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MiR-199a-5p suppresses proliferation and invasion of human laryngeal cancer cells

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Abstract. – OBJECTIVE: To explore the roles of micro ribonucleic acid (miR)-199a-5p in the proliferation, apoptosis, invasion and metastasis of laryngeal cancer cells, and its molecular mechanisms.

PATIENTS AND METHODS: The expression of miR-199a-5p in 25 cases of laryngeal cancer tissues and paracancerous tissues was detected via quantitative real-time polymerase chain reaction (qRT-PCR). Its expression in TU212, TU686 and human epithelial type 2 (HEp-2) laryngeal cancer cell lines and normal nasopharyngeal epithelial cell line NP69 was also detected via qRT-PCR. HEp-2 cells were transiently transfected with miR-199a-5p mimic or miR-199a-5p inhibitor, and the expression of miR-199a-5p was verified using RT-PCR after transfection. The regulatory effects of miR-199a-5p on the proliferation, apoptosis, invasion and migration abilities of HEp-2 cells were observed through methyl thiazolyl tetrazolium (MTT) assay, flow cytometry, wound healing assay and transwell assay, respectively. Then, the mechanisms of miR-199a-5p in regulating Caspase-3 activity and epithelial-mesenchymal transition (EMT)-related proteins were further explored.

RESULTS: The qRT-PCR results revealed that miR-199a-5p was significantly lowly expressed in the laryngeal cancer tissues and tumor cell lines, and overexpression of miR-199a-5p substantially inhibited the proliferation of HEp-2 cells. According to the results of flow cytometry, overexpression of miR-199a-5p promoted the apoptosis of HEp-2 cells, whereas down-regulating miR-199a-5p suppressed their apoptosis. It was found that the activity of Caspase-3 was notably enhanced after overexpression of miR-199a-5p, which was evidently weakened after down-regulating miR-199a-5p. Wound healing assay and transwell assay results manifested that overexpressing miR-199a-5p weakened the invasion and migration abilities of HEp-2 cells, which were facilitated by down-regulating miR-199a-5p. Based on Western blotting results, miR-199a-5p regulated the expressions of E-cadherin, N-cadherin and vimentin. Overexpression of miR-199a-5p could inhibit EMT process, whereas suppressing miR-199a-5p could accelerate the process.

CONCLUSIONS: The expression of miR-199a-5p in laryngeal cancer tissues is substantially lower than that in the paracancerous tissues. MiR-199a-5p suppresses proliferation, invasion and migration in laryngeal cancer cell proliferation, while triggers cell apoptosis.

Key Words:

MiR-199a-5p, Laryngeal cancer, Invasion, Proliferation.

Introduction

Head and neck tumors belong to the major life-threatening cancers in humans, and rank 6th in tumor morbidity rate. Laryngeal cancer is one the most prevalent malignancies in the head and neck[1]. The cause of laryngeal cancer remains elusive. It is generally considered that smoking, drinking, exposure to hazardous dust and papillomavirus infection are major pathogenic factors of laryngeal cancer. The development and progression of laryngeal cancer is a complex process involving multi-factor, multi-gene and multi-pathway changes. Primary site, differentiation degree and tumor size of laryngeal cancer largely determine the infiltration and metastasis. However, the molecular mechanism of the development and progression of laryngeal cancer have not yet been clarified. Although new surgical methods, chemotherapeutic drugs and advanced radiotherapeutic strategies have been applied to the treatment of laryngeal cancer in the past three decades, the overall survival rate of laryngeal cancer fails to be significantly improved. Therefore, elucidating the key molecular mechanisms of cell proliferation, apoptosis and metastasis of laryngeal cancer will provide theoretical basis for

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illustrating the pathogenesis of laryngeal cancer and references for the development of novel therapeutic targets.

Micro ribonucleic acids (miRNAs), a class of non-coding small RNAs with 18-24 nt in length, complementarily pair with the 3' untranslated region of their target mRNAs to regulate the translation of the target mRNA molecules or directly degrade them[2]. As the understanding of miRNA functions is being deepened, miRNAs are found to be involved in regulating cell growth and differentiation, energy metabolism, apoptosis and other important and fundamental cellular processes. Besides, they exert extremely vital effects on individual development and disease occurrence[3]. MiR-199a-5p is lowly expressed in multiple tumor tissues or cells, such as breast cancer, lung cancer, and bladder cancer tissues or cells[4-6]. However, there are few reports on the role of miR-199a-5p in laryngeal cancer currently. The present study, therefore, aims to explore the expression of miR-199a-5p in laryngeal cancer and its influences on the proliferation, apoptosis, invasion and migration abilities of laryngeal cancer cells, as well as the possible mechanisms therein.

Patients and Methods

Collection of Laryngeal Cancer Samples

Laryngeal cancer tissue and paracancerous tissue samples were collected from 25 patients undergoing surgery in our hospital from January 2016 to December 2018, and all cases were pathologically confirmed. None of them were preoperatively treated with neoadjuvant chemotherapy, radiotherapy or other anti-cancer treatment. After being surgically resected, the specimens were immediately cryopreserved in liquid nitrogen and then in a refrigerator at -80°C. All the patients signed the informed consent, and this investigation was approved by the Ethics Committee of the First Hospital of Jilin University.

Materials

Laryngeal cancer cell lines TU212, TU686 and human epithelial type 2 (HEp-2) and normal nasopharyngeal epithelial cell line NP69 were purchased from Shanghai Institute of Biochemistry and Cell Biology, CAS (Shanghai, China). Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), penicillin 100 UI/mL, streptomycin 100 μg/mL, and trypsin from Gibco (Rockville, MD, USA), hematoxylin staining solution from Fuzhou Maxim Biotechnology Co., Ltd. (Fuzhou, China), primary antibodies used for Western blotting from BD (Franklin Lakes, NJ, USA), horseradish peroxidase (HRP)-labeled secondary antibodies from Beyotime Biotechnology (Shanghai, China), and TRIzol reagent from Invitrogen (Carlsbad, CA, USA). The sequences were synthesized by Shanghai GenePharma Co., Ltd. (Shanghai, China).

Cell Culture and Transfection

All cell lines were routinely cultured using DMEM containing 10% FBS in an incubator with 5% CO_2 at 37°C. The medium was replaced every 1-2 d, and the cells were sub-cultured when growing to 80-90% confluence.

The HEp-2 cells in the logarithmic growth phase were inoculated into a 6-well plate at 4×10^4 cells/well. When the cell density reached to 30-50%, the cells were transfected with miR-199a-5p mimic, mimic negative control (NC), miR-199a-5p inhibitor or inhibitor NC using Lipofectamine 2000 in accordance with the instructions. After being cultured in the incubator with 5% CO_2 at 37°C for 4-6 h, the mixture medium in each well was removed and replaced with fresh medium. Cells were cultured for another 24 h for subsequent experiments.

Cell Proliferation Assay

The above-mentioned transfected HEp-2 cells were seeded into a 96-well plate at 1×10^3 cells/well. 10 μL of CCK-8 reagent was applied per well. Then, the cells were cultured in the incubator with 5% CO_2 at 37°C. Finally, the absorbance in each well was measured using a microplate reader at 1, 2, 3 and 4 d after culture, and the cell growth curve was plotted.

Caspase-3 Activity Measurement

After transfection for 24 h, the density of HEp-2 cells was adjusted to 4×10^3 cells/mL. The activity of Caspase-3 was measured according to the instructions of the Caspase-3 activity measurement kit. Finally, the absorbance was determined at 405 nm.

Flow Cytometry

The transfected HEp-2 cells were prepared into single-cell suspension with 8×10^3 cells/mL. 1 mL of cell suspension was centrifuged at 4°C and 1,000 rpm for 10 min. With the supernatant discarded, the cells were added with 1 mL of pre-

cold phosphate-buffered saline (PBS), slightly shaken, and centrifuged as above. The supernatant was removed, and the precipitant was re-suspended in 200 μL of binding buffer 10 μL of Annexin V-FITC (fluorescein isothiocyanate) and 5 μL of Propidium Iodide (PI) were applied. Subsequently, the mixture was moderately shaken evenly and reacted in the dark at room temperature for 15 min, followed by detection using a flow cytometer.

RNA Isolation and Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Total RNAs were isolated from the tissues and cells using TRIzol, and 1 g of RNAs were reversely transcribed to complementary deoxyribose nucleic acids (cDNAs). With 2 μL of reverse transcription products as the template of PCR, the content of miR-199a-5p was measured using fluorescence qRT-PCR. PCR was performed using the following primer sequences (U6 Forward: 5'-CTTCGGCAGCACATATAC-3', Reverse: 5'-GAACGCTTCACGAATTTGC-3', and miR-199a-5p Forward: 5'- TTATTACCCAGGCAG-ACACCG -3', Reverse: 5'-AGTGCGAACTGT-GGCGAT-3') in a reaction system (20 μL). The reaction conditions were as follows: denaturation at 95°C for 10 s, annealing at 58°C for 10 s and extension at 72°C for 10 s, for 40 cycles. With U6 as the loading control for the quantification of miRNAs, the relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method.

Wound Healing Assay

After transfection, the HEp-2 cells were inoculated into a 6-well plate, and the adherent cells were cultured in the serum-free DMEM until the cell confluence reached up to 90-100%. Then, a scratch was made slowly using a 10 μL pipette tip perpendicular to the bottom of the 6-well plate. Subsequently, the cells were washed using PBS 3 times to remove the un-adherent cells. After culture for 48 h, the migration distance of cells in the scratch area was observed under a microscope, and several different fields were randomly selected and photographed.

Transwell Assay

Transwell chamber was pre-coated with 6 μL of Matrigel diluted in 60 μL of DMEM and inserted in each well of a 24-well plate for at least 5-h incubation at 37°C. 1×10^4 cells were applied on the upper chamber. 48 h later, cells invade to the bottom were fixed, stained, photographed and recorded under a microscope in at least 5 selected fields.

Western Blotting Analysis

Following concentration determination, 30 μg of total proteins from each group were subjected to vertical sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis, with the separation gel concentration of 10%. Protein samples were transferred onto a polyvinylidene difluoride (PVDF) membrane at 400 mA for 90 min, blocked using 5% skim milk for 2 h, and incubated with E-cadherin, N-cadherin, vimentin and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primary antibodies diluted in Tris-buffered saline and Tween-20 (TBST) at 4°C overnight. After being washed with TBST for 3 times, the membrane was incubated with the secondary antibodies for 2 h and washed again with TBST for 3 times. Band exposure was achieved by enhanced chemiluminescence (ECL). Finally, the optical density of target bands was measured.

Statistical Analysis

Statistical analysis was performed using Statistical Product and Service Solutions (SPSS) 22.0 software (IBM, Armonk, NY, USA). Data were represented as mean ± SD (Standard Deviation). The *t*-test was used for analyzing measurement data between two groups. Comparison between multiple groups was done using One-way ANOVA test, followed by Post-Hoc Test (Least Significant Difference). $p<0.05$ indicated a significant difference.

Results

MiR-199a-5p Was Lowly Expressed in Laryngeal Cancer Tissues and Cells

To explore the potential role of miR-199a-5p in the development and progression of laryngeal cancer, the expression of miR-199a-5p in laryngeal cancer tissues and paracancerous tissues was first detected *via* qRT-PCR. According to the results, the expression level of miR-199a-5p in the laryngeal cancer tissues was significantly lower than that in the paracancerous tissues, showing a statistically significant difference (Figure 1A). Meanwhile, it was found that miR-199a-5p was downregulated in the three laryngeal cancer cell lines TU212, TU686 and HEp-2 compared to the

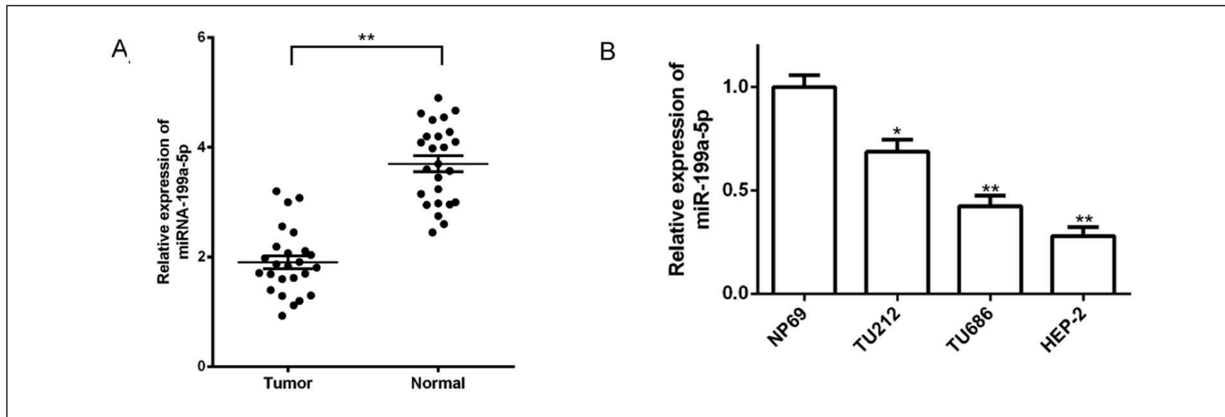

Figure 1. MiR-199a-5p expression was downregulated in laryngeal cancer tissue and cells. **A**, qRT-PCR analysis of miR-199a-5p expression levels in 25 pairs of laryngeal cancer tissues and adjacent normal tissues. **B,** qRT-PCR analysis of miR-199a-5p expression levels in laryngeal cancer cell lines (TU212, TU686 and HEp-2) and normal nasopharyngeal epithelial cell line NP69. Data are presented as means ± SD (n = 3). *p<0.05, **p<0.01 *vs.* control group.

normal nasopharyngeal epithelial cell line NP69 (Figure 1B). The above results suggested that miR-199a-5p may inhibit the development and progression of laryngeal cancer.

MiR-199a-5p Suppressed the Proliferation and Induced Apoptosis of HEp-2 Cells

It was discovered through the qRT-PCR that the expression level of miR-199a-5p was the lowest in HEp-2 cell lines among the tested laryngeal cancer cell lines, so the HEp-2 cells were selected for the exploration of specific mechanisms in this study. First, transfection efficacy of miR-199a-5p mimic and inhibitor was verified in HEp-2 cells (Figure 2A, 2B). Subsequently, MTT assay was performed to explore the regulatory role of miR-199a-5p in the proliferation of laryngeal cancer cells. The results manifested that the proliferation ability of the HEp-2 cells transfected with miR-199a-5p mimic was worse than that in control group (Figure 2C). Conversely, the proliferation ability of those transfected with miR-199a-5p inhibitor was substantially enhanced compared with that in control group (Figure 2D). Furthermore, it was found through the cell apoptosis assay that overexpressing miR-199a-5p markedly promoted the apoptosis of HEp-2 cells (Figure 2E), while down-regulating miR-199a-5p remarkably inhibited their apoptosis (Figure 2F). Additionally, the activity of Caspase-3 in HEp-2 laryngeal cancer cells was enhanced after up-regulating miR-199a-5p, but weakened after down-regulating its expression (Figure 2G, 2H).

MiR-199a-5p Modulated the Migration and Invasion Abilities of HEp-2 Cells

Since invasion and metastasis are the major characteristics of malignant tumors, the effects of miR-199a-5p on the migration and invasion of laryngeal cancer cells were further analyzed. According to the results of the wound healing assay, the migration distance of cells in miR-199a-5p mimic group was markedly shorter than that in control group, while the distance in miR-199a-5p inhibitor group was longer than that in control group (Figure 3A, 3B). It is indicated that miR-199a-5p can weaken the migration ability of HEp-2 cells. Then, transwell assay discovered that the invasion ability of cells in miR-199a-5p mimic group was significantly inhibited, while was enhanced after knockdown of inhibitor (Figure 3C, 3D). Further, the wound healing assay also showed the similar results.

MiR-199a-5p Inhibited the Epithelial-Mesenchymal Transition (EMT) Process of HEp-2 Cells

The expressions of EMT-related molecules were detected using Western blotting. Of note, the Western blotting results revealed that after overexpressing miR-199a-5p, the expression of E-cadherin was substantially up-regulated, while the expressions of N-cadherin and vimentin were remarkably down-regulated. Knockdown of miR-199a-5p achieved the opposite results (Figure 4). These results imply that miR-

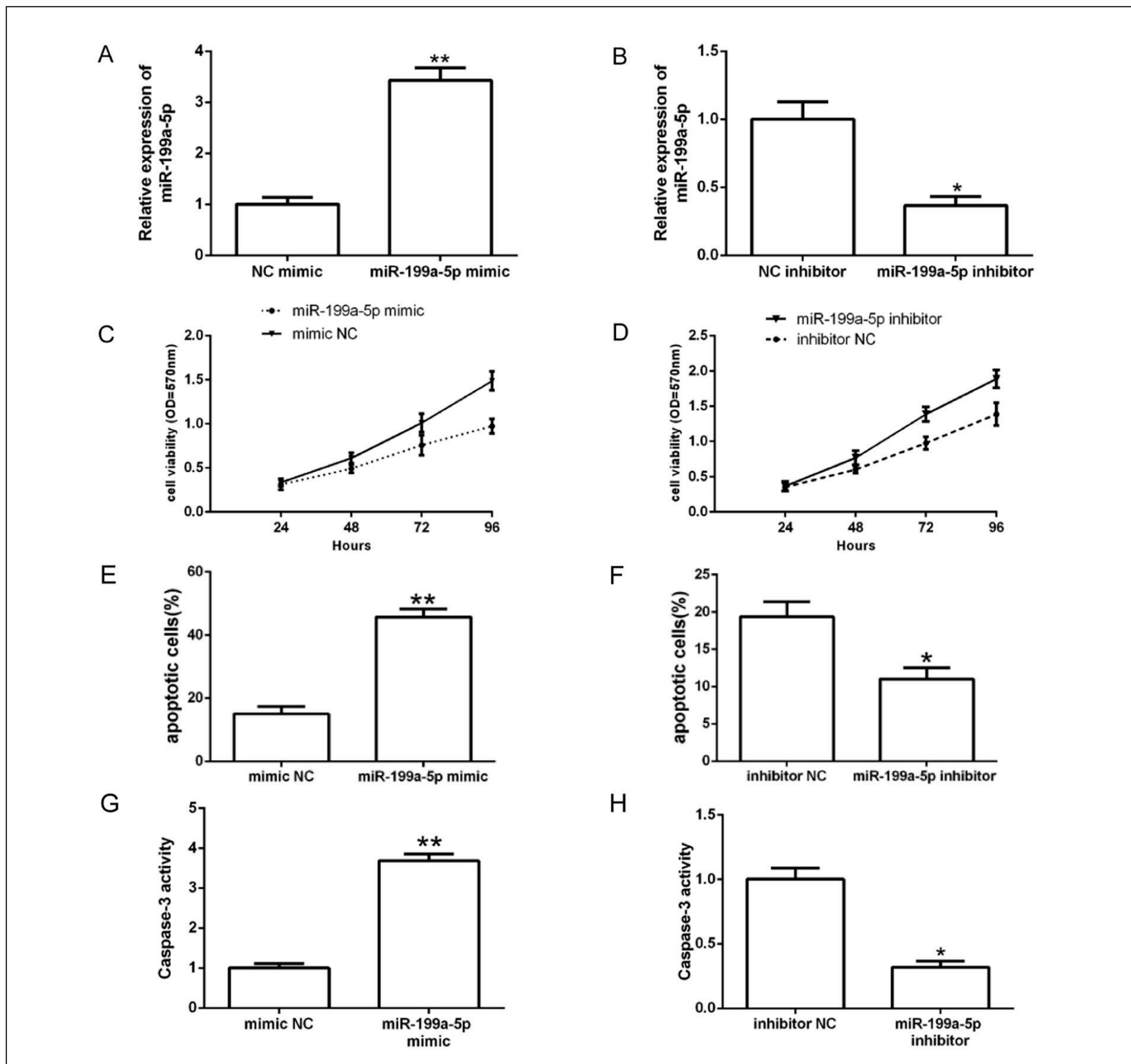

Figure 2. MiR-199a-5p inhibited laryngeal cancer cell proliferation and induces apoptosis in HEp-2 cells. **A, B,** qRT-PCR analysis of miR-199a-5p expression levels in HEp-2 cells transfected with miR-199a-5p mimic or miR-199a-5p inhibitor. **C, D,** Proliferation of HEp-2 cells after transfection was detected by MTT assay. **E, F,** Percentage of apoptotic HEp-2 cells among two different groups was detected by flow cytometry. **G, H,** Activity of Caspase-3 in HEp-2 laryngeal cancer cells was enhanced after transfected with miR-199a-5p mimic and weakened after transfected with miR-199a-5p inhibitor. Data are presented as means ± SD (n = 3). *p<0.05, **p<0.01 *vs.* control group.

199a-5p inhibits the EMT process of HEp-2 cells and modulates the expressions of EMT-related proteins therein.

Discussion

The roles of miRNAs in malignant tumors have been growingly concerned and researched. Current studies have proven that several miRNAs are involved in the development and progression of laryngeal cancer. Cai et al[7] detected the expression of miR-34c in 10 pairs of laryngeal cancer and paracancerous normal tissues. They found that miR-34c is substantially down-regulated in the laryngeal cancer tissues, which inhibits proliferation and invasion, and induces apoptosis in cancer cells by targeting C-Met. Li et al[8] discovered that miR-194 functions as a tumor-suppressor

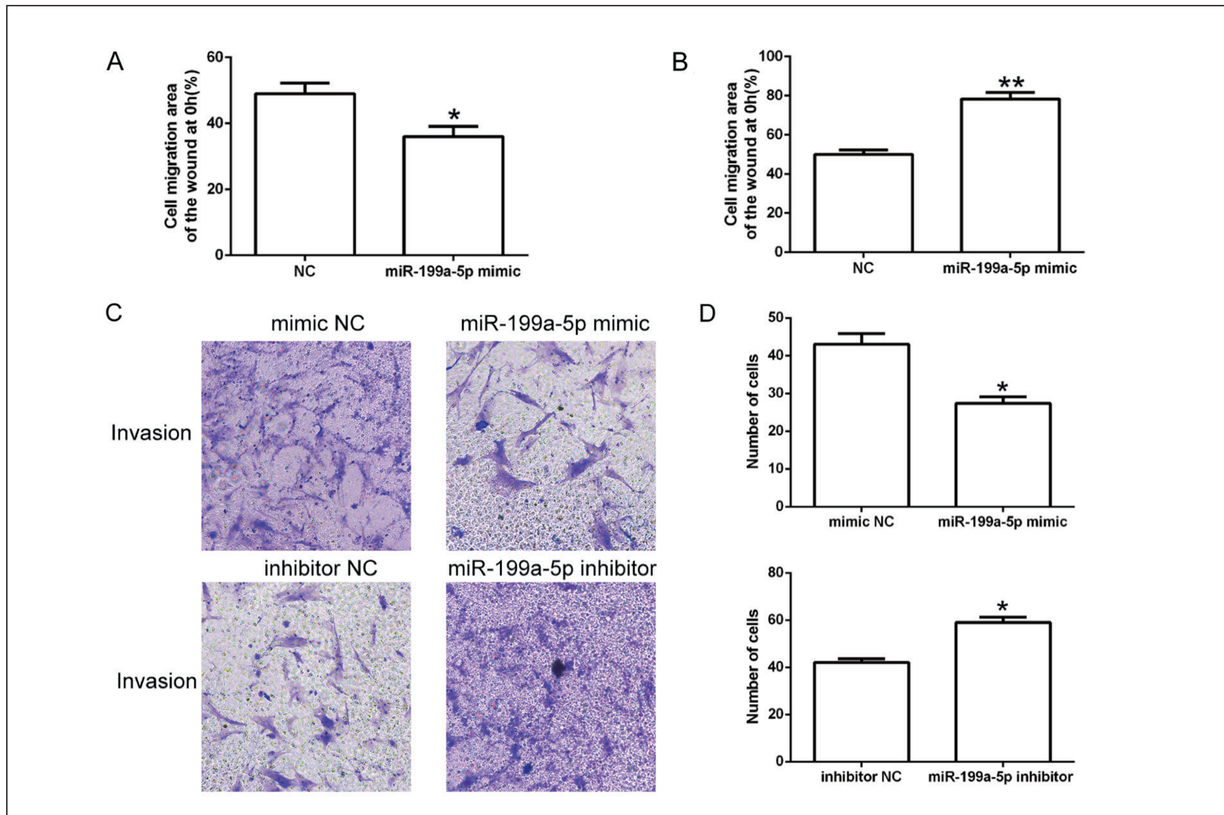

Figure 3. MiR-199a-5p inhibited the migration and invasion of HEp-2 cells. **A, B,** Migration capability of HEp-2 cells transfected with miR-219-5p mimic or miR-199a-5p inhibitor was detected by wound-healing assay (magnification: 40×). **C, D,** Invasion capability of HEp-2 cells transfected with miR-219-5p mimic and miR-199a-5p inhibitor was detected by transwell assay (magnification: 40×). Data are presented as means ± SD (n = 3). *p<0.05, **p<0.01 *vs.* control group.

gene in laryngeal squamous cell carcinoma. MiR-194 weakens the proliferation and invasion abilities of laryngeal cancer cells through the targeted inhibition on Wee1 expression. Several studies[9-11] have established that miR-199a-5p serves as a tumor-suppressor gene in multiple malignancies, such as lung cancer, glioma cancer and ovarian cancer. Considering the above study results, we seek to explore the role of miR-199a-5p in laryngeal cancer and its mechanism.

First, the difference in the expression of miR-199a-5p between laryngeal cancer tissues and paracancerous tissues was verified through qRT-PCR. It was found that the expression level of miR-199a-5p in laryngeal cancer tissues was considerably lower than that in paracancerous tissues, indicating that miR-199a-5p plays a potential role as a tumor-suppressor gene in laryngeal cancer tissues. To explore the specific role of miR-199a-5p in laryngeal cancer, the HEp-2 cells with the lowest expression level of miR-199a-5p were selected from three tested cell lines for *in vitro* assays. MiR-199a-5p mimic and inhibitor were employed to intervene miR-199a-5p expression. It was discovered in the cell proliferation assay that overexpressing miR-199a-5p remarkably inhibited the proliferation ability of HEp-2 cells, while down-regulating miR-199a-5p promoted their proliferation, implying that miR-199a-5p facilitates the proliferation of HEp-2 cells. Anti-apoptosis is one of the features of tumor cells. According to the findings in this study, overexpressing miR-199a-5p markedly promoted the apoptosis of laryngeal cancer cells, whereas down-regulating miR-199a-5p inhibited their apoptosis. The further Caspase-3 activity measurement results showed that the transfection with miR-199a-5p mimic up-regulated the activity of Caspase-3 activity, but transfection with miR-199a inhibitor weakened the activity of Caspase-3. There-

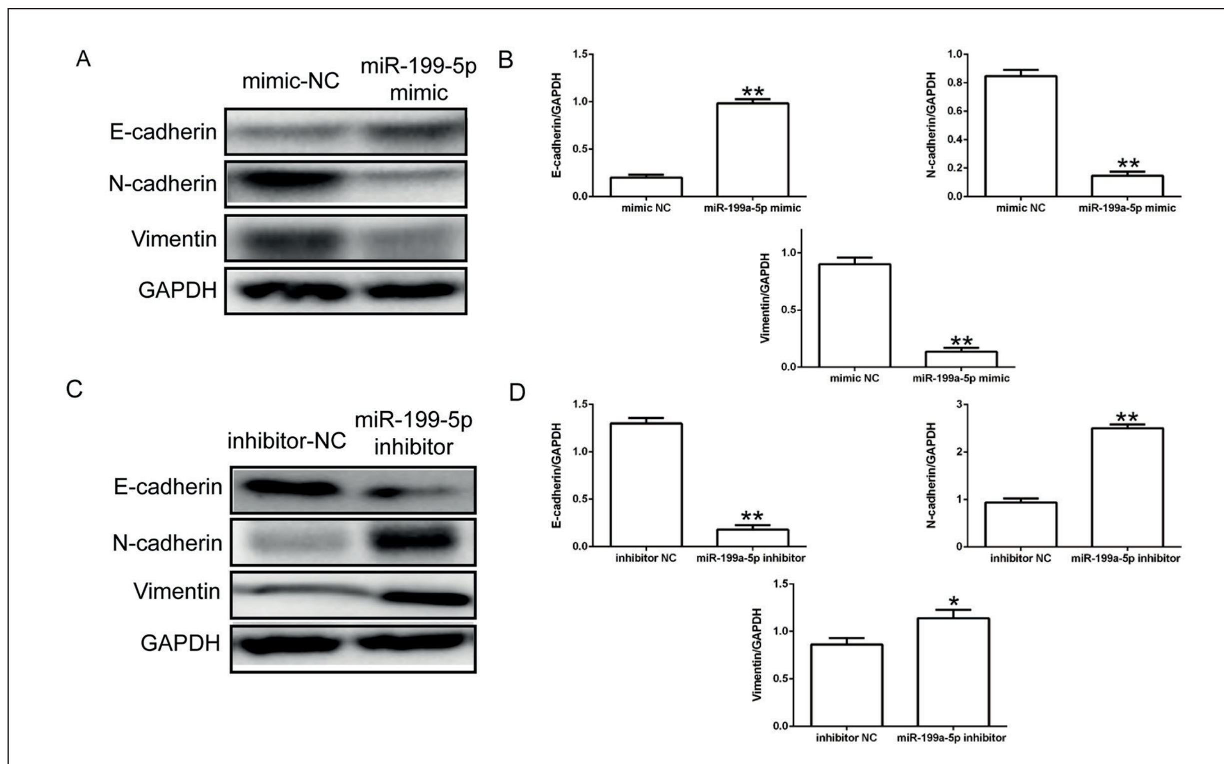

Figure 4. MiR-199a-5p inhibited the epithelial-mesenchymal transition process of HEp-2 cells. **A, B,** Expression of EMT-related proteins (E-cadherin, N-cadherin and Vimentin) were detected by Western blotting after transfected with miR-219-5p mimic and mimic NC. **C, D,** Expression of EMT-related proteins (E-cadherin, N-cadherin and Vimentin) were detected by Western blotting after transfected with miR-219-5p inhibitor and inhibitor NC. Data are presented as means ± SD (n = 3). *p<0.05, **p<0.01 *vs.* control group.

fore, miR-199a-5p can regulate the activity of Caspase-3 to accelerate cell apoptosis.

Local infiltration and distant metastasis are the leading causes of death in laryngeal cancer patients[12]. The influence of miR-199a-5p on the invasion and metastasis abilities of laryngeal cancer cells was determined *via* wound healing assay and transwell assay, respectively. The results suggested that miR-199a-5p can suppress the invasion and metastasis of laryngeal cancer cells. EMT is a biological phenomenon that epithelial cells gradually lose epithelial phenotypes and are transformed into mesenchymal cells, thereby obtaining mesenchymal cell phenotypes[13]. Metastasis is a multi-step cellular biological process. Of note, cancerous cells spread to distant organs and subsequently adapt to tissue microenvironment. The invasion and metastasis abilities of tumors depend on the interaction between tumor cells and internal environment[14]. Moreover, EMT is a vital step in tumor metastasis[15]. In the present study, it was found that transfection of miR-199a-5p mimic in HEp-2 cells considerably inhibited EMT, while transfection of miR-199a-5p promoted EMT. The above results suggested that miR-199a-5p can regulate EMT to repress the metastasis of laryngeal cancer cells.

Conclusions

We demonstrated that knockdown of miR-199a-5p facilitates the development and progression of laryngeal cancer by inhibiting proliferation and metastasis and inducing apoptosis. Our findings provide a theoretical reference that miR-199a-5p may be a therapeutic target for laryngeal cancer.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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