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LncRNA WTAPP1 promotes proliferation of laryngeal carcinoma cells through regulating microRNA-592

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Abstract. – OBJECTIVE: The aim of this work was to investigate the mechanism by which long non-coding RNA (lncRNA) WTAPP1 promotes the malignant progression of laryngeal cancer.

PATIENTS AND METHODS: In this study, quantitative real-time polymerase chain reaction (qRT-PCR) examined the expression of lncRNA WTAPP1 in 49 pairs of tumor tissue specimens and paracancerous normal ones collected from laryngeal cancer patients. Subsequently, in the laryngeal squamous cell carcinoma cell lines AMC-HN-8 and Hep-2, WTAPP1 overexpression and knockdown vectors were constructed using lentivirus, and cell counting kit-8 (CCK-8), cell colony formation and 5-ethynyl-2'-deoxyuridine (EdU) assays were carried out to analyze the impact of lncRNA WTAPP1 on the function of laryngeal cancer cells. Finally, Luciferase reporting assay and recovery experiments were carried out to further explore whether lncRNA WTAPP1 has an impact on the malignant progression of laryngeal cancer via modulating microRNA-592.

RESULTS: QRT-PCR results revealed a significantly higher expression of lncRNA WTAPP1 in tumor tissues of patients with laryngeal cancer than that in adjacent normal ones. Compared with patients with low expression of WTAPP1, those with higher expression had a more advanced pathological stage. Meanwhile, the proliferation ability of cells in sh-WTAPP1 group was remarkably attenuated when compared with that in sh-NC group. In addition, microRNA-592 and WTAPP1 mRNA levels were found negatively correlated in laryngeal carcinoma tissue specimens. Luciferase reporter gene assay indicated that WTAPP1 can be targeted by microRNA-592 through certain binding sites. Moreover, we demonstrated through some recovery experiments that WTAPP1 can indeed serve as an oncogene accelerating the malignant progression of laryngeal cancer through the modulation of microRNA-592.

CONCLUSIONS: LncRNA WTAPP was markedly highly expressed both in laryngeal carcinoma tissues and cell lines, which was also found to be closely relevant to the pathological stage of laryngeal cancer patients. Additionally, lncRNA WTAPP1 is able to enhance the proliferation capacity of laryngeal carcinoma cells via regulating microRNA-592.

Key Words:
LncRNA WTAPP1, MicroRNA-592, Laryngeal cancer, Proliferation

Introduction

Laryngeal cancer is one of the most common malignant tumors in head and neck cancer, of which 85% to 90% of pathological types are squamous cell carcinoma (LSC). Studies[1-3] have shown that more than 150,000 patients are diagnosed with LSCC each year. At present, laser resection, partial laryngectomy or radiotherapy are mainly used for the treatment of early laryngeal cancer. After treatment, vocal cords and swallowing function can be retained, and the 5-year survival rate can exceed 90%[4,5]. However, for advanced laryngeal cancer, total laryngectomy or total laryngectomy plus chemoradiotherapy is mostly used, and the surgical treatment is usually accompanied by permanent tracheotomy openings, dysphagia, and loss of natural voice, leading to the 5-year survival rate less than 60%. Therefore, early diagnosis of laryngeal cancer is particularly important[6,7]. At present, the biopsy of primary tumor is generally accepted as the gold standard for the diagnosis of laryngeal cancer, which is mostly performed under general or

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local anesthesia with poor sensitivity and easy missed diagnosis. However, CT, MRI, B-mode ultrasound, and other non-invasive examinations cannot serve as effective diagnosis methods. This often leads to the delay of diagnosis and treatment[8]. To prevent advanced patients with voice loss and dysphagia and improve the prognosis and long-term quality of life of patients with laryngeal cancer, it is extremely urgent to search for new biomarkers to advance the early diagnosis rate of laryngeal cancer and improve treatment efficiency[9,10].

Most of the transcriptional RNA in the human genome, identified as non-coding RNA (ncRNA), may be involved in the complex regulation of human gene expression[11,12]. Long non-coding RNA (lncRNA) is a class of RNA molecules with a length of more than 200 nucleotides and no protein-coding function, but it can interact with DNA, RNA, and proteins to regulate gene expression and cell growth[13,14]. LncRNA plays an essential role in the progression of tumors. Overexpressed lncRNA HOTAlR is engaged in the malignant progression of breast cancer, colon cancer, liver cancer, and gastrointestinal stromal tumor; in addition, lncRNA TARlD can prevent tumor formation by methylating some tumor suppressor genes[15-17]. LncRNA can regulate the occurrence of tumors through epigenetic regulation, DNA damage and cell cycle regulation, microRNA modulation, and participation in signal transduction pathways[18]. LncRNAs possess good tissue specificity and can be used as a potential biomarker for early diagnosis, prognosis, and therapeutic targets of a variety of cancers, which provides a great prospect for finding new diagnostic markers and therapeutic targets for laryngeal cancer[15,16]. LncRNA WTAPP1 plays a key role in human cell differentiation, proliferation, and genome repair[19]. Specifically, the dysregulation of lncRNA WTAPP1 may break heterochromatin stability by inducing abnormal expression of some genes, and thus affect tumor occurrence and progression.

LncRNAs can also be used as competitive endogenous RNA (ceRNA) or miRNA sponge, and competitively bind to miRNAs through miRNA response elements (MREs) to inhibit their function and activity, and thus regulate the expression of target mRNAs for corresponding miRNAs at the post-transcriptional level[20,21]. Recent studies have found that lncRNA WTAPP1 can specifically bind to microRNA-592. Therefore, we explored whether lncRNA WTAPP1 regulates laryngeal cancer proliferation through microRNA-592, so as to provide experimental basis for its clinical application.

Patients and Methods

Patients and Laryngeal Disease Samples

The surgically resected tumor tissue samples and corresponding adjacent ones were collected from 49 laryngeal carcinoma patients, and stored at -80°C. This investigation was approved by the Ethics Committee of The Second Children & Women's Healthcare of Jinan City. Signed written informed consents were obtained from all participants before the study.

Cell Lines and Reagents

Human laryngeal squamous cell carcinoma cell lines (AMC-HN-8 and Hep-2) were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA) and cultured with high glucose Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA), penicillin (100 U/mL), and streptomycin (100 μg/mL) in a 37°C, 5% CO_2 incubator. They were passaged with 1% trypsin + ethylenediaminetetraacetic acid (EDTA) for digestion when grown to 80%-90% confluence.

Transfection

The control sequences (sh-NC) and the lentivirus containing the WTAPP1 knockdown sequences (sh-WTAPP1) were obtained from Shanghai GenePharma Company (Shanghai, China). Cells were plated in 6-well plates and grown to a cell density of 40%, and then transfection was carried out according to the manufacturer's instructions. After 48 h, cells were harvested for quantitative real-time polymerase chain reaction (qRT-PCR) analysis and cytofunctional experiments.

Cell Counting Kit-8 (CCK-8) Assay

After 48 h of transfection, cells were collected and plated into 96-well plates at 2000 cells per well. After cultured for 24 h, 48 h, 72 h, and 96 h respectively, 10 μL of CCK-8 solution (Dojindo, Molecular Technologies, Kumamoto, Japan) was added per well for incubation for 2 h. Then, the optical density (OD) value of each well was measured in the microplate reader at 490 nm absorption wavelength.

Colony Formation Assay

After 48 h of transfection, cells were collected, and 200 cells were seeded in each well of a 6-well plate and cultured with complete medium for 2 weeks. The medium was changed after one week and then twice a week. The medium should not be replaced as much as possible in the previous week to avoid cell adhesion. After 2 weeks, the cells were cloned and then fixed in 2 mL of methanol for 20 min. After the methanol was aspirated, the cells were stained with 0.1% crystal violet staining solution for 20 min, washed 3 times with phosphate-buffered saline (PBS), photographed, and counted under a light-selective environment.

5-Ethynyl-2′-Deoxyuridine (EdU) Proliferation Assay

EDU proliferation assay was performed according to the manufacturer's requirements. After transfection for 24 h, the cells were incubated with 50 μM EDU for 2 h and then stained with AdoLo and 4',6-diamidino-2-phenylindole (DAPI), and the number of EDU-positive cells was detected by fluorescence microscopy. The display rate of EDU positive is shown as the ratio of the number of EDU positive cells to the total DAPI chromogenic cells (blue cells).

QRT-PCR

1 mL of TRIzol (Invitrogen, Carlsbad, CA, USA) was used to lyse the cells, and total RNA was extracted. The initially extracted RNA was treated with DNase I to remove genomic DNA and repurify the RNA. RNA reverse transcription was performed according to the Prime Script Reverse Transcription Kit (TaKaRa, Otsu, Shiga, Japan) instructions, and real-time PCR was performed according to the SYBR® Premix Ex Taq™ (TaKaRa, Otsu, Shiga, Japan) kit instructions using the StepOne Plus Real-time PCR System. The Bio-Rad PCR instrument was used to analyze and process the data with the software iQ5 2.0 (Bio-Rad, Hercules, CA, USA). The glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and U6 genes were used as internal parameters. The following primers were for qPCR reaction: lncRNA WTAPP1: forward: 5'-GGGCGACTCCAAGGAAAC-3', reverse: 5'-CCTGCGGCAAAACCCAAC-3'; GAPDH: forward: 5'-GTCAAGGCTGAGAACGGGAA-3', reverse: 5'-AAATGAGCCCCAGCCTTCTC-3'; microRNA-592: forward: 5'-TTGTGTCAATATCGATGATGT-3', reverse: 5'-GCGAGCACAGAATTAATAATACGAC-3'; U6: forward: 5'-CTCGCTTCGGCAGCACA-3', reverse: 5'-AACGCTTCACGAATTTGCG-3'.

Dual-Luciferase Reporter Assay

HEK293T cells were seeded in 24-well plates and co-transfected with microRNA-592 mimic/NC and pMIR Luciferase reporter plasmids. The plasmid was then introduced into the cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. After 48 h of transfection, the Luciferase activity of each group was detected and normalized by a Dual-Luciferase reporter assay system (Promega, Madison, WI, USA).

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 5 V5.01 software (La Jolla, CA, USA). Differences between two groups were analyzed by using the Student's *t*-test. Comparison between multiple groups was done using One-way ANOVA test followed by Post-Hoc Test (Least Significant Difference). Independent experiments were repeated at least three times for each experiment and data were expressed as mean ± standard deviation. Kaplan-Meier method with the log-rank test was used for the survival analysis. $p<0.05$ was considered as statistically significant.

Results

LncRNA WTAPP1 Was Highly Expressed In Laryngeal Cancer Tissues and Cell Lines

We collected a total of 49 pairs of tumor tissue specimens and paracancerous ones from laryngeal carcinoma patients to examine the expression of WTAPP1 by qPCR. The results revealed that tumor tissues contained significantly higher expression of lncRNA WTAPP1 than adjacent ones (Figure 1A), suggesting that WTAPP1 may act as an oncogene in laryngeal cancer. To explore the interplay between WTAPP1 and the prognosis of laryngeal cancer patients, we plotted Kaplan-Meier survival curve to reveal that high expression of WTAPP1 was remarkably correlated with poor prognosis of laryngeal carcinoma, namely, the higher the WTAPP1 level, the worse the prognosis ($p<0.05$; Figure 1B). In addition, according to the mRNA expression of WTAPP1. We divided the above 49 pairs tissue samples into two groups, high and low expression group, and analyzed the

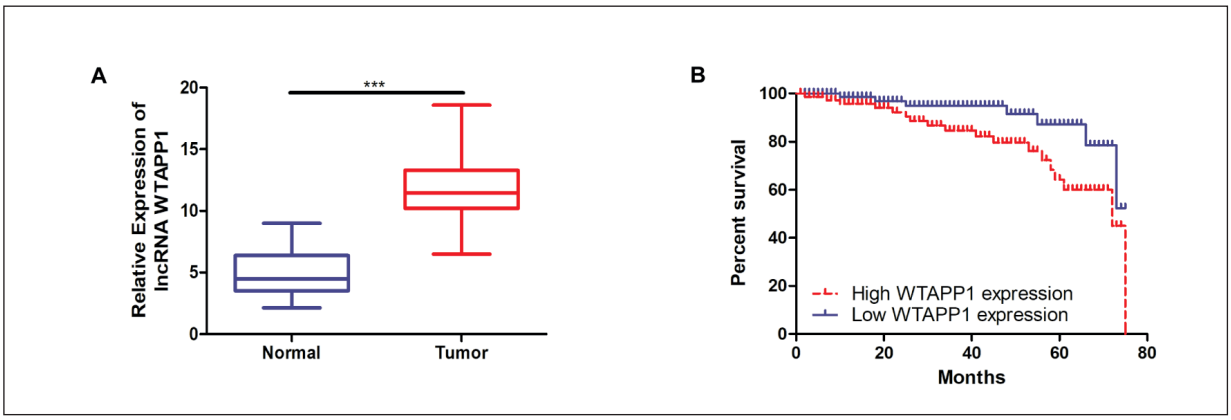

Figure 1. LncRNA WTAPP1 is highly expressed in laryngeal carcinoma tissues and cell lines. **A,** qRT-PCR was used to detect the difference in expression of WTAPP1 in laryngeal carcinoma and adjacent tissues; **B,** Kaplan Meier survival curve of laryngeal cancer patients was plotted based on lncRNA WTAPP1 expression; the prognosis of patients with high expression of WTAPP1 was significantly worse than that of those in low expression group. Data are mean ± SD, ***p<0.001.

correlation between WTAPP1 and clinical indexes of laryngeal cancer patients. Consequently, as shown in Table I, WTAPP1 was closely relevant to the pathological stage of laryngeal cancer patients.

Knockdown of LncRNA WTAPP1 Inhibited Cell Proliferation In Laryngeal Cancer

We subsequently constructed a knockdown WTAPP1 model in AMC-HN-8 and Hep-2 cell lines using lentiviral as a vector to clarify the impact of WTAPP1 on laryngeal carcinoma cell function and verified the interference efficiency by qPCR (Figure 2A). CCK-8, cell colony formation, and EdU assays were carried out to detect the proliferation capacity of AMC-HN-8 and Hep-2 cell lines; as a result, the knockdown of WTAPP1 dramatically attenuated that when compared to the sh-NC group (Figure 2B-2D).

LncRNA WTAPP1 Was Bound to MicroRNA-592

To further explore the ways in which WTAPP1 promotes the malignant progression of laryngeal cancer, we predicted by bioinformatics analysis that a relationship could exist between WTAPP1 and microRNA-592. As shown in Figure 3A, Luciferase reporter assay demonstrated that lncRNA WTAPP1 can be targeted by microRNA-592 *via* a specific binding site. Subsequently,

Table I. Association of lncRNA WTAPP1 expression with clinicopathologic characteristics of laryngeal cancer.

Parameters	No. of cases	lncRNA WTAPP1 expression		*p*-value
		Low (%)	High (%)	
Age (months)				0.761
< 60	31	15	11	
≥ 60	18	15	13	
Gender				0.484
Male	29	16	13	
Female	20	9	11	
T stage				0.001
T1-T2	24	18	6	
T3-T4	25	7	18	
Lymph node metastasis				0.830
No	34	17	17	
Yes	15	8	7	
Distant metastasis				0.686
No	34	18	16	
Yes	15	7	8	

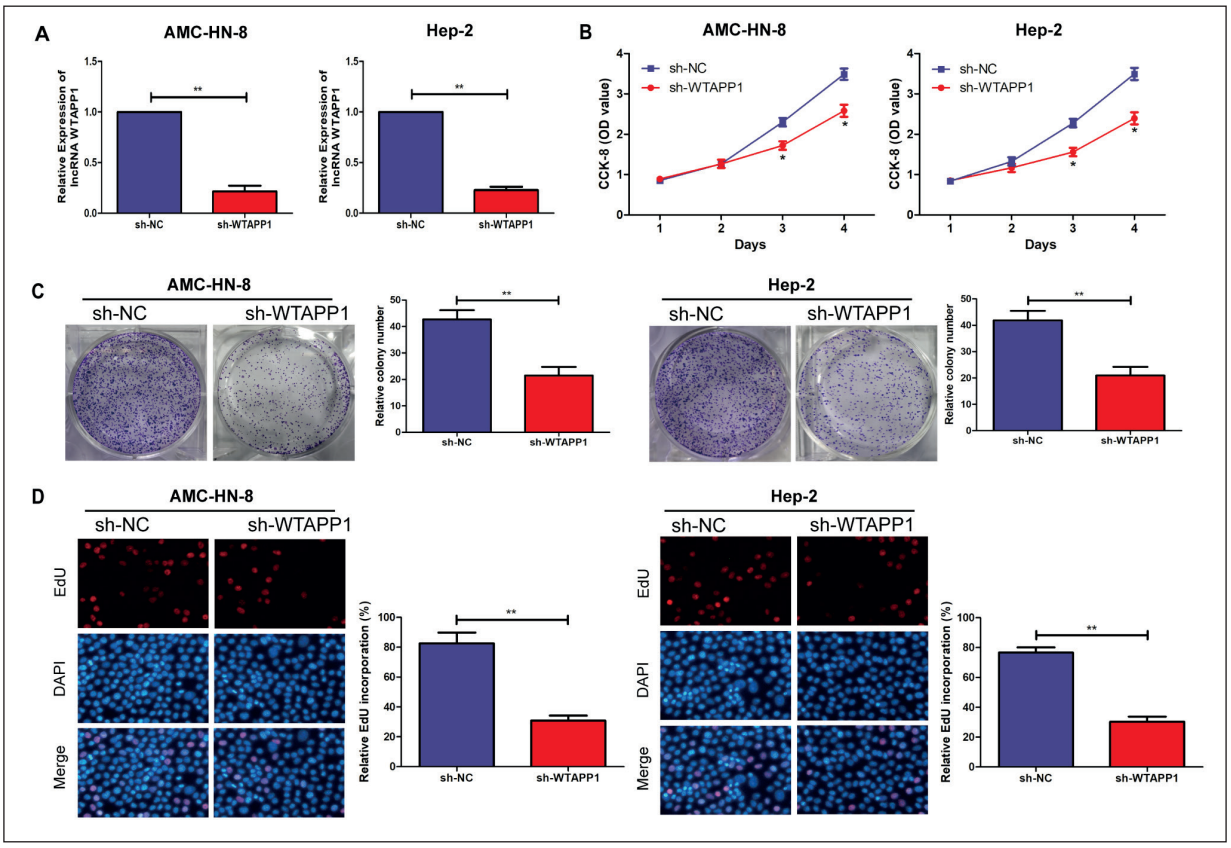

Figure 2. Silencing lncRNA WTAPP1 inhibits the proliferation of laryngeal carcinoma cells. **A,** qRT-PCR verified the interference efficiency after transfection of the knockdown WTAPP1 vector in laryngeal carcinoma AMC-HN-8 and Hep-2 cell lines; **B,** CCK-8 assay detected the effect of WTAPP1 knockdown on laryngeal carcinoma cell proliferation in AMC-HN-8 and Hep-2 cell lines; **C,** Plate cloning experiments were performed to detect the number of laryngeal carcinoma proliferation-positive cells transfected with WTAPP1 knockdown vector in laryngeal carcinoma AMC-HN-8 and Hep-2 cell lines (magnification: 10×); **D,** EdU assay detected the proliferation of laryngeal carcinoma cells in AMC-HN-8 and Hep-2 cell lines after knockdown of WTAPP1 (magnification: 40×). Data are mean ± SD, *p<0.05, **p<0.01.

qPCR experiments revealed that microRNA-592 was remarkably reduced in above-mentioned tumor tissues compared with the paracancerous normal ones (Figure 3B). Furthermore, to specify the association between microRNA-592 and prognosis of laryngeal cancer patients, we also depicted a Kaplan-Meier survival curve, which revealed that low expression of microRNA-592 was remarkably relevant to the poor prognosis of laryngeal cancer (p<0.05; Figure 3C). As a result, according to above qPCR results, we concluded that the expression levels of WTAPP1 and microRNA-592 were negatively correlated in laryngeal carcinoma tissues (Figure 3D).

Knockdown of MicroRNA-592 Promoted Cell Proliferation In Laryngeal Cancer

We subsequently transfected a microRNA-592 knockdown vector into AMC-HN-8 and Hep-2 cell lines to further explore the impact of microRNA-592 on the cell functions of laryngeal carcinoma, and also detected the interference efficiency through performing qPCR (Figure 4A). Consequently, the results of CCK-8, plate cloning, and EdU assays revealed that the proliferation capacity of laryngeal carcinoma cells in the microRNA-592 inhibitor group was remarkably enhanced compared with the NC group (Figure 4B-4D).

LncRNA WTAPP1 Modulated MicroRNA- 92 In Laryngeal Cancer Cell Lines

After simultaneous knockdown of lncRNA WTAPP1 and microRNA-592 in AMC-HN-8 and Hep-2 cell lines, we examined the expression level of WTAPP1 and found that microRNA-592 reversed the expression of WTAPP1 (Figure 5A). In addition, knockdown of microRNA-592 was

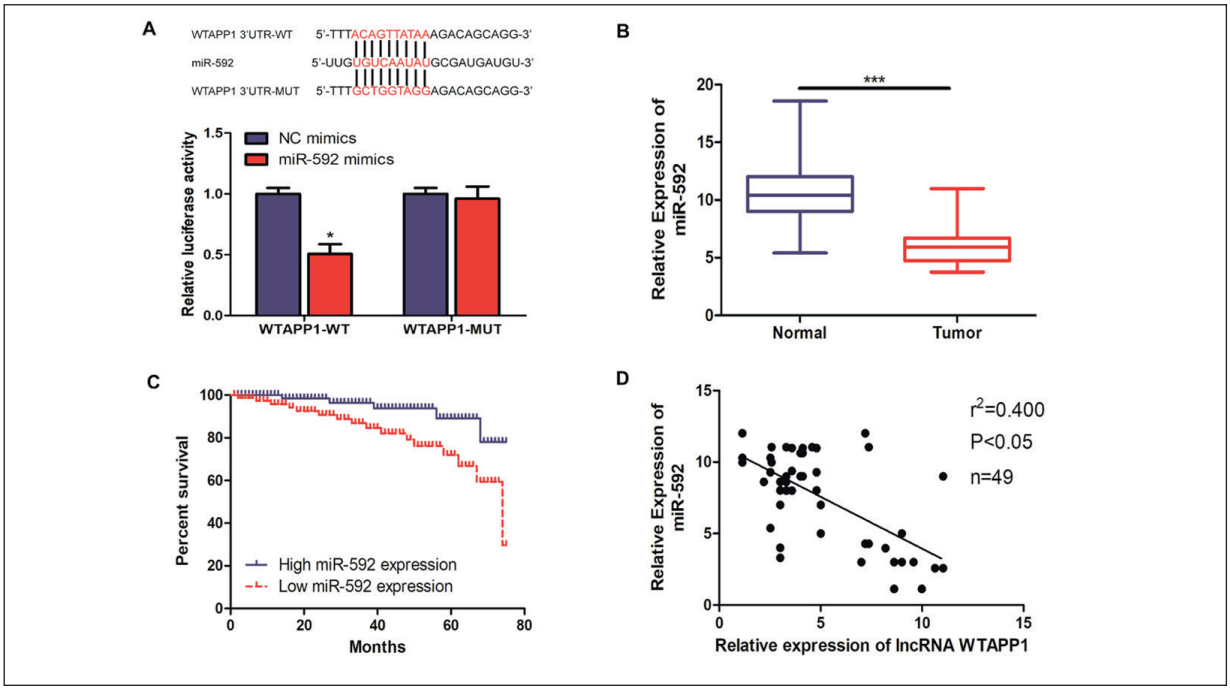

Figure 3. LncRNA WTAPP1 direct targeting of miR-592. **A,** Dual-Luciferase reporter gene assay verified the direct targeting of lncRNA WTAPP1 and miR-592; **B,** qRT-PCR was used to detect the differential expression of miR-592 in tumor tissues and paracancerous tissues of patients with laryngeal cancer; **C,** Kaplan Meier survival curve of laryngeal cancer patients based on miR-592 expression was shown; patients with low expression group had significantly worse prognosis than those in high expression group; **D,** There was a significant negative correlation between the expression levels of WTAPP1 and miR-592 in laryngeal carcinoma tissues. Data are mean ± SD, *p<0.05, ***p<0.001.

found to partially counteract the enhanced proliferation ability of laryngeal carcinoma cells induced by knockdown of WTAPP1 (Figure 5B-5C).

Discussion

Laryngeal cancer is a common malignant tumor of otolaryngology head and neck surgery,

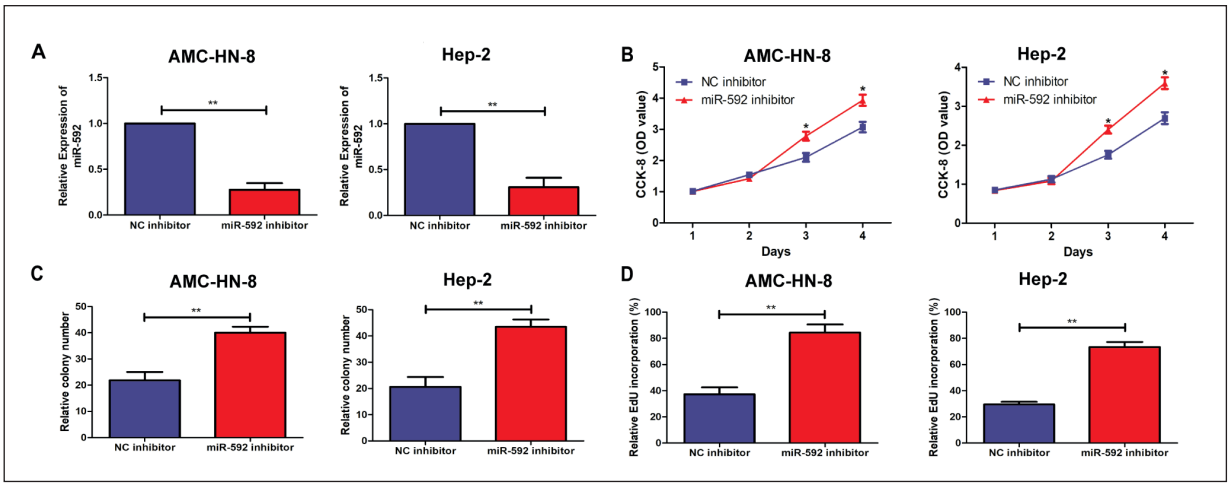

Figure 4. Silencing miR-592 promotes the proliferation of laryngeal cancer cells. **A,** qRT-PCR verified the interference efficiency of miR-592 knockdown vector in AMC-HN-8 and Hep-2 cell lines; **B,** CCK-8 assay detected the effect of transfection of miR-592 knockdown vector on laryngeal carcinoma cell proliferation in AMC-HN-8 and Hep-2 cell lines; **C,** Plate cloning experiments were performed to detect the number of laryngeal carcinoma proliferation-positive cells transfected with miR-592 knockdown vector in AMC-HN-8 and Hep-2 cell lines; **D,** The EdU assay detected the proliferation of laryngeal carcinoma cells in AMC-HN-8 and Hep-2 cell lines transfected with miR-592 knockdown vector. Data are mean ± SD, *p<0.05, **p<0.01.

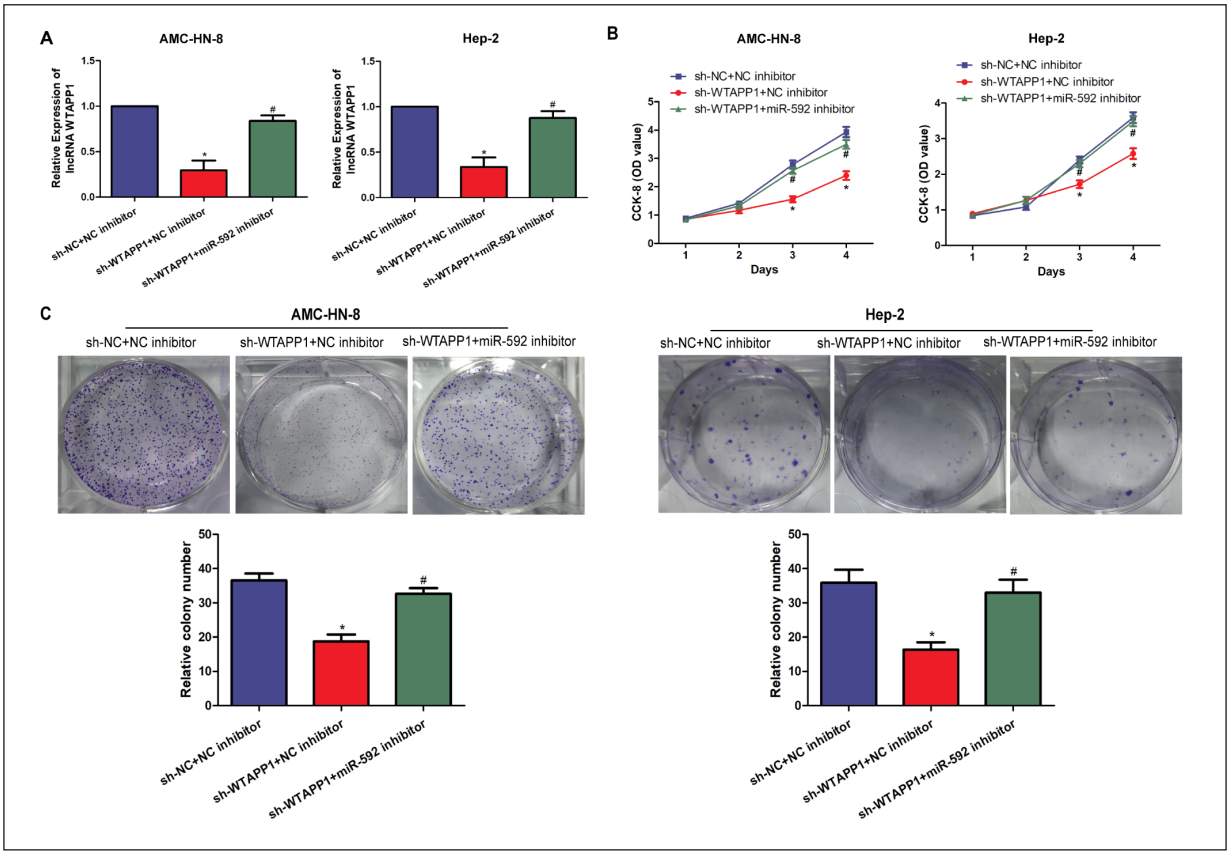

Figure 5. LncRNA WTAPP1 regulates the expression of miR-592 in laryngeal carcinoma tissues and cell lines. **A,** Expression level of lncRNA WTAPP1 was detected by qRT-PCR after co-transfection of lncRNA WTAPP1 and miR-592 knockdown vector in laryngeal carcinoma cell lines; **B,** CCK-8 assay detected the proliferation of laryngeal carcinoma cells after co-transfection of lncRNA WTAPP1 and miR-592; **C,** Plate cloning experiments detected the number of laryngeal carcinoma proliferation positive cells after co-transfection of lncRNA WTAPP1 and miR-592 (magnification: 10×). The data are average ± SD, *#$p<0.05$.

ranking second in epithelial malignant tumors of the head and neck and accounting for 5.7%-7.6% of systemic tumors[1-4]. In recent years, an evident growth trend has been found in the incidence of laryngeal cancer, which seriously threatens people's health[4,5]. The early diagnosis rate of laryngeal cancer still remains relatively low, especially for supraglottic and subglottic laryngeal cancers, which are usually observed at advanced stage and lymph node metastasis also occur, seriously affecting the prognosis and survival rate of patients suffered from laryngeal cancer[6-9]. At present, the research on the relationship between lncRNA and laryngeal cancer still remains at an initial stage, but has gradually attracted increasing attention from scholars[9,10].

LncRNAs are transcripts that do not code proteins. They do not have open reading frames and can be widely found in multicellular organisms. They were once considered as by-products of the transcription process, but several studies[11,12] have shown that lncRNAs are involved in biological processes such as gene imprinting, chromatin modification, mRNA degradation, and DNA methylation. Some lncRNAs have been discovered to be correlated with the diagnosis and prognosis prediction of various tumors, which opens up a new direction for tumor research[13-15]. LncRNA has been found to be associated with the occurrence of tumors, coronary heart disease, diabetes mellitus, Alzheimer's disease, and other diseases[13,14]. Similar to short chain ncRNA, lncRNA is abnormally expressed in most tumor cells and can act as oncogenes or tumor suppressor genes[16-18]. The dysregulation of lncRNA WTAPP1 may directly induce tumor occurrence; however, the relationship between the dysregulation of lncRNA WTAPP1 and laryngeal cancer development still remains to be accurately clarified[19]. Therefore, in this study,

we investigated and further determined the correlation between lncRNA WTAPP1 and the progression of laryngeal cancer. The expression of lncRNA WTAPP1 in the tumor tissues of laryngeal cancer patients was found to be remarkably higher than that in the adjacent ones and was positively correlated with the poor prognosis of patients, suggesting that lncRNA WTAPP1 may act as a cancer-promoting gene in this disease. To further clarify the molecular mechanism of how lncRNA WTAPP1 affects the progression of laryngeal cancer, we conducted an *in vitro* experiment and found that silencing WTAPP1 dramatically reduced the proliferation ability of laryngeal cancer cells.

LncRNAs can act as ceRNA or miRNA sponge, competitively bind to miRNAs and inhibit their functions and activity, and therefore regulate the expression of target mRNAs of certain miRNAs at the post-transcriptional level. In this way, lncRNAs can be involved in a variety of biological behavior processes, including tumor cell proliferation, invasion, metastasis, and angiogenesis[20,21]. By bioinformatics analysis, our previous studies predicted an interaction between microRNA-592 and lncRNA WTAPP1. In this report, we have indicated the direct binding of lncRNA WTAPP1 to downstream microRNA-592 through Dual-Luciferase reporter gene assay. MicroRNA-592 was found less expressed in tumor tissues of laryngeal cancer patients and negatively correlated with lncRNA WTAPP1 expression. In summary, the above findings suggested that lncRNA WTAPP1 could down-regulate the expression of microRNA-592, thus promoting the proliferation of laryngeal squamous cell carcinoma cells.

Conclusions

Taken together, lncRNA WTAPP1 expression was remarkably increased both in laryngeal cancer tissues and cell lines and it was also remarkably correlated with the incidence of lymph node metastasis of laryngeal cancer patients. In addition, it was found that lncRNA WTAPP1 may promote malignant progression of laryngeal cancer by regulating microRNA-592.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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