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Circular RNA hsa_circ_0000285 acts as an oncogene in laryngocarcinoma by inducing Wnt/β-catenin signaling pathway

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Abstract. – OBJECTIVE: Laryngocarcinoma is one of the most ordinary head and neck cancers worldwide. Recent studies have revealed that circular RNAs (circRNAs) act as an important role in malignant tumors and participate in tumorigenesis. The purpose of our work is to uncover how hsa_circ_0000285 functions in laryngocarcinoma.

PATIENTS AND METHODS: In this research, the Real Time-quantitative Polymerase Chain Reaction (RT-qPCR) was performed to detect hsa_circ_0000285 expression in laryngocarcinoma samples. Besides, function assays were performed in laryngocarcinoma cells transfected with hsa_circ_0000285 shRNA or lentivirus. Furthermore, the RT-qPCR and Western blot assay were conducted to explore Wnt-signaling pathway of hsa_circ_0000285.

RESULTS: Hsa_circ_0000285 expression was found to be upregulated in laryngocarcinoma samples compared with adjacent tissues. The function assays showed that the inhibition of the cell proliferation was induced *via* knockdown of hsa_circ_0000285 in laryngocarcinoma in vitro, while the promotion of cell apoptosis was induced *via* the knockdown of hsa_circ_0000285 in laryngocarcinoma in vitro. On the other hand, the overexpression of hsa_circ_0000285 had the opposite function. In addition, the Wnt/β-catenin signaling pathway was repressed by knockdown of hsa_circ_0000285 in laryngocarcinoma, while the Wnt/β-catenin signaling pathway was promoted *via* overexpression of hsa_circ_0000285 in laryngocarcinoma.

CONCLUSIONS: In our study, hsa_circ_0000285 was identified as a novel oncogene and could induce the Wnt/β-catenin signaling pathway in laryngocarcinoma.

Keywords:
Circular RNA, Hsa_circ_0000285, Wnt/β-catenin signaling pathway, Laryngocarcinoma.

Introduction

Laryngocarcinoma is one of the most aggressive cancers in head and neck, which originates in the squamous cells of the laryngeal epithelium. Laryngocarcinoma brings a huge health burden and affects the quality of life to the patients. Approximately 12,500 new cases were diagnosed with laryngocarcinoma every year[1]. Due to late metastases and the resistance to chemotherapy and radiotherapy, the overall survival time of patients remains dismal in patients with laryngocarcinoma. Moreover, for the past decades, no significant improvement has been found in the overall survival rate of this disease[2,3]. Therefore, uncovering the molecular mechanism of tumorigenesis in laryngocarcinoma and figuring out an effective therapeutic strategy is very important.

Circular RNAs (circRNAs) are formed by a covalently closed loop without the ability of coding proteins. Circular RNAs (circRNAs) are tissue-specific, ubiquitously expressed noncoding RNAs. Serving as microRNA (miRNA) sponges is the first described function of cellular circRNAs. Recently, circRNAs have been indicated to be important regulators in multiple physiological and pathological processes of the tumorigenesis. For example, circ VPS13C-has-circ-001567 is upregulated in ovarian cancer and promotes cell proliferation and cell invasion[4]. through sponging miR-424-5p and modulating the expression of LATS1, circ_LARP4 suppresses cell proliferation and cell invasion in gastric cancer[5]. CircRNA ciRS-7 correlates with advanced disease and poor prognosis and its down-regulation inhibits cells proliferation while induces cells apoptosis in non-small cell

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lung cancer[6]. Through regulating the expression of mir-29a, circ MYLK functions as an oncogene and promoting progression of prostate cancer[7]. Recently, hsa_circ_0000285 is reported to be a novel circRNA and promotes tumor metastasis and proliferation in multiple cancers. However, the role of hsa_circ_0000285 in laryngocarcinoma remains unknown.

In our research, we first discovered that hsa_circ_0000285 was involved in cell proliferation and apoptosis by inducing the Wnt/β-catenin signaling pathway in laryngocarcinoma cells, which might offer new insight into the therapy of laryngocarcinoma.

Patients and Methods

Clinical Samples

Totally, we collected 56 paired laryngocarcinoma samples from patients who had undergone surgeries at the Heping Hospital Affiliated to Changzhi Medical College from June 2016 to December 2018. This investigation was approved by the Ethics Committee of Heping Hospital Affiliated to Changzhi Medical College. All patients were diagnosed with laryngocarcinoma by two independent pathologists without any controversial. Written informed consent was offered by each patient before surgery.

Cell Culture

Four laryngocarcinoma cell lines (HEp-2, HLC3, J82, BIU, and SW780) were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). The culture medium Dulbecco's Modified Eagle Medium (DMEM; Hyclone, South Logan, UT, USA) and 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA) were used to incubate the cells in an incubator containing 5% CO_2 at 37°C.

Cell Transfection

For cell transfection, lentivirus expressing shRNA (shRNA) and lentivirus targeting hsa_circ_0000285 were compounded and then cloned to pLenti-EF1a-EGFP-F2A-Puro vector (Inc., San Diego, CA, USA). Hsa_circ_0000285 shRNA or negative control shRNA (NC), and hsa_circ_0000285 lentivirus or scramble vector (SV) were used for transfection in laryngocarcinoma cells with lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol.

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

TRIzol reagent (Invitrogen, Carlsbad, USA) was utilized to isolate total RNA from tissues and cells. SYBR green (Roche, Basel, Switzerland) was conducted to measure the relative expression levels by normalized to β-actin. The primers were designed as following: hsa_circ_0000285, forward 5'-TATGTTC GATCCTGTTCGGCA and reverse: 3'-TG GGGTAGACCAAG TGTC; β-actin, forward 5'-GATGG AT GAGG and reverse 5'-TC ACTT GGA TGC-3'. The thermal was as follows: 95°C at 95°C, 5 sec for 4 at 95°C, 35 s at 60°C. The relative expression was calculated by performing the $2^{-\Delta\Delta CT}$ method.

MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyl Tetrazolium Bromide) Assay

Following the manufacturer's protocol, 2×10^3 transfected cells were seeded in 96-well plates and proliferation was assessed by the Cell Proliferation Reagent Kit I (MTT; Roche, Basel, Switzerland) at 0 h, 24 h, 48 h, 72 h. Absorbance at 490 nm was assessed using an enzyme-linked immunosorbent assay (ELISA) reader system (Multiskan Ascent, LabSystems, Helsinki, Finland).

Flow Cytometry Assay

Flow cytometry binding buffer (100 μL) was added after harvested cells were washed twice using ice-cold. The mixture containing 5 μL Annexin V/FICC (fluorescein isothiocyanate) and 5 μL Propidium Iodide (PI; BD, Franklin Lakes, NJ, USA) was used for staining these cells for 15 min in dark. Then, they were added with 400 microliters binding buffer. FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) was performed to analyze cell apoptosis.

Western Blot Analysis

The cell samples were washed with precooled phosphate-buffered saline (PBS) and then lysed with cell lysis solution (RIPA; Beyotime, Shanghai, China). Protein concentration was detected using bicinchoninic acid (BCA; Thermo Fisher Scientific Inc., Waltham, MA, USA). The proteins were transferred on to a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA), blocked in Tris Buffered Saline-Tween (TBST) (25 mM Tris, 140 mM NaCl,

and 0.1% Tween 20, pH 7.5) containing 5% skimmed milk and incubated for 2 h. The proteins were incubated with the primary antibody of target proteins including Wnt3a, β-catenin, C-my, and Survivin (Abcam, Cambridge, MA, USA) in the Wnt/β-catenin signaling pathway and β-actin (Abcam, Cambridge, MA, USA) and incubated at 4°C overnight. After being washed (3×10 min) with TBST, the secondary antibody was added and incubated at room temperature for 1 h. Results were analyzed by the Image J software (NIH, Bethesda, MD, USA).

Statistical Analysis

All statistical analyses were performed by Statistical Product and Service Solutions (SPSS) 20.0 (SPSS, Chicago, IL, USA). Independent-sample *t*-test was selected when appropriate. Moreover, $p<0.05$ was considered to indicate a statistically significant difference.

Results

Hsa_circ_0000285 in Laryngocarci Tissues and Cells

Before our study, RT-qPCR was conducte detect hsa_circ_0000285 expression in 50 tients' tissues and four laryn noma ce lines. As was shown in Fig gulated hsa_circ_0000285 was vi in la gocarcinoma tissue samples co d wit jacent tissues. Moreo expression level of lary cinoma cell lines was shown i ure 1B.

Hsa_circ_0000285 Knockdown Repressed Cell Growth Ability and Promotes Cell Apoptosis in Laryngocarcinoma Cells

To determine whether hsa_circ_0 285 a vital function in laryngocarci a, SW780 cells were chosen for the kno of hsa_circ_0000285. The hsa_cir 000 hRNA and negative control shR were sy zed and transduced into SW ells. Next, the fection efficiency was ermin y RT-q (Figure 2A). As wa n i ure 2B, MTT assay results sho t inhibiti cell viability in lar carcino lls nduced by knockdo hsa_circ_0 o further confirm th f hsa_circ_ 285 on cell apoptosis lary cinoma, we performed flow cytometry assay yngocarcinoma cells. As own in Fig C, the percentage o apoptosis rate was increased after hsa_circ_0000285 w nocked down.

Hsa_circ_000 5 Overexpression Pro Growth Ability and Inhibi Apoptosis in aryngocarcinoma Cells

ther confirm the role of hsa_circ_0000285 gocarcinoma, the UM-UC3 cells were transfected with hsa_circ_0000285 lentivirus and scramble vector were synthesized and transduced into UM-UC3 cells. Then, the transfection efficiency was determined by RT-qPCR (Figure 3A). As was shown in Figure 3B, MTT assay results showed that the promotion of cell viability in laryngocarcinoma cells was induced by overexpression of hsa_circ_0000285. As was shown

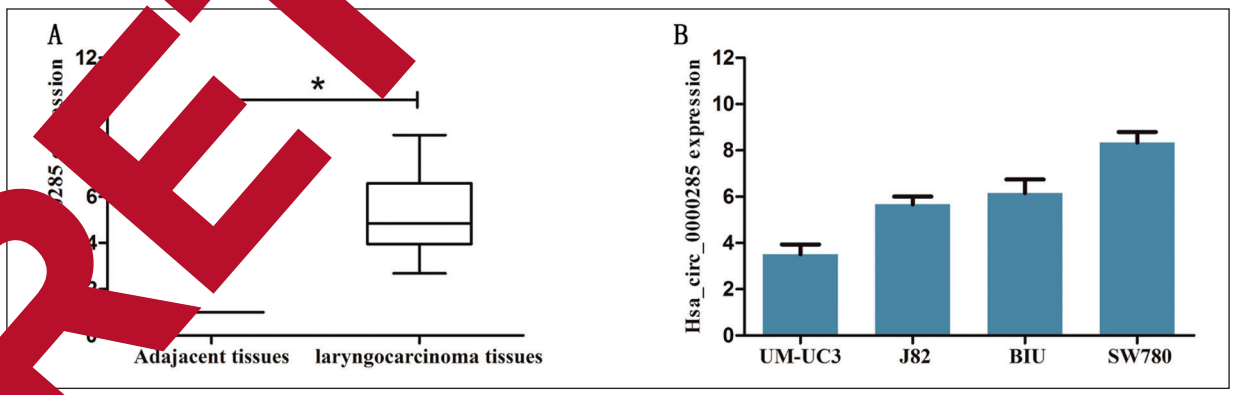


Figure 1. Expression levels of hsa_circ_0000285 in laryngocarcinoma tissues and cell lines. **A,** Hsa_circ_0000285 expression was significantly upregulated in the laryngocarcinoma tissues compared with adjacent tissues. **B,** Expression levels of hsa_circ_0000285 relative to β-actin were determined in the human laryngocarcinoma cell lines by RT-qPCR. Data are presented as the mean ± standard error of the mean. *$p<0.05$.

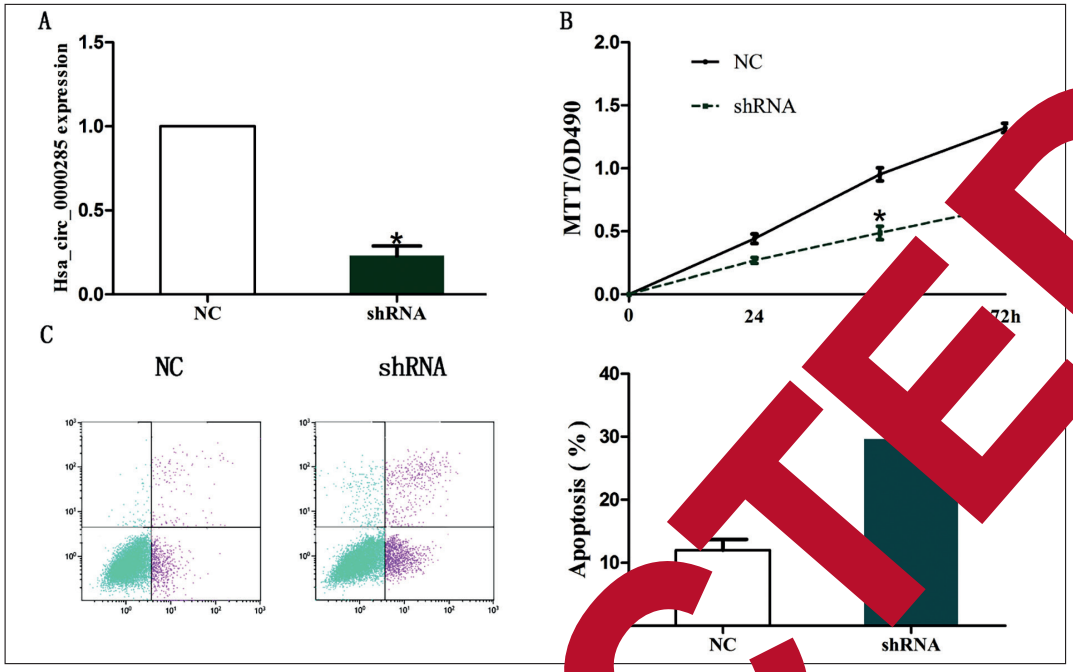
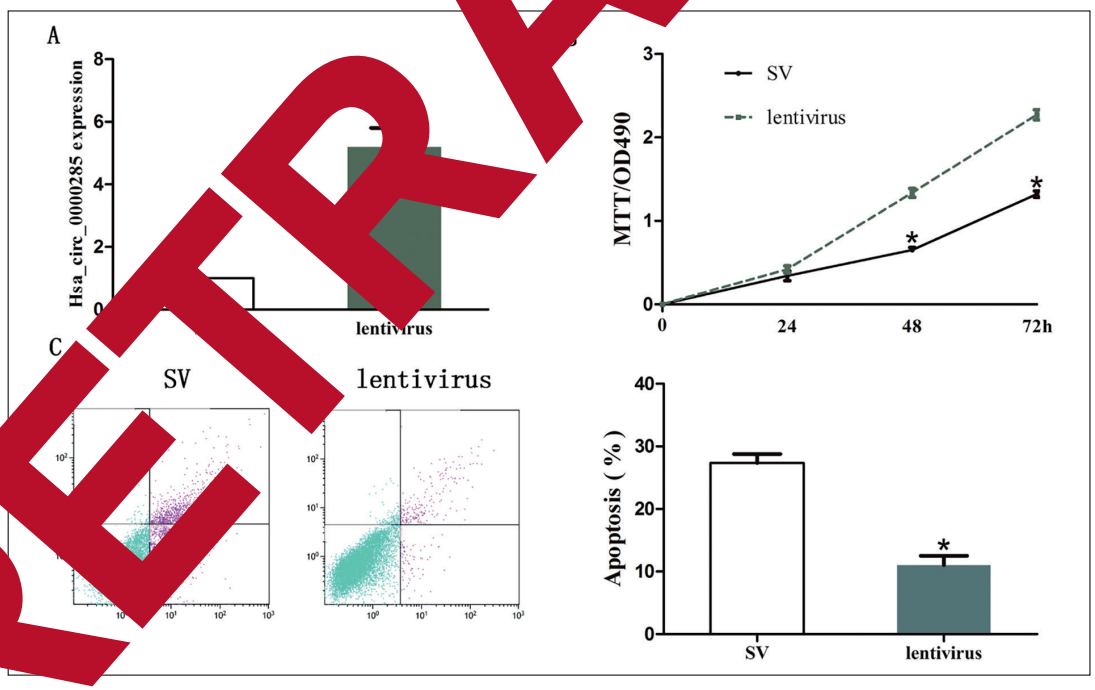


Figure 2. Knockdown of hsa_circ_0000285 inhibited laryngoca ma cell prolif n and promoted cell apoptosis. **A,** Hsa_circ_0000285 expression in laryngocarcinoma cells transduced egative c shRNA (NC) or hsa_circ_0000285 shRNA(shRNA) was detected by RT-qPCR. β-actin was used as an in MTT assay showed that the inhibition of cell viability in laryngocarcinoma cells was induc knockdown of 000285. **C,** Flow cytometry assay results indicated that the percentage of cell apoptosis ra ed after hsa_circ_0000285 was knocked down. The results represent the average of three independent experime error of the mean). *p<0.05.

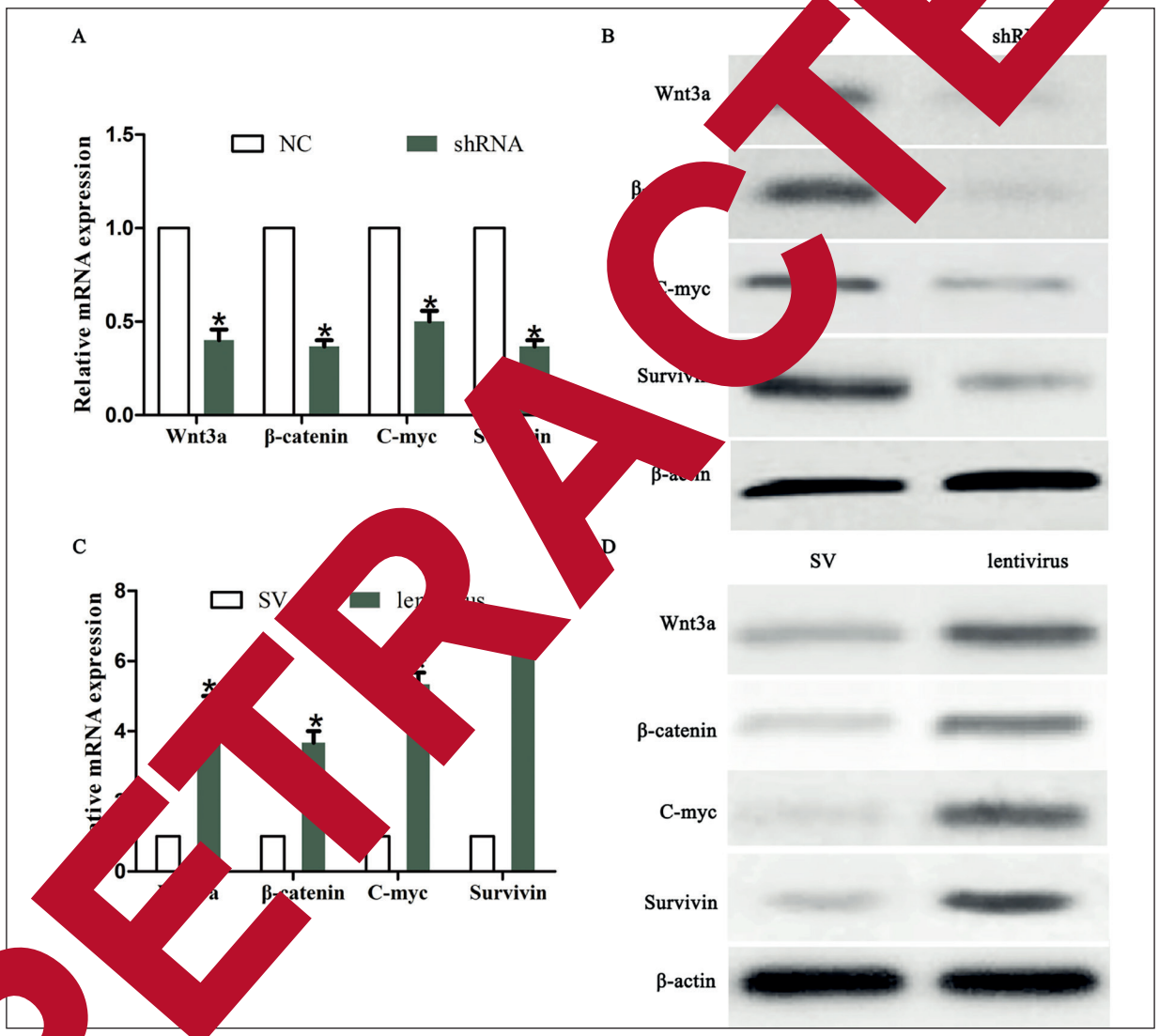


ure 3. Overexpression of hsa_circ_0000285 promoted laryngocarcinoma cell proliferation and inhibited cell apoptosis. **A,** irc_0000285 expression in laryngocarcinoma cells transduced with scramble vector (SV) or hsa_circ_0000285 lentivirus (s) was detected by RT-qPCR. β-actin was used as an internal control. **B,** MTT assay showed that the promotion of cell ability in laryngocarcinoma cells was induced by overexpression of hsa_circ_0000285. **C,** Flow cytometry assay results indicated that the percentage of cell apoptosis rate was decreased after hsa_circ_0000285 was overexpressed. The results represent the average of three independent experiments (mean ± standard error of the mean). *p<0.05.

in Figure 3C, the percentage of cell apoptosis rate was decreased after hsa_circ_0000285 was overexpressed.

The Interaction Between Wnt/β-Catenin Signaling Pathway and Hsa_circ_0000285 in Laryngocarcinoma Cells

To explore the underlying mechanism how hsa_circ_0000285 functioned in laryngocarcinoma, RT-qPCR and Western blot assay were conducted to detect Wnt3a, β-catenin, C-myc, and Survivin, which were the target proteins of Wnt/β-catenin signaling pathway. As was shown in Figure 4A, the mRNA expression of [illegible] teins could be downregulated *via* k[illegible]down hsa_circ_0000285. As was shown [illegible] Figure 4B, the protein levels of those protein[illegible] be downregulated *via* knockdown of hsa_c[illegible]00285. As was shown in Figures [illegible] and Figu[illegible] the mRNA expression and [illegible]teins of thos[illegible]



F[illegible]tion between Wnt/β-catenin signaling pathway and hsa_circ_0000285 in laryngocarcinoma. **A,** RT-qPCR [illegible]lts revealed that the expression of target proteins in Wnt/β-catenin signaling pathway was downregulated in shRNA group [illegible]pared with NC group. **B,** Western blot assay results revealed that the expression of target proteins in Wnt/β-catenin signaling [illegible]ay was downregulated in shRNA group compared with NC group. **C,** RT-qPCR results showed that the expression of target pr[illegible]n Wnt/β-catenin signaling pathway was upregulated in lentivirus group compared with SV group. **D,** Western blot assay result[illegible] indicated that the expression of target proteins in Wnt/β-catenin signaling pathway was upregulated in lentivirus group compared with SV group. The results represent the average of three independent experiments. Data are presented as the mean ± standard error of the mean. *$p<0.05$.

teins could be upregulated *via* overexpression of hsa_circ_0000285. These results suggested that hsa_circ_0000285 participated in the regulation of Wnt/β-catenin signaling pathway and further promoted laryngocarcinoma development.

Discussion

Laryngocarcinoma is one of the most general malignancies in the world. The survival rate is significantly poorer in patients with metastasis than those with non-metastatic disease. Developing effective and targeted therapies for laryngocarcinoma are depended on gaining an improved understanding of the molecular mechanisms underlying laryngocarcinoma-genesis, proliferation, invasion, and metastasis.

Evidence has proved that noncoding RNAs participate in the regulation of cancer development and are used to predict the treatment response, assess the disease state and clinical outcome. For example, RBM6 functions as a tumor suppressor and inhibits the growth and progression of laryngocarcinoma cells[8]. Long noncoding RNA TUG1 is upregulated in laryngocarcinoma which contributes to the progression of laryngocarcinoma *via* targeting the miR-145-5p/ROCK signaling[9]. Low-expression of miR-9 promotes cell proliferation and cell metastasis in laryngocarcinoma by downregulating the expression of miR-9[10]. CircRNAs, as one of the new types of noncoding RNAs, also play vital roles in tumorigenesis. However, the function of circRNAs in laryngocarcinoma remains unclear.

Recently, a novel circRNA hsa_circ_0000285 was found dysregulated in nasopharyngeal carcinoma and identified as a prognostic biomarker in radio-sensitivity[11]. In our study, we researched the function of hsa_circ_0000285 on the proliferation and apoptosis of laryngocarcinoma. Results showed that hsa_circ_0000285 was upregulated in laryngocarcinoma tissues. After hsa_circ_0000285 was knocked down, the cell proliferation was suppressed and cell apoptosis was promoted in laryngocarcinoma cells. Meanwhile, after hsa_circ_0000285 was overexpressed, the cell proliferation was promoted, and apoptosis was inhibited in laryngocarcinoma cells. These data indicated that hsa_circ_0000285 functions as an oncogene and promotes tumorigenesis of laryngocarcinoma.

The Wnt/β-catenin signaling pathway is one of the most important pathways in the progression of tumor proliferation and metastasis, modulating the integrity of the stem cell, stem cell division and migration. Wnt proteins mediate a variety of processes during embryogenesis. Wnt/β-catenin signaling in metastasis-initiating cells is suggested to be an important regulatory role in the progression of several cancers, which may be a promising therapeutic target. For instance, through the downregulation of Wnt/β-catenin signaling and FZD4 expression induced by miR-516b, circRNA_100290 enhances the progression of colorectal cancer[12]. Downregulation of circ-ITCH inhibits cell proliferation and metastasis in triple-negative breast through regulating the Wnt/β-catenin pathway[13]. Through the regulation of miR-135a-5p/SIRT1 pathway, has_circ_0001946 promotes cell growth of lung adenocarcinoma *via* activation of Wnt/β-catenin axis[14]. Circ_0067934 promotes tumor growth and cell proliferation in hepatocellular carcinoma by regulating miR-1324/FZD5/Wnt/β-catenin signaling[15].

In our research, we further explored the association between Wnt/β-catenin pathway and hsa_circ_0000285. Hsa_circ_0000285 knockdown reduced target proteins in the Wnt/β-catenin signaling pathway *in vitro*. Hsa_circ_0000285 overexpression increased target proteins in the Wnt/β-catenin signaling pathway *in vitro*. The above results suggested that hsa_circ_0000285 might promote tumorigenesis of laryngocarcinoma *via* activating the Wnt/β-catenin signaling pathway.

Conclusions

It has been demonstrated in this study that hsa_circ_0000285 is a novel biomarker in the development of laryngocarcinoma and could enhance laryngocarcinoma proliferation and inhibit apoptosis through the activation of the Wnt/β-catenin signaling pathway.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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