studied. Thyroid cancer (TC) is one of the most ordinary malignant tumors. Regulatory effect of hsa_circ_0000285 on metastatic TC was explored in this research.

PATIENTS AND METHODS: Real Time-quantitative Polymerase Chain Reaction (RT-qPCR) was performed to detect hsa_circ_0000285 expression in TC tissues. Knockdown and overexpression of Hsa_circ_0000285 models were established in TC cells. Moreover, wound healing assay and transwell assay were conducted to identify the role of hsa_circ_0000285 in regulating cellular phenotypes of TC cells. Furthermore, the interaction between hsa_circ_0000285 and miR-599 in TC cells was uncovered by the Dual-Luciferase reporter gene assay.

RESULTS: Hsa_circ_0000285 level was significantly higher in TC samples than that in adjacent samples. Migration and invasion of TC were reduced after silence of hsa_circ_000 while their metastatic abilities were enha d by overexpression of hsa_circ_0000285. M over, RT-qPCR results revealed miR-5 was downregulated via overexp of hsa circ_0000285, while miR-599 s u lated via knockdown of hsa_cir more, the Dual-Luciferas epor ene assay showed that miR-599 directly eted by hsa_circ_0000285 in .

CONCLUSIONS: Hsa_ci 00285 could enhance cell meta sis of TC argeting miR-599. Hsa_circ 0028 /miR-59 may be utilized as potential c targets in TC.

Key Wo

Ci ar A, Hsa 0000285, Thyroid cancer, Mi

Introduction

Thyroid cancer (TC) originates from follicular or parafollicular thyroid cells. Among several subtypes of TC, papillary thyroid carcinoma (PTC) accounts for approximately 90% of all the cases[1]. The incidence of TC has substantially increased globally over the pa few decades[2]. Extensive application and e uracy of ultrasonography are consi d to be import- ant reasons for the increas cidence TC. In China, TC is the ei h mo mn cancer, and it brings a hu ourde n for d patients and society[3,4]. S y i e stand d therapeutic approach to thy ancer while radioactive iodine is erred t cases of follicular cell-de roid ca s. The prognosis for the p rly di tiated thyroid cancer remains di al[5]. In addit lthough the mortality is low differentiated thyroid cancer, its recurrence is appro imately 20-30% or even higher[6]. fore s urgent to uncover the underlying mo mechanisms of TC and identify new biomarkers for early diagnosis of aggressive TC.

Early as the 1970s, scientists observed many circular structures in RNA viruses by electron microscopy. The circular closed RNA structures in these cells were mostly considered to be viral-related substances or "noise" of gene splicing. CircRNAs are covalently closed circular structures. In recent years, through deep sequencing technology, it has been found that about 1/8 of the genes can be transcribed into a large number of circRNAs which are 10 times more than linear transcripts, suggesting that they are highly expressed *in vivo*[7]. This may be due to the fact that circRNAs are different from conventional intracellular RNA structures which lack of 3'-terminal poly-A endings and 5'-terminal cap structures. Compared with linear transcripts, circRNAs in the cytoplasm are less susceptible to the cleavage of debranching enzymes and exonucleases, so they are more abundant and more stable.

The functional recognition of circRNAs is still limited. The mechanisms of circRNAs all ready known include the following: (1). com-

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petitive adsorption of microRNA. It has been found out that circRNAs can adsorb specific microRNAs in cytoplasm through their microRNAs adsorption sites to interfere with the biological regulation mediated by microRNAs. (2). Working through RNA-binding proteins. CircRNAs can bind to RNA-binding proteins or pair with RNAs to form RNA-protein complexes, which regulate linear classical transcripts. (3). Regulation of gene transcription level. It has been suggested that circRNA may play an indirect role by competing to suppress the linear transcript level of its corresponding genes. (4). Protein translation. Although most circRNAs are considered as non-coding RNAs, it has been found that some circRNAs contain translation initiation codons and have highly conserved termination codons near the splicing sites. These longer circRNAs also have the ability to translate into biologically active proteins. Among these mechanisms, competitive adsorption of microRNA is a new hot topic in the noncoding RNAs network which has been indicated to be play an important role in the processes of tumorigenesis. Acting as a s of miR-153-3p, circ_0084043 accelera proliferation and migration in malignant noma *via* up-regulating the expression of Si In breast cancer, circ-ABCB10 facilitates c proliferation and tumorigenesis b sponging miR-1271[9]. Hsa_circ_0005986 s as a tumor suppressor in hepatoc ar inoma by serving as a miR-129-5p ge, w be a novel biomarker for l carc noma[10].

A novel circRNA, _circ_0 has been reported to be a ated with tu ize, differentiation, lym metastasis, distant metastasis and Tu No tasis stage in nasopharyngeal carcinoma a r cancer which may be p nostic biomarke different cancers[11, 12]. Th xpres ion of hsa_circ_0000285 was signific . Ho r, the function of hsa_circ_0000 TC d the ntial molecular mechanism been ed so far, which drives us to unco hsa_circ_0000285 in TC

nd Methods

Tissue les

Paired noma and adjacent non-tumor tissues were sequentially gathered from 52 TC patients undergoing surgery i lospital of China Medical Universi 6 to December 2018. No radi apy or ch er-apy was performed bef rgery. Tissu ar-vested from the surgery d imm tely at –80°C. This study was ap thics Committee of the Hospital Medical University. Si writ informe consents were obtained all icipants before the study.

Cell Cult

Huma cell lines (K C-1, SW579), and a norm thyroid cell line (Nthy-ori 3-1) were the Chinese Academy of Scienc (Shangh). Cells were cultured in D co's Modifie gle's Medium (DMEM; co, Rockville, MD, USA) containing with % fetal bovine serum (FBS; Gibco, Rockville, , USA) and enicillin in an incubator con-ing 5% CO 37°C.

Ca tion

After TC cells were cultured for 24 h in well plates, cells were transfected with hsa_ 285 lentivirus (Biosettia Inc., San Di-, USA), hsa_circ_0000285 shRNA (Bio-ttia Inc., San Diego, CA, USA) or empty vector using Lipofectamine 3000 (Invitrogen, Carlsbad, A, USA). GFP-positive cells were chosen for the llowing experiments.

RNA Extraction and Real Time-Quantitative Polymerase Chain Reaction (RT-qPCR)

The total RNA was separated by TRIzol reagent (Invitrogen, Carlsbad, CA, USA). RNA was reversely transcribed to complementary deoxyribose nucleic acids (cDNAs) through reverse Transcription Kit (TaKaRa Biotechnology Co., Ltd., Dalian, China). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was utilized as the internal reference. Primers used for qRT-PCR were as follows: hsa_circ_0000285 primers forward 5'-TATGTTGGTGGATCCTGTTCGGCA-3', reverse 5'-TGGTGGGTAGACCAAGACTTGTGA-3'; Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primers forward 5'-CCAAAACCAGATGGGGCAATGCTGG-3' and reverse 5'-TGATGGCATGGACTGTGGCCATCCA-3'. The thermal cycle was set as follows: 30 sec at 95°C, 5 sec at 95°C, and 35 sec at 60°C, for a total of 40 cycles. The relative expression level of the target gene was expressed by $2^{-\Delta\Delta Ct}$.

Wound Healing Assay

After transfection, TC cells were seeded in 6-well plates and incubated in DMEM overnight. Then, cells were scratched with a plastic tip and cultured in serum-free DMEM. Each assay was repeated in triplicate independently. Wound distance was viewed under a light microscope (Olympus Corp., Tokyo, Japan) at 48 h.

Transwell Assay

For detecting TC cell migration, 2×10^5 transfected cells in 100 µL of serum-free DMEM were applied on the top chamber of an 8-µm culture insert (Corning, Corning, NY, USA). DMEM containing 20% FBS was added to the bottom chamber. 24 h later, these inserts were treated by methanol for 30 min and stained by hematoxylin for 20 min. An inverted microscope (×20) was utilized for counting migratory cells in three random fields. For detecting TC cell invasion, experimental procedures were the same as those in transwell migration assay, except for pre-coating with 50 µg Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) on the insert.

Dual-luciferase Reporter Gene Ass

First, the 3′-untranslated region (3′-U f hsa_circ_0000285 was cloned into the p vector (Promega, Madison, WI, USA). Site-d rected mutagenesis of the miR-599 binding site in hsa_circ_0000285 3′-UTR formed using Quick-change site-dire m genesis kit (Stratagene, La Jolla, CA A). C transfected with hsa_circ_0 -U

or hsa_circ_0000285 MUT-3′ iR-ctrl or miR-599 for 48 h. Then Dua rase reporter assay system (Pr ga, Madis VI, USA) was utilized for lu se assays.

Statistical Analysis

All statistical ana s were pe by Statistical Product an rvice olutions (SS) 20.0 (SPSS, Chicago US Independent-sample *t*-test was appl propri $p<0.05$ was considered as stat ignif t.

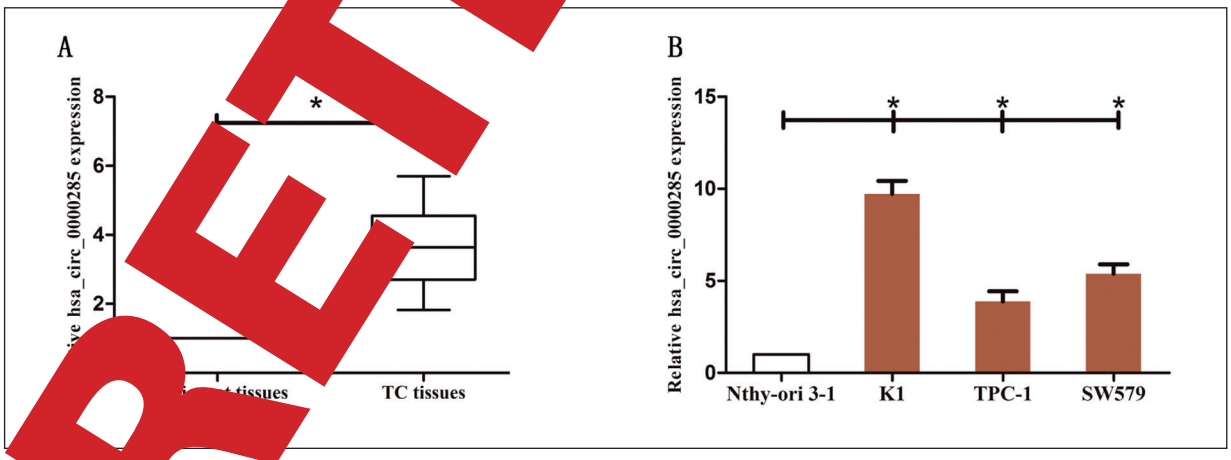
Results

Hsa_circ_000 xpression Level in TC sues and C

-qPCR analysis showed that in 52 paired tissues, hsa_circ_0000285 expression was egulated rela to adjacent normal tissues ure 1A). M over, its expression in human l line TPC-1, SW579, and the normal hu cell line Nthy-ori 3-1 was detected. Hsa_circ_0000285 expression in TC cells was higher than that of Nthy-ori 3-1 cells (Figure 1B).

own of Hsa_circ_0000285 uppressed Cell Migration and nvasion of TC Cells

According to hsa_circ_0000285 expression the tested TC cells, K1 cell line was used for knockdown of hsa_circ_0000285. RT-qPCR was utilized for detecting the transfection efficiency (Figure 2A). Hsa_circ_0000285 knock-



Figu irc_0000285 was upregulated in TC tissues and cell lines. **A,** Hsa_circ_0000285 expression was significantly upregulat tissues compared with adjacent tissues. **B,** Hsa_circ_0000285 relative to GAPDH was determined in the human TC cel d Nthy-ori 3–1 by RT-qPCR. GAPDH was used as an internal control. Data are presented as the mean ± standard error o e mean. *$p<0.05$.

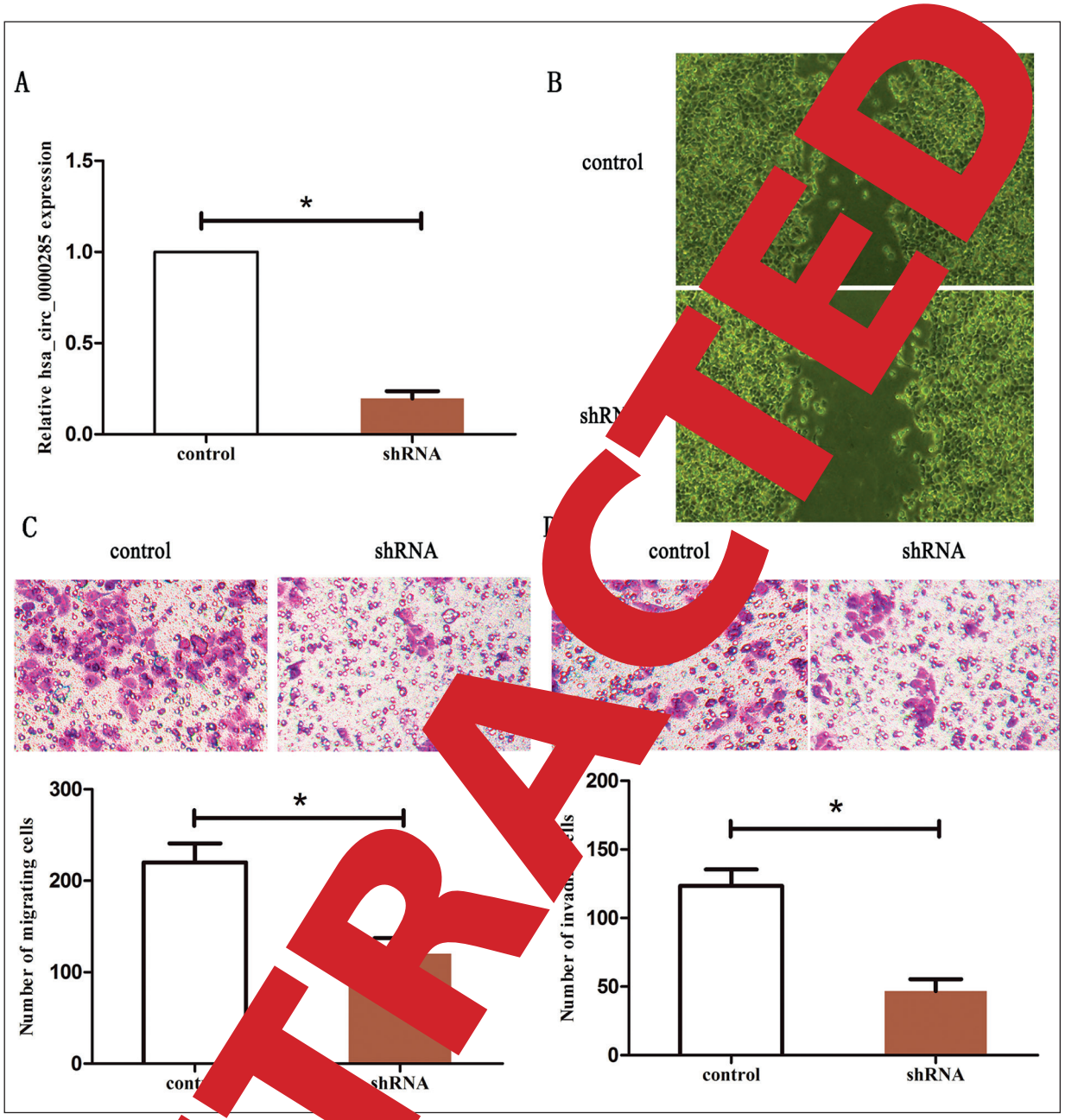


Figure 2. Knockd of hsa_circ_0000285 inhibited K1 cell migration and invasion. **A,** Hsa_circ_0000285 expression in K1 cells transfected with shRNA or control was detected by RT-qPCR. **B,** Wound healing assay showed that the wound closure percentage of K cells in shRNA group significantly decreased compared with that in control group (magnification:40×). **C,** Transwell assay showed that knockdown of hsa_circ_0000285 significantly repressed cell migration in K1 cells (magnification: 40×). **D,** Transwell assay showed that knockdown of hsa_circ_0000285 significantly repressed cell invasion in K1 cells (magnification: 4 results represent the average of three independent experiments (mean ± standard error of the mean). *p<0.05, as compared with control cells.

down wound closure percentage of TC cells (Figure 2B). The number of migratory cells remarkably decreased after silence of hsa_circ_0000285 in TC cells (Figure 2C). The number of invasive cells remarkably decreased after knockdown of hsa_circ_0000285 in TC cells as well (Figure 2D).

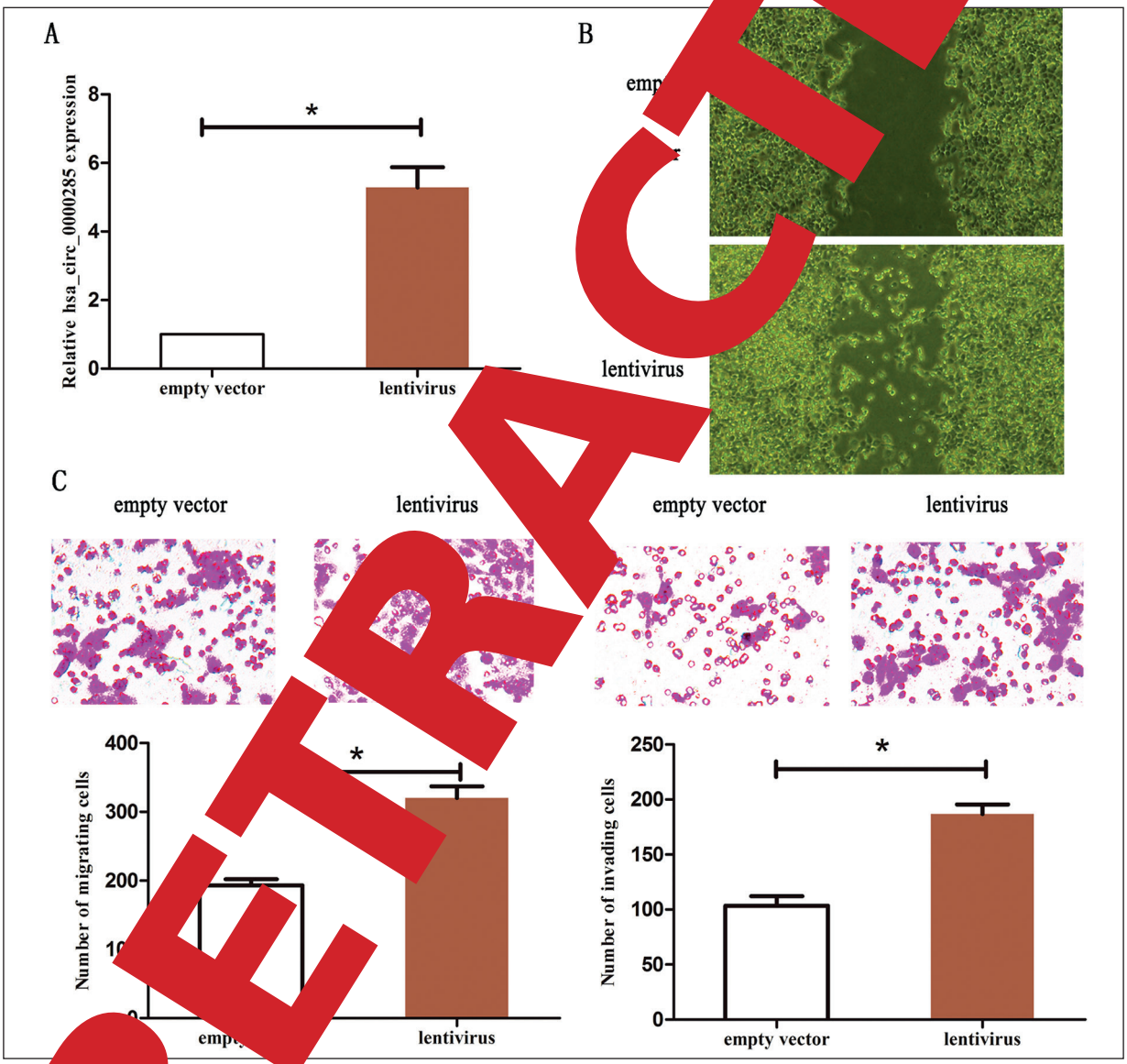
Overexpression of Hsa_circ_0000285 Promoted Cell Migration and Invasion of TC Cells

According to hsa_circ_0000285 expression in TC cells, TPC-1 cell line was used for overexpression of hsa_circ_0000285. RT-qPCR was utilized for detecting the transfection efficiency (Figure

3A). Hsa_circ_0000285 overexpression increased wound closure percentage of TC cells (Figure 3B). The numbers of migratory and invasive cells remarkably increased after the overexpression of hsa_circ_0000285 in TC cells (Figure 3C, D).

The Interaction Between MiR-599 and Hsa_circ_0000285 in TC

As shown in Figure 4A, miR-599 was predicted to be the target miRNA of hsa_circ_0000285 through Circular RNA Intera[illegible]s://circinteractome.nia.nih.gov/). [illegible]and Figure 4C, RT-qPCR a[illegible] showed [illegible]the expression of miR-599 [illegible]egatively re[illegible]ted by hsa_circ_0000285 i[illegible]. Furth[illegible]ore, Dual-Luciferase repo[illegible]rter ge[illegible]ealed that co-transfecti[illegible] of hsa_[illegible]00285-WT and miR-59[illegible]rgely decreased[illegible]e lucif-erase activity, [illegible]e co[illegible]nsfection of hsa_circ_0000285[illegible]iR-59[illegible]d no effect



Fig[illegible] 3. C[illegible]f hsa_circ_0000285 promoted TPC-1 cell migration and invasion. **A,** Hsa_circ_0000285 e[illegible]n in[illegible]nsfected with hsa_circ_0000285 lentivirus or empty vector was detected by RT-qPCR. **B,** Wound h[illegible]owed that the wound closure percentage of TPC-1 cells in lentivirus group significantly increased compared with t[illegible]y vector group (magnification: 40×). **C,** Transwell assay showed that overexpression of hsa_circ_0000285 significant[illegible]d cell migration in TPC-1 cells (magnification: 40×). **D,** Transwell assay showed that overexpression of hsa_circ_0000[illegible]nificantly promoted cell invasion in TPC-1 cells (magnification: 40×). The results represent the average of three independe[illegible] experiments (mean ± standard error of the mean). *p<0.05, as compared with the control cells.

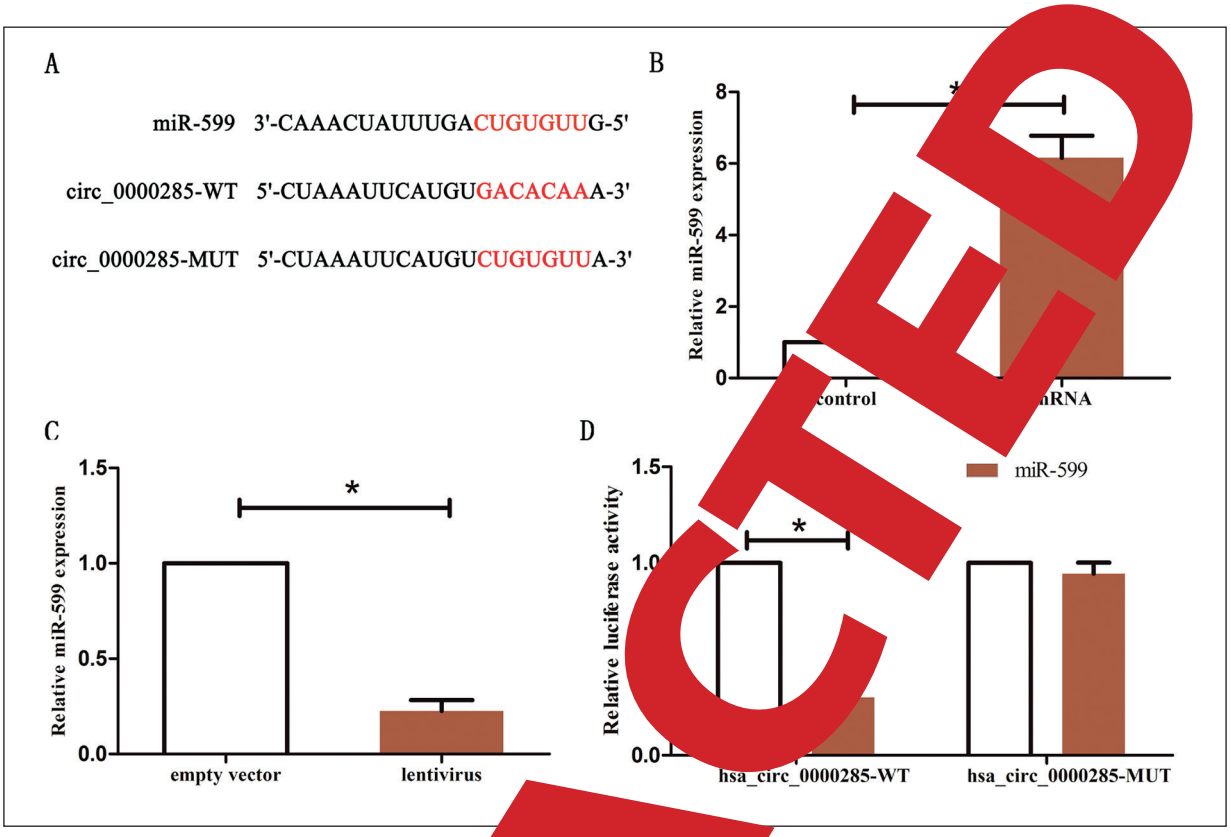


Figure 4. Reciprocal repression between hsa_circ_0000285 and miR-599. **A,** The binding sites of miR-599 on hsa_circ_0000285. **B,** MiR-599 expression was upregulated in shRNA group compared with that in control group. **C,** MiR-599 expression was downregulated in hsa_circ_0000285 lentivirus group compared with that in empty vector group. **D,** Co-transfection of miR-599 and hsa_circ_0000285-WT strongly decreased the luciferase activity, while co-transfection of miR-599 and hsa_circ_0000285-MUT did not change the luciferase activity either. The results represent the average of three independent experiments. Data are presented as mean ± standard error of the mean. *p<0.05.

on the luciferase activity (Figure 4D), suggested that miR-599 was the target binding to hsa_circ_0000285.

Discussion

TC is one of the most common malignant tumors in the endocrine system. The incidence of TC is increasing year by year. Invasion and migration is a complex multi-stage process. The degradation of extracellular matrix, the decrease of adhesion between cells and matrix, and the enhancement of cell motility can all affect the metastasis of TC.

CircRNA, a large class of newly identified non-coding RNA in recent years, has the characteristics of high expression, high conservativeness, relative stability, suggesting that circRNA may play an important role in maintaining cell life function and homeostasis. With the development of bioinformatics technology and high-throughput sequencing, it has been found that circRNAs are abnormally expressed in many pathological states, such as tumors, autoimmune diseases, neurological diseases, and may be a potential indicator of disease activity. Moreover, many circRNAs have been identified as important regulators in the progression of TC, such as circ-ITCH, circ_0025033, circRNA_102171 and so on[13-15].

In this study, we found that hsa_circ_0000285 was upregulated in TC samples. Besides, silence of hsa_circ_0000285 repressed cell migration and invasion of TC cells, while overexpression of hsa_circ_0000285 achieved the opposite trends. Above results indicated that hsa_circ_0000285 promoted metastasis of TC and might act as an oncogene.

MicroRNAs (MiRNAs) are small non-coding RNA molecules. There is increasing evidence that microRNAs play an important role in var-

ious biological and pathological processes. The function of competitive adsorption of microRNA has been widely studied in circRNA. It has been found out that circRNAs can adsorb specific microRNAs in cytoplasm through their microRNAs adsorption sites to interfere with the biological regulation mediated by microRNAs[16, 17]. MicroRNA-599 is one of the most common microRNAs in mammals. It exists in many kinds of cells and tissues. Tian et al[18] used microarray analysis of microRNAs to find thathsa-miR-599 was significantly down-regulated in hepatocellular carcinoma and modulated cell proliferation, migration and invasion by targeting the oncogene, MYC[18]. They found[19] that the expression of miR-599 was up-regulated in non-small cell lung cancer patients, and that miR-599 mimics could promote the proliferation, invasion and migration of NSCLC cells which speculated that miR-599 might be a potential therapeutic target for NSCLC[19].

In the present work, miR-599 could directly bind to hsa_circ_0000285. In addition, miR-599 expression was negatively regulated by hsa_circ_0000285. All these results indicated hsa_circ_0000285 might promote tumori of TC by directly targeting miR-599.

Conclusions

We showed that Hsa_circ_ as remarkably upregulated in TC ents facilitate TC cell migration by ta geting miR-599. These fi ding ted that hsa_circ_0000285 may tribute t for TC as a candidate targ

Conflict of Inte

The Authors decla at they have no conflict of interests.

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