European Review for Medical and Pharmacological Sciences 2018; 22: 6350-6357

MicroRNA-203 inhibits invasion and induces apoptosis of laryngeal cancer cells via targeting LASP1

J. TAN, Y.-Y. JING, L. HAN, H.-W. ZHENG, J.-X. SHEN, L.-H. ZHANG, L.-S. YU

Department of Otorhinolaryngology and Head and Neck Surgery, Peking University People's Hospital, Beijing, China

Abstract. – OBJECTIVE: To explore the role of microRNA-203 in laryngeal cancer and its underlying mechanism in regulating cell invasion and apoptosis.

PATIENTS AND METHODS: MicroRNA-203 expression in laryngeal cancer tissues and paracancerous tissues was detected by quantitative real time-polymerase chain reaction (qRT-PCR). The regulatory effects of microRNA-203 on the invasion and apoptosis of laryngeal cancer cells were detected by transwell assay and flow cytometry, respectively. Dual-Luciferase reporter gene assay was performed to access the binding condition of microRNA-203 and LASP1. Both mRNA and protein levels of LASP1 in laryngeal cancer cells were detected after transfection with microRNA-203 mimic or microRNA-203 inhibitor by qRT-PCR and Western blot, respectively. Rescue experiments were finally performed to detect whether microRNA-203 regulates laryngeal cancer development via targeting LASP1.

RESULTS: MicroRNA-203 was lowly expressed in laryngeal cancer tissues and cell lines. MicroRNA-203 knockdown in Hep-2 cells can promote the invasion and inhibit the apoptosis of laryngeal cancer cells. Subsequently, LASP1 was predicted to be the target gene of microRNA-203, which was further verified by the Dual-Luciferase reporter gene assay. LASP1 expression was negatively regulated by microRNA-203. Furthermore, rescue experiments showed that the regulatory effects of microRNA-203 on the invasion and apoptosis of laryngeal cancer cells were reversed by LASP1.

CONCLUSIONS: We showed that lowly expressed microRNA-203 could promote the invasion and inhibit apoptosis of laryngeal cancer cells via inhibiting LASP1.

Key Words:
MicroRNA-203, Laryngeal carcinoma, Cell invasion, Apoptosis, LASP1.

Introduction

Head and neck neoplasms rank sixth in the incidence of all cancers that severely endanger human health. As a kind of common head and neck neoplasms, laryngeal cancer ranks second in the incidence of respiratory tract tumors. Based on the pathological types of laryngeal cancer, squamous cell carcinomas are the most common ones in clinical patients[1]. Although the diagnosis and treatment for laryngeal cancer have been greatly advanced, the 5-year survival is still unsatisfactory due to the frequent recurrence and lymph node metastasis[2]. The potential pathogenic factors of laryngeal cancer include smoking, alcohol, air pollution, occupational factors, etc.[3]. It is reported that some certain oncogenes (such as bcl-2, c-myc, etc.) and tumor-suppressor genes (such as p53, Rb, p16, p21, etc.) are involved in the occurrence and development of laryngeal cancer[4]. The specific mechanism of laryngeal cancer has not been fully elucidated and is required for further in-depth investigations[5].

MicroRNA is a type of non-coding RNAs with approximately 19-25 nt in length. It degrades mRNA level or inhibits the translation of the target gene by pairing with the 3'UTR of target gene[6]. MicroRNA exerts diverse functions in cell proliferation, differentiation, apoptosis and migration[7,8]. Recent studies have suggested that microRNAs could be served as oncogenes or tumor-suppressor genes, and are involved in oncogenesis, tumor cell invasion and angiogenesis[9]. Some certain microRNAs have been served as biomarkers and therapeutic targets of laryngeal cancer[10,11]. For example, microRNA-203 is lowly expressed in laryngeal cancer tissues[12]. The specific role of microRNA-203 in laryngeal cancer still needs to be comprehensively studied.

Corresponding Author: Lisheng Yu, MD; e-mail: yulish68@163.com

This study aims at exploring the regulatory effect of microRNA-203 on invasion and apoptosis of laryngeal cancer cells, as well as its underlying mechanism.

Patients and Methods

Sample Collection

Laryngeal cancer tissues and paracancerous tissues were surgically resected from laryngeal cancer patients treated in our hospital. Tissues were immediately preserved in liquid nitrogen for the following experiments. Enrolled patients were pathologically diagnosed as laryngeal cancer and informed consent has been signed. This study was approved by the Ethics Committee of Peking University People's Hospital.

Cell Culture

M2E, TU212, Hep-2, and M4E cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640; Gibco, Grand Island, NY, USA, USA) containing 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin (Hyclone, South Logan, UT, USA). Cells were incubated in a 5% CO_2 incubator at 37°C.

Cell Transfection

Cells in logarithmic growth phase were seeded in the 6-well plates and transfected with microRNA-203 mimics, microRNA-203 inhibitor, pcDNA-LASP1 or negative control following the instructions of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Transfection plasmids were provided by GenePharma (Shanghai, China). Culture medium was replaced 4-6 h later.

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

Total RNA in tissues was extracted using TRIzol method (Invitrogen, Carlsbad, CA, USA) for reverse transcription according to the instructions of PrimeScript RT reagent Kit (TaKaRa, Otsu, Shiga, Japan). RNA concentration was detected using a spectrometer and those samples with A260/A280 ratio of 1.8-2.0 were selected for the following qRT-PCR reaction. QRT-PCR was then performed based on the instructions of SYBR Premix Ex Taq TM (TaKaRa, Otsu, Shiga, Japan) at 94°C for 15 s, 55°C for 30 s, and 72°C for 30 s, for a total of 40 cycles. Primers used in the study were as follows: MicroRNA-203, F: 5'-GTGAAATGTTTAGGACCACTAGAA-3', R: 5'-GCTGTCAACGATACGCTACGT-3'; U6, F: 5'-CGCTTCGGCAGCACATATAC-3', R: 5'-TTCACGAATTTGCGTGTCAT-3'; GAPDH, F: 5'-GGAATCCACTGGCGTCTTCA-3', R: 5'-GGTTCACGCCCATCACAAAC-3'; LASP1, F: 5'-TGCGGCAAGATCGTGTATCC-3', R: 5'-GCAGTAGGGCTTCTTCTCGTAG-3'.

Cell Apoptosis Detection

Hep-2 cells were washed with phosphate-buffered saline (PBS), digested with trypsin and centrifuged at 1000 rpm/min for 5 min. After washing with pre-cooled PBS twice, cells were resuspended in PBS at a density of 1×10^5/mL. Subsequently, 1 μL of propidium iodide (PI) and 5 μl of Annexin V-fluorescein isothiocyanate (FITC) were added in 100 μL of cell suspension, followed by incubation at room temperature in the dark for 15 min. Finally, cell apoptosis was analyzed by flow cytometry (Partec AG, Arlesheim, Switzerland).

Transwell Assay

Transwell chamber was pre-coated with 1 mg/mL Matrigel at 37°C for 3-5 h. Cells were adjusted at a density of 2×10^5/mL. 100 μL of cell suspension and 600 μL of culture medium containing 10% FBS was added in the upper and lower chamber, respectively. 24 h later, non-adherent cells were cleaned. Cells were fixed with formaldehyde for 30 min and stained with violet crystal for 15-30 min. Finally, penetrating cells were observed and captured under a microscope.

Western Blot

The RIPA (radioimmunoprecipitation assay) protein lysate (Beyotime, Shanghai, China) was used to extract the total protein in each group of cells and tissues. The BCA (bicinchoninic acid) method was performed to quantify the protein concentration. Protein samples were electrophoresed on polyacrylamide gels and then transferred to polyvinylidene difluoride (PVDF) membranes (Merck Millipore, Billerica, MA, USA). After blocking with 5% skimmed milk, the membranes were incubated with primary antibody (LASP1, Cell Signaling Technology, Danvers, MA, USA) at 4°C overnight. The membrane was incubated with the secondary antibody after rinsing with Tris-buffered saline and Tween 20 (TBST). Chemiluminescence was used to expose the protein bands on the membrane.

Dual-Luciferase Reporter Gene Assay

Wild-type and mutant-type LASP1 were constructed. Cells were co-transfected with microRNA-203 mimics or negative control and wild-type or mutant-type LASP1, respectively. Luciferase activity was detected according to the instructions of Dual-Luciferase detection kit (Promega, Madison, WI, USA).

Statistical Analysis

Statistical Product and Service Solutions SPSS 20.0 statistical software (IBM, Armonk, NY, USA) was used for data analysis. Measurement data were expressed as mean ± standard deviation ($\bar{x} \pm s$) and compared using the *t*-test. $p < 0.05$ was considered statistically significant.

Results

MicroRNA-203 Was Lowly Expressed in Laryngeal Cancer

We detected the expression level of microRNA-203 in laryngeal cancer tissues and corresponding paracancerous tissues by qRT-PCR. The results showed that microRNA-203 was lowly expressed in laryngeal cancer tissues compared with that of paracancerous tissues (Figure 1A). Similarly, microRNA-203 expression was also downregulated in laryngeal cancer cell lines (LL212, M2E, Hep-2, and M4E) compared with that of controls (Figure 2B). Hep-2 cells were selected for subsequent experiments. To further develop the specific mechanism of microRNA-203 in regulating the laryngeal cancer development,

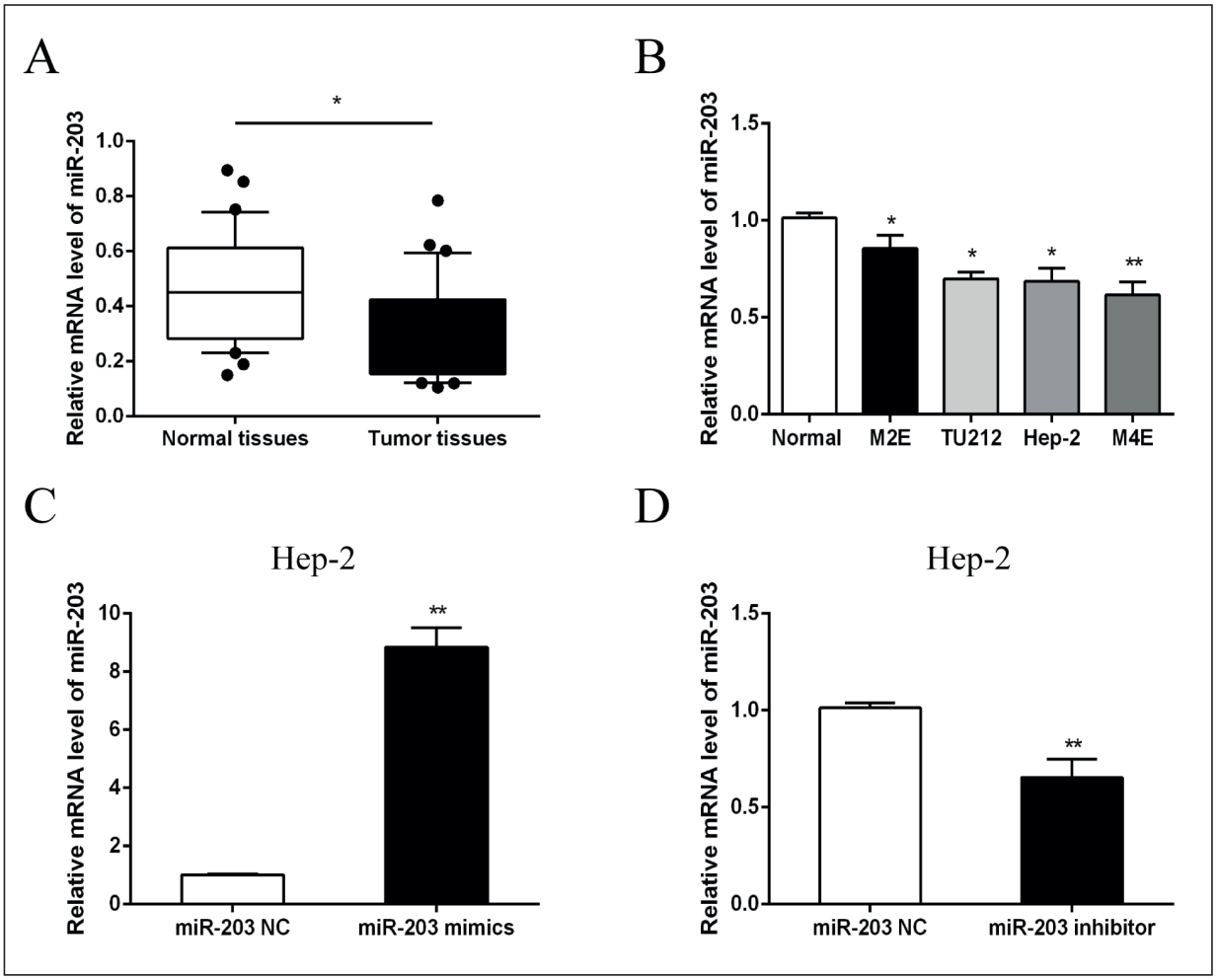

Figure 1. MicroRNA-203 was lowly expressed in laryngeal cancer. ***A,*** MicroRNA-203 was lowly expressed in laryngeal cancer tissues compared with that of paracancerous tissues. ***B,*** MicroRNA-203 was downregulated in laryngeal cancer cell lines (LL212, M2E, Hep-2, and M4E) compared with that of controls. ***C, D,*** Transfection efficacies of microRNA-203 mimic and microRNA-203 inhibitor in Hep-2 cells were verified by qRT-PCR.

microRNA-203 mimic and microRNA-203 inhibitor were constructed. Transfection efficacies of microRNA-203 mimic and microRNA-203 inhibitor in Hep-2 cells were verified by qRT-PCR (Figure 1C and 1D).

Overexpression of MicroRNA-203 Inhibited Invasion and Induced Apoptosis of Laryngeal Cancer

We detected the effect of microRNA-203 on regulating invasion of Hep-2 cells by transwell assay. The data indicated that microRNA-203 overexpression remarkably inhibited invasion of Hep-2 cells, manifesting as a lower amount of penetrating cells than that of controls (Figure 2A and 2B). We subsequently examined the effect of microRNA-203 on apoptosis of Hep-2 cells by flow cytometry. It is showed that microRNA-203 overexpression induced cell apoptosis of laryngeal cancer cells (Figure 2C).

MicroRNA-203 Directly Bound to LASP1

Bioinformatics predicted that LASP1 was the target gene of microRNA-203. Furthermore, we constructed wild-type and mutant-type LASP1

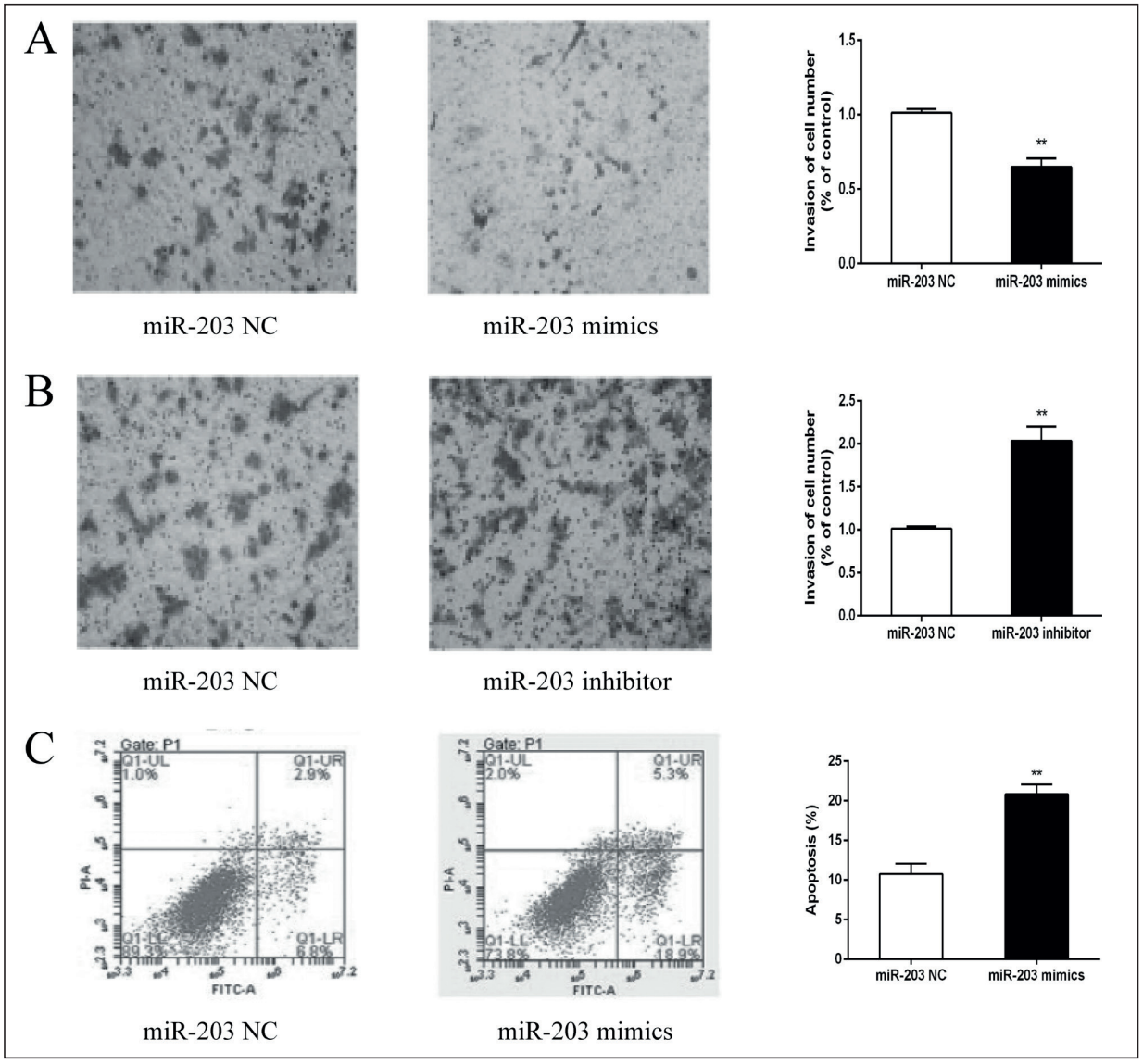

Figure 2. Overexpression of microRNA-203 inhibited invasion and induced the apoptosis of laryngeal cancer. ***A,*** MicroRNA-203 overexpression remarkably inhibited invasion of Hep-2 cells. ***B,*** MicroRNA-203 knockdown remarkably promoted invasion of Hep-2 cells. ***C,*** MicroRNA-203 overexpression induced cell apoptosis of laryngeal cancer cells.

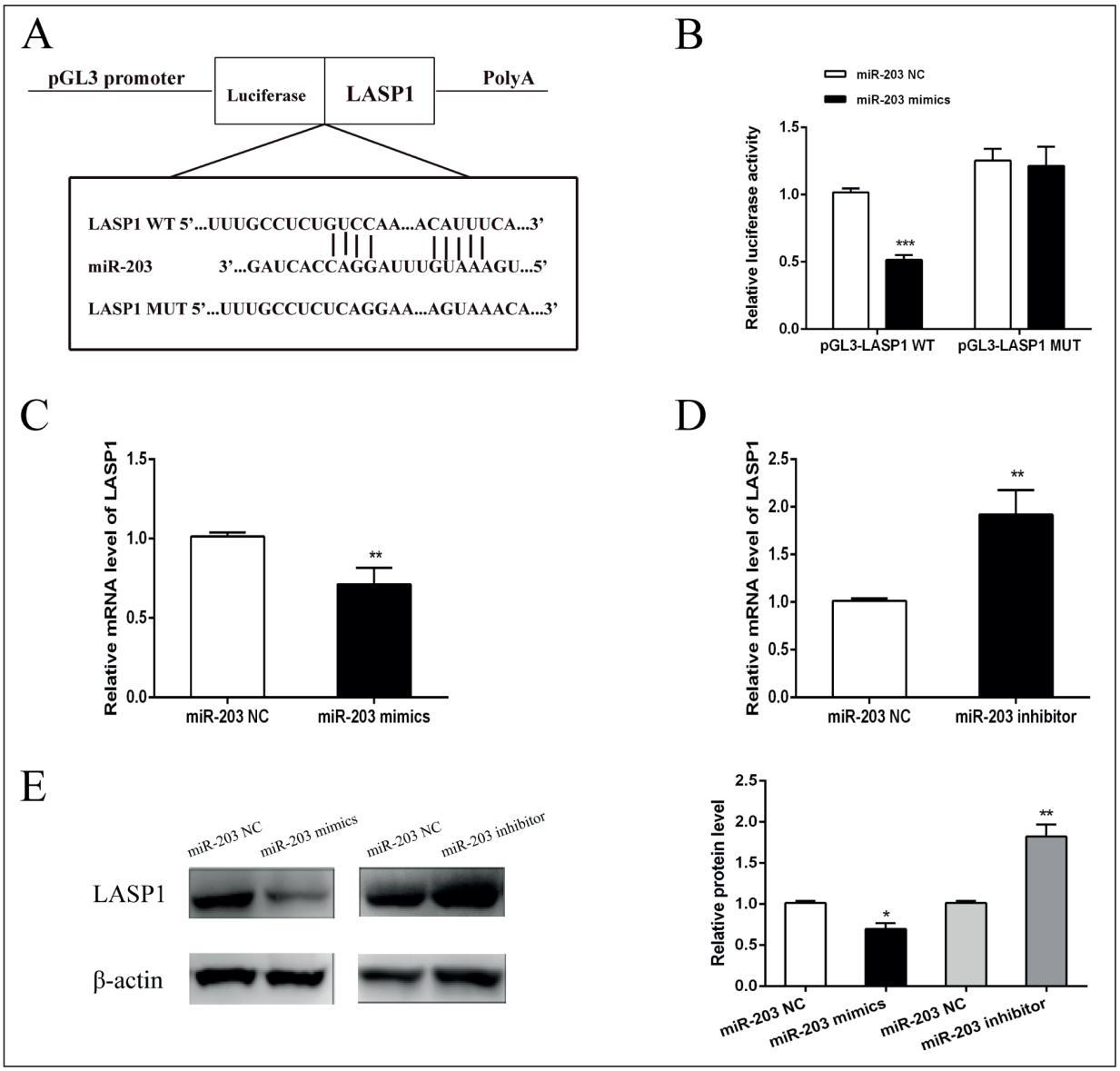

Figure 3. MicroRNA-203 directly bound to LASP1. ***A,*** Bioinformatics predicted that LASP1 was the target gene of microRNA-203. ***B,*** Dual-Luciferase reporter gene assay showed that the Luciferase activity in cells co-transfected with microRNA-203 mimic and wild-type LASP1 decreased. However, no significant difference in Luciferase activity was seen in cells co-transfected with mutant-type LASP1. ***C-E,*** Both mRNA and protein levels of LASP1 were negatively regulated by microRNA-203.

(Figure 3A). Dual-Luciferase reporter gene assay showed that the luciferase activity in cells co-transfected with microRNA-203 mimic and wild-type LASP1 decreased. However, no significant difference in Luciferase activity was seen in cells co-transfected with microRNA-203 mimic and mutant-type LASP1 (Figure 3B). Both mRNA and protein levels of LASP1 were downregulated after microRNA-203 overexpression in Hep-2 cells. On the contrary, microRNA-203 knockdown upregulated LASP1 expression (Figure 3C-3E).

LASP1 Reversed the Biological Function of MicroRNA-203

To investigate the potential role of LASP1 in regulating laryngeal cancer development, rescue experiments were conducted. We found that the inhibited invasion and promoted apoptosis of Hep-2 cells induced by microRNA-203 overexpression were partially reversed by LASP1 overexpression (Figure 4A and 4B). Our data demonstrated that microRNA-203 inhibited invasion and induced apoptosis of Hep-2 cells *via* suppressing LASP1 expression.

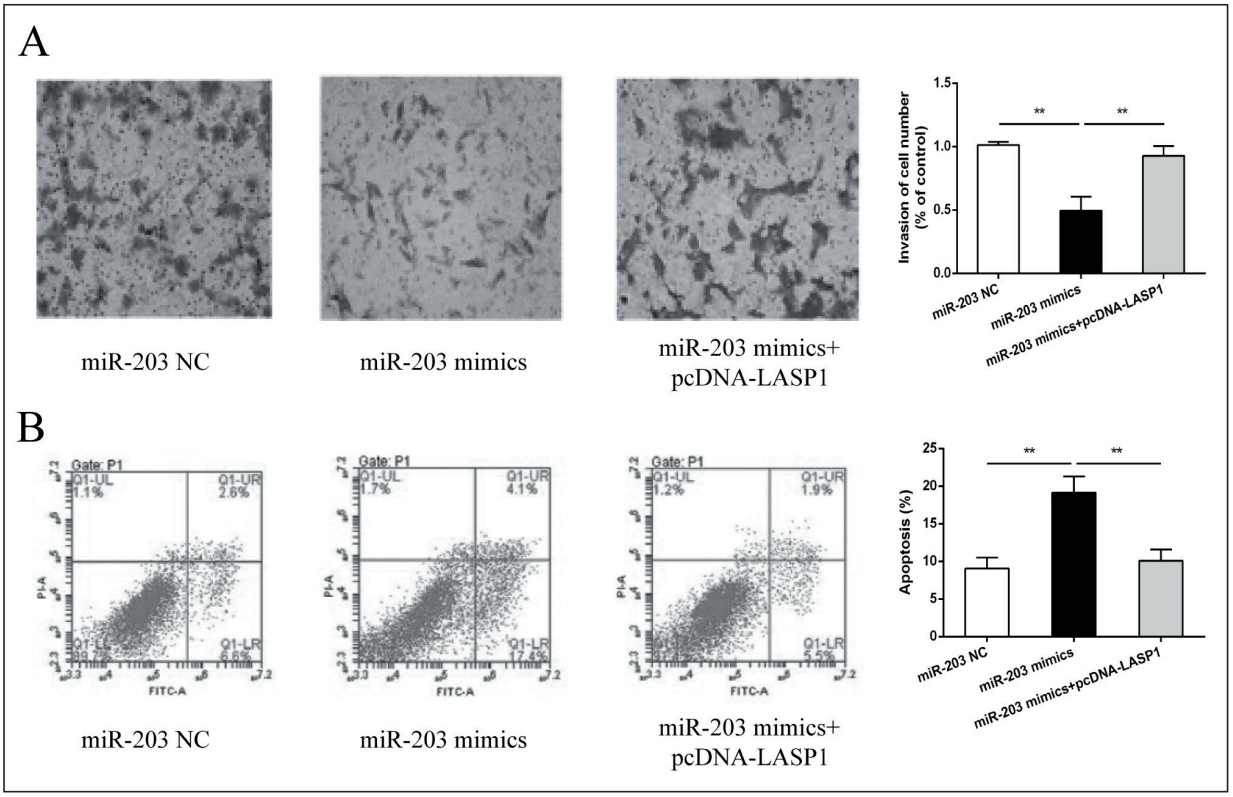

Figure 4. LASP1 reversed the biological function of microRNA-203. ***A, B,*** Inhibited invasion of Hep-2 cells induced by microRNA-203 overexpression was partially reversed by LASP1 overexpression. ***B,*** Promoted apoptosis of Hep-2 cells induced by microRNA-203 overexpression was partially reversed by LASP1 overexpression.

Discussion

Some studies have found that microRNAs are closely related to the occurrence, development, invasion, metastasis, and apoptosis of various tumors[13]. MicroRNAs regulate biological processes through cell apoptosis, proliferation and differentiation[14]. The dynamic balance of invasion and apoptosis is necessary for maintaining homeostasis of the internal environment. Abundant studies have shown the regulatory effects of microRNAs on important pathways involving in tumorigenesis[15,16]. As a common malignancy in the head and neck neoplasms, laryngeal cancer is a result of abnormal apoptosis and invasion of tumor cells.

MicroRNA-203 is located at 14q32.33, where encodes approximately 12% of the human genome. MicroRNA-203 is specifically expressed in epithelial tissues. Malignancies originated from different tissues present different expression level of microRNA-203. Relative studies have pointed out that microRNA-203 is lowly expressed in NSCLC (non-small cell lung cancer) tissues than that of paracancerous tissues. Its expression in NSCLC patients is correlated with lymph node metastasis, tumor size and TNM stage[10,11]. Lower expression of microRNA-203 in liver cancer patients predicts a shorter overall survival[17]. The expression level of microRNA-203 is negatively correlated with tumor size of gastric cancer[18]. MicroRNA-203 negatively regulates LASP1 expression in esophageal cancer and downregulates invasion and migration of esophageal cancer cells[10].

LASP1 is mainly expressed in immunocompetent cells (such as DC cells, NK cells) and muscle tissues. Cytoplasmic LASP1 regulates cell migration and adhesion *via* binding to F-actin and Zyxin[19,20]. LASP1, as an important structural protein of the cytoskeleton, participates in multiple biological processes through protein interactions mediated by LIM and SH3 domains[21,22]. LASP1 specifically interacts with palladin, pro-IL-16, VASP and Zyxin. It is reported that LASP1 regulates the binding condition to actin tension fiber bundles by interacting with palladin[23,24]. Recent

studies have reported that LASP1 is upregulated in a variety of tumors, such as NSCLC and prostate cancer. Particularly, LASP1 positively regulates the development and metastasis of malignancies[25,26].

Our study found that microRNA-203 is lowly expressed in laryngeal cancer tissues and cell lines. MicroRNA-203 knockdown can promote the invasion and inhibit the apoptosis of laryngeal cancer cells. Subsequently, LASP1 was predicted to be the target gene of microRNA-203 by bioinformatics method, which was further verified by Dual-Luciferase reporter gene assay. LASP1 expression was negatively regulated by microRNA-203 both at mRNA and protein levels. Furthermore, rescue experiments showed that microRNA-203 regulates invasion and apoptosis of laryngeal cancer cells *via* targeting LASP1.

Conclusions

We showed that the lowly expressed microRNA-203 could promote the invasion and inhibit apoptosis of laryngeal cancer cells *via* inhibiting LASP1.

Conflict of Interest

The Authors declare that they have no conflict of interests.

References

1) Genden EM, Ferlito A, Silver CE, Jacobson AS, Werner JA, Suarez C, Leemans CR, Bradley PJ, Rinaldo A. Evolution of the management of laryngeal cancer. Oral Oncol 2007; 43: 431-439.
2) Marioni G, Marchese-Ragona R, Cartei G, Marchese F, Staffieri A. Current opinion in diagnosis and treatment of laryngeal carcinoma. Cancer Treat Rev 2006; 32: 504-515.
3) Dias FL, Lima RA, Kligerman J, Cernea CR. Therapeutic options in advanced laryngeal cancer: an overview. ORL J Otorhinolaryngol Relat Spec 2005; 67: 311-318.
4) de Miguel-Luken MJ, Chaves-Conde M, Carnero A. A genetic view of laryngeal cancer heterogeneity. Cell Cycle 2016; 15: 1202-1212.
5) Makitie AA, Monni O. Molecular profiling of laryngeal cancer. Expert Rev Anticancer Ther 2009; 9: 1251-1260.
6) Karnati HK, Panigrahi MK, Gutti RK, Greig NH, Tamargo IA. MiRNAs: key players in neurodegenerative disorders and epilepsy. J Alzheimers Dis 2015; 48: 563-580.
7) Ivey KN, Muth A, Arnold J, King FW, Yeh RF, Fish JE, Hsiao EC, Schwartz RJ, Conklin BR, Bernstein HS, Srivastava D. MicroRNA regulation of cell lineages in mouse and human embryonic stem cells. Cell Stem Cell 2008; 2: 219-229.
8) Li B, Wu N, Zhang XJ, Wei ZL, Shang LX. MicroRNA-409 inhibits the proliferative ability of cervical carcinoma cells by regulating AKT. Eur Rev Med Pharmacol Sci 2018; 22: 936-942.
9) Shen J, Xiao Z, Wu WK, Wang MH, To KF, Chen Y, Yang W, Li MS, Shin VY, Tong JH, Kang W, Zhang L, Li M, Wang L, Lu L, Chan RL, Wong SH, Yu J, Chan MT, Chan FK, Sung JJ, Cheng AS, Cho CH. Epigenetic silencing of miR-490-3p reactivates the chromatin remodeler SMARCD1 to promote Helicobacter pylori-induced gastric carcinogenesis. Cancer Res 2015; 75: 754-765.
10) Yuan Y, Zeng ZY, Liu XH, Gong DJ, Tao J, Cheng HZ, Huang SD. MicroRNA-203 inhibits cell proliferation by repressing ΔNp63 expression in human esophageal squamous cell carcinoma. BMC Cancer 2011; 11: 57.
11) Wang C, Wang X, Liang H, Wang T, Yan X, Cao M, Wang N, Zhang S, Zen K, Zhang C, Chen X. miR-203 inhibits cell proliferation and migration of lung cancer cells by targeting PKCα. PLoS One 2013; 8: e73985.
12) Yu X, Li Z. The role of microRNAs expression in laryngeal cancer. Oncotarget 2015; 6: 23297-23305.
13) Kunz M, Xiao K, Liang C, Viereck J, Pachel C, Frantz S, Thum T, Dandekar T. Bioinformatics of cardiovascular miRNA biology. J Mol Cell Cardiol 2015; 89: 3-10.
14) Zhang J, Li S, Li L, Li M, Guo C, Yao J, Mi S. Exosome and exosomal microRNA: trafficking, sorting, and function. Genomics Proteomics Bioinformatics 2015; 13: 17-24.
15) Zhang LY, Liu M, Li X, Tang H. miR-490-3p modulates cell growth and epithelial to mesenchymal transition of hepatocellular carcinoma cells by targeting endoplasmic reticulum-Golgi intermediate compartment protein 3 (ERGIC3). J Biol Chem 2013; 288: 4035-4047.
16) Cho KR, Shih leM. Ovarian cancer. Annu Rev Pathol 2009; 4: 287-313.
17) Wan D, Shen S, Fu S, Preston B, Brandon C, He S, Shen C, Wu J, Wang S, Xie W, Chen B, Liya A, Guo Y, Zheng D, Zhi Q, Peng B. miR-203 suppresses the proliferation and metastasis of hepatocellular carcinoma by targeting oncogene ADAM9 and oncogenic long non-coding RNA HULC. Anticancer Agents Med Chem 2016; 16: 414-423.
18) Chiang Y, Song Y, Wang Z, Chen Y, Yue Z, Xu H, Xing C, Liu Z. Aberrant expression of miR-203 and its clinical significance in gastric and colorectal cancers. J Gastrointest Surg 2011; 15: 63-70.
19) Butt E, Gambaryan S, Gottfert N, Galler A, Marcus K, Meyer HE. Actin binding of human LIM and SH3 protein is regulated by cGMP- and

cAMP-dependent protein kinase phosphorylation on serine 146. J Biol Chem 2003; 278: 15601-15607.

20) Orth MF, Cazes A, Butt E, Grunewald TG. An update on the LIM and SH3 domain protein 1 (LASP1): a versatile structural, signaling, and biomarker protein. Oncotarget 2015; 6: 26-42.

21) Stolting M, Wiesner C, van Vliet V, Butt E, Pavenstadt H, Linder S, Kremerskothen J. Lasp-1 regulates podosome function. PLoS One 2012; 7: e35340.

22) Traenka C, Remke M, Korshunov A, Bender S, Hielscher T, Northcott PA, Witt H, Ryzhova M, Felsberg J, Benner A, Riester S, Scheurlen W, Grunewald TG, von Deimling A, Kulozik AE, Reifenberger G, Taylor MD, Lichter P, Butt E, Pfister SM. Role of LIM and SH3 protein 1 (LASP1) in the metastatic dissemination of medulloblastoma. Cancer Res 2010; 70: 8003-8014.

23) Grunewald TG, Butt E. The LIM and SH3 domain protein family: structural proteins or signal transducers or both? Mol Cancer 2008; 7: 31.

24) Li B, Zhuang L, Trueb B. Zyxin interacts with the SH3 domains of the cytoskeletal proteins LIM-nebulette and Lasp-1. J Biol Chem 2004; 279: 20401-20410.

25) Zhang X, Liu Y, Fan C, Wang L, Li A, Zhou H, Cai L, Miao Y, Li Q, Qiu X, Wang E. Lasp1 promotes malignant phenotype of non-small-cell lung cancer via inducing phosphorylation of FAK-AKT pathway. Oncotarget 2017; 8: 75102-75113.

26) Hailer A, Grunewald TG, Orth M, Reiss C, Kneitz B, Spahn M, Butt E. Loss of tumor suppressor mir-203 mediates overexpression of LIM and SH3 Protein 1 (LASP1) in high-risk prostate cancer thereby increasing cell proliferation and migration. Oncotarget 2014; 5: 4144-4153.