

miR-203 is involved in the laryngeal carcinoma pathogenesis via targeting VEGFA and Cox-2

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Abstract: The development of laryngeal squamous cell carcinoma (LSCC) is a multistep process involving multiple factors. MicroRNAs, a group of important negative regulators of gene expression, have also been confirmed to be involved in the LSCC pathogenesis. In the present study, we compared the expression of nine selected microRNAs in the LSCC tissues and adjacent nontumor tissues. We found that the expression of *miR-203* was significantly reduced in the LSCC tissues. Predicted by using bioinformatics tools, we found that VEGFA and cyclooxygenase-2 (Cox-2) may be direct targets of *miR-203*. By subsequent determination through dual-luciferase assay and Western blot, we confirmed that *miR-203* suppresses the expression of VEGFA and Cox-2 by directly targeting 3'-untranslated region. Meanwhile, by analyzing the relationship between *miR-203* and VEGFA in clinical tissue samples, we found that a negative correlation existed in the expression of *miR-203* and VEGFA ($P=0.0096$, $r=-0.33$). Similarly, the expression of *miR-203* and Cox-2 also has a negative correlation ($P=0.0019$, $r=-0.46$). Subsequently, in vitro functional study indicated that *miR-203* played as a tumor suppressor by repressing proliferation, migration, and invasion of Hep-2 cells. The overexpression of VEGFA partially rescued the effect of overexpressed *miR-203*. Overexpressed Cox-2 partially rescued the effect of *miR-203* on Hep-2 cell proliferation but not on the cell migration and invasion capacity. These findings suggest that *miR-203* plays as a tumor suppressor in LSCC, partially by regulating VEGFA and Cox-2, and may serve as a potential target for therapeutic intervention.

Keywords: non-coding RNA, laryngeal squamous cell carcinoma, correlation analysis

Introduction

Head and neck cancer is the seventh most common form of cancer in the world, which is composed of oral cavity, oropharynx, hypopharynx, and larynx cancers.^{1,2} Laryngeal cancer is considered to be the most common malignant neoplasm in the head and neck region. In 2008, an estimate of 151,000 new cases of laryngeal cancer was diagnosed worldwide. In the same year, it was responsible for 82,000 deaths worldwide.² Meanwhile, although early stage laryngeal cancer is often cured by surgery or radiotherapy, for the majority of patients with the advanced disease, the outcome has not improved in the last three decades. This implies that significant health benefits could be achieved via effective prevention and treatment strategies.

MicroRNA (miRNA) is a kind of short noncoding RNAs that suppress the expression of protein-coding genes by partial complementary binding, especially to the 3'-untranslated regions (UTRs) of messenger RNAs. Alterations in miRNA expression are involved in the initiation, progression, and metastasis of human cancer, and it is believed that miRNAs function both as tumor suppressors and as oncogenes in cancer development.^{3,4} Accumulating studies have shown that disturbed expression

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of miRNAs is involved in the cancer pathogenesis and drug resistance.^{5,6} Recently, increased reports indicated that disturbed miRNA levels existed in the laryngeal cancer and played very important roles in the pathogenesis of laryngeal cancer.⁷⁻⁹ However, the functional study is at the early stage and almost all the results needed to be confirmed in larger populations. Therefore, in this study, we detected the expression of nine miRNAs in laryngeal cancer tissues and examined the function of *miR-203* on cell proliferation, migration, and invasion.

Patients and methods

Tissue samples

Patients were enrolled in the period between July 2010 and June 2013. A total of 60 patients with laryngeal squamous cell carcinoma (LSCC) who underwent partial or total laryngectomy at the Department of Otolaryngology – Head & Neck Surgery, Shanghai Jiao Tong University Affiliated First People's Hospital were included in the study. No chemotherapy and radiation were applied to the patients before the operation. The matched specimens of LSCC and the corresponding adjacent nonneoplastic tissues obtained from the patients were preserved in liquid nitrogen within 5 minutes of excision and then transported frozen to the laboratory and stored briefly at -80°C . The study was approved by the Ethics Committee of Shanghai Jiao Tong University Affiliated First People's Hospital, and written informed consent was obtained from all the participants.

RNA isolation and quantitative reverse transcription-polymerase chain reaction

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis was used to determine the relative expression levels of selected miRNAs. Total RNA was extracted from the tissue samples by using TRIzol (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Random six RNA samples were mixed as one sample for the detection of target molecules as the preliminary experiment. The levels of *miR-203* in the samples of every patient were detected subsequently. The miRNAs expression was detected by TaqMan miRNA RT-Real-Time PCR. Single-stranded complementary DNA was synthesized by using TaqMan MicroRNA Reverse Transcription Kit (Thermo Fisher Scientific) and then amplified by using TaqMan Universal PCR Master Mix (Thermo Fisher Scientific) together with miRNA-specific TaqMan MGB probes (Thermo Fisher Scientific). The U6 snRNA was used for normalization. Each sample in each group was measured in triplicate, and

the experiment was repeated at least three times for the detection of miRNAs.

Cell culture

Hep-2 and HEK293T cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (HyClone, Logan, UT, USA), 100 IU/mL penicillin, and 10 mg/mL streptomycin. All cells were maintained at a temperature of 37°C under an atmosphere of 5% CO_2 .

Western blot

Protein extracts were boiled in sodium dodecyl sulfate/ β -mercaptoethanol sample buffer, and 30 μg samples were loaded into each lane of 10% polyacrylamide gels. The proteins were separated by electrophoresis, and the proteins in the gels were blotted onto polyvinylidene difluoride membranes (Amersham Pharmacia Biotech, St Albans, Herts, UK) by electrophoretic transfer. The membranes were incubated with rabbit anti-vascular endothelial growth factor A VEGFA polyclonal antibody (ab51745; Abcam, Cambridge, MA, USA) with a dilution of 1:2,000, rabbit anti-Cox-2 polyclonal antibody (Abcam) with a dilution of 1:5,000, or mouse anti- β -actin monoclonal antibody (sc-47778; Santa Cruz Biotechnology Inc., Dallas, TX, USA) with a dilution of 1:3,000 for 2 hours at 37°C . The specific protein-antibody complex was detected by using horseradish peroxidase-conjugated goat antirabbit or rabbit antimouse IgG. Detection by the chemiluminescence reaction was carried out using the ECL kit (Pierce, Appleton, WI, USA). The β -actin signal was used as a loading control.

Dual-luciferase assay

A 435 bp segment of VEGFA 3'-UTR and a 1132 bp segment of Cox-2 were cloned into downstream of firefly luciferase coding region in the pGL3 control vector (Promega Corporation, Fitchburg, WI, USA) to generate luciferase reporter vector. For luciferase reporter assays, HEK293T cells were seeded in 48-well plates. One of the luciferase reporter vectors was cotransfected with *miR-203* into HEK293T cells by using lipofectamine 2000 (Thermo Fisher Scientific). After 48 hours, the cells were harvested and assayed with the Dual-Luciferase Assay (Promega Corporation). Each treatment was performed in triplicate in three independent experiments. The results were expressed as relative luciferase activity (Firefly luciferase/Renilla luciferase).

Cell proliferation assay

Hep-2 cells were seeded in 96-well plates at low density (5×10^3) in DMEM culture and allowed to attach overnight. The cells

were then transfected with *miR-203* mimic or inhibitor, with sequence scrambled single-strand or double-strand short RNA as control. In all, 20 μ L of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; 5 mg/mL; Sigma-Aldrich Co., St Louis, MO, USA) was added into each well after 48 hours of transfection, and the cells were incubated for further 4 hours. The absorbance was recorded at 570 nm with a 96-well plate reader after the dimethyl sulfoxide addition.

Cell migration and invasion assays

Hep-2 cells were transfected with the *miR-203* mimic or inhibitor with scramble RNA as control. The transfected cells were harvested and subjected to the following assays after 48 hours of transfection. For migration assays, the transfected cells (5×10^4 cells/mL) were seeded in the top of an 8.0 mm-pore membrane chamber (Corning Incorporated, Corning, NY, USA). Following a 12-hour incubation period, the cells that passed through the membrane to attach to the bottom of the membrane were fixed and stained with hematoxylin and eosin (Sigma-Aldrich Co.). The cells were scraped and removed from the top of the chamber. The membranes were mounted on cover slides, and cells were counted. Cell migration was quantified by counting the amount of cells passing through the pores from five different fields per sample at $100\times$ selected in a random manner. Cell invasion assays were carried out using modified Boyden chambers in 24-well tissue culture plates at 5×10^4 cells/well (BD Biosciences, San Jose, CA, USA). All experiments were performed in duplicate.

Statistical analysis

The results of independent two groups were analyzed by using *t*-test, and the correlation analysis was analyzed by χ^2 test. All the data were analyzed by using SPSS Statistical Package Version 16 (SPSS Inc., Chicago, IL, USA). $P < 0.05$ was considered statistically significant.

Results

Expression of *miR-203* is reduced in LSCC tissues

MiRNAs is a group of gene expression suppressors, and disturbed miRNAs expression has been found in the LSCC tissues. To investigate the roles of miRNAs in the pathogenesis of LSCC, we first selected nine miRNAs that were reported to have disturbed expression in the LSCC patients.⁷⁻⁹ Subsequently, total RNA was extracted from the LSCC tissues and adjacent nontumor tissues and every six RNA samples were mixed into one group. The expressions of miRNAs were detected by using stem-loop qRT-PCR.

As shown in Figure 1, the expression of *miR-203* was reduced significantly ($P=0.0020$) compared with the adjacent nontumor control. Since *miR-203* has significant lower expression in tumor samples, the level of *miR-203* in every tumor and adjacent nontumor samples was detected by qRT-PCR subsequently. The correlation of *miR-203* and clinic pathological parameters was analyzed. Lower *miR-203* expression was also found in the T3–T4 stage LSCC compared with T1–T2, and more reduced *miR-203* expression was related to lymph node metastasis (Table 1).

miR-203 suppresses the expression of VEGFA and Cox-2 by targeting 3'-UTR

To understand the roles of reduced *miR-203* in the pathogenesis of LSCC, we first predicted the target genes of *miR-203* by using online bioinformatics tool: TargetScan (<http://www.targetscan.org>).¹⁰ To our surprise, VEGFA and Cox-2, the overexpression of whom has been found to be positively correlated with the malignancy of LSCC, are the direct feasible targets of *miR-203* (Figures 2 and 3).¹⁰⁻¹³ To examine whether these two genes are the real target of *miR-203*, we employed the dual-luciferase reporter system. A 334 bp segment of VEGFA or 1138 bp segment of Cox-2 containing the putative target sites of *miR-203* was subcloned into pGLO3 control vector, under the firefly luciferase coding region to generate the reporter plasmids. HEK293T cells were cotransfected with one of the reporter vectors and *miR-203* mimics or antagonist, with pRL-TK which expression renilla luciferase as transfection control. The expression of *miR-203* was detected by qRT-PCR (Figures 2B and 3B). Compared with the miRNA control, the luciferase activity was significantly suppressed by the *miR-203*, $\sim 45.7\%$ ($P < 0.01$). Furthermore, the luciferase activity was significantly upregulated by the *miR-203* inhibitor compared with the inhibitor control, $\sim 12.5\%$ ($P < 0.05$). These results indicate that *miR-203* targets the 3'-UTR of VEGFA, leading to the change in the translation of firefly luciferase. Similar results were obtained when using Cox-2 reporter vector. Compared with the miRNA control, the luciferase activity was significantly suppressed by the *miR-203*, $\sim 44.1\%$ ($P < 0.05$). Furthermore, the luciferase activity was significantly upregulated by the *miR-203* inhibitor compared with the inhibitor control, $\sim 18.5\%$ ($P < 0.05$).

Seed sequence mutation clones were also used to further confirm the binding site for *miR-203* (Figures 2D and 3D). The vector contains putative *miR-203* binding region in the 3'-UTR of VEGFA with four mutant nucleotides or the 3'-UTR region of Cox-2 with six mutant nucleotides. The histogram in Figures 2D and 3D showed that the enzyme activity was not significantly reduced in cells transfected with

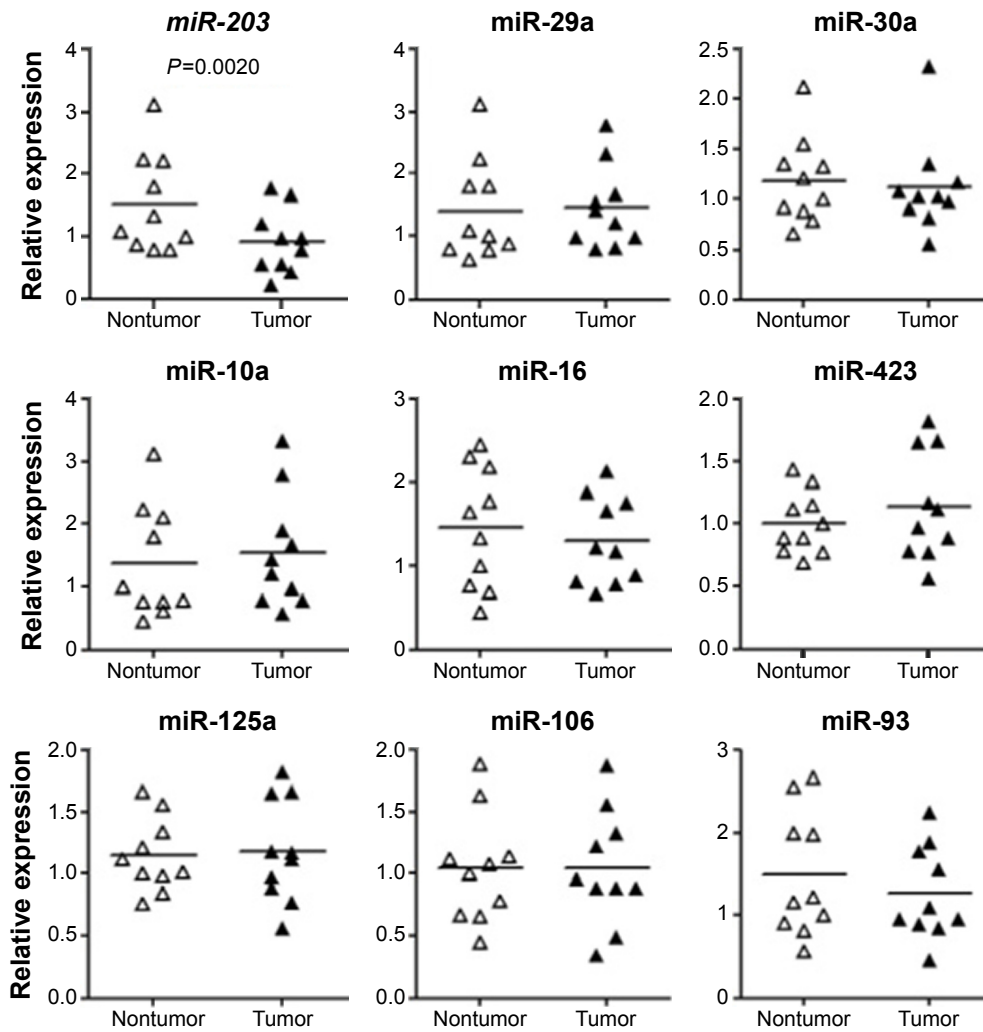


Figure 1 The expression of *miR-203* was downregulated in LSCC tissues.

Notes: Total RNA was extracted from tissues samples by using TRIzol reagent according to the manufacturer's instructions. Random six RNA samples were mixed as one sample for the detection of target molecules. The expression of miRNAs was detected by TaqMan miRNA RT-Real-Time PCR. The U6 snRNA was used for normalization. Each sample in each group was measured in triplicate, and the experiment was repeated at least three times for the detection of miRNAs. Student's t-test was used to analyze the expression of candidate miRNAs in tumor and nontumor groups, and $P < 0.05$ was considered as statistically significant.

Abbreviations: LSCC, laryngeal squamous cell carcinoma; miRNA, microRNA; PCR, polymerase chain reaction; snRNA, small nuclear RNA.

Table 1 Correlation between *miR-203* and clinicopathological parameters

Characteristics (n)	<i>miR-203</i> expression (T/N ratio)	P-value
Sex		0.194
Male (41)	0.613±0.073	
Female (19)	0.822±0.174	
Age (years)		0.913
≥60 (35)	0.665±0.086	
<60 (25)	0.699±0.134	
T classification		0.037
T1–2 (28)	0.851±0.132	
T3–4 (32)	0.529±0.0703	
Lymph node metastasis		0.0469
Negative (29)	0.832±0.119	
Positive (31)	0.537±0.0852	
Primary site		0.873
Glottic (27)	0.693±0.126	
Supraglottic (33)	0.669±0.0890	

miR-control compared with *miR-203* mimic ($P > 0.05$). These data indicate that *miR-203* may suppress the expression of VEGFA and Cox-2 through binding to seed sequence at the 3'-UTR of VEGFA and Cox-2.

To further examine whether endogenous expression of VEGFA and Cox-2 is suppressed by *miR-203*, Hep-2 cells were transfected with *miR-203* mimics or inhibitor. VEGFA and Cox-2 protein levels were detected by Western blot after 48 hours of transfection. Compared with the corresponding control, the levels of VEGFA and Cox-2 proteins were significantly suppressed by *miR-203* mimics and upregulated by *miR-203* inhibitor in Hep-2 cells (Figures 2E and 3E). These results indicated that *miR-203* repressed the endogenous expression of VEGFA and Cox-2 in the laryngeal cancer cells by directly targeting 3'-UTR.

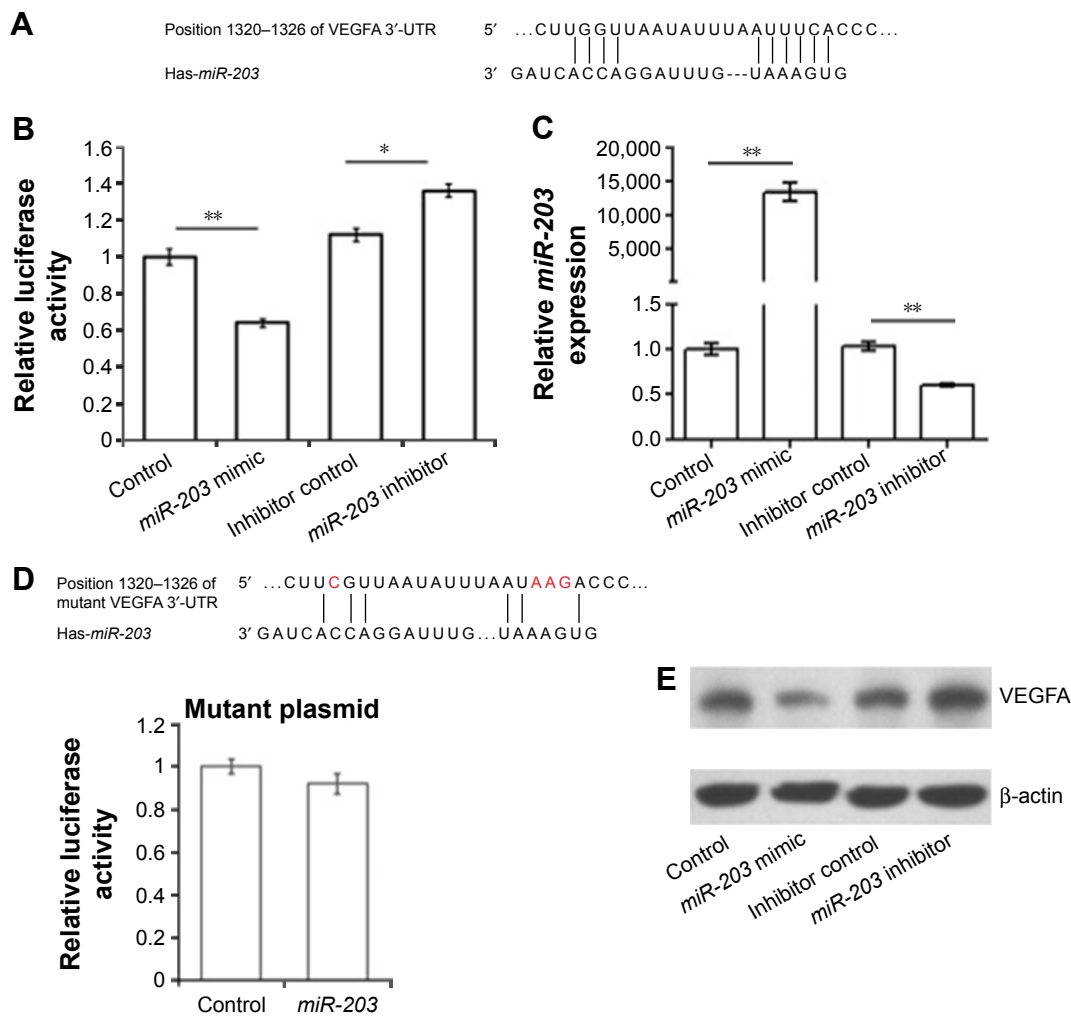


Figure 2 VEGFA is a direct target of *miR-203*.

Notes: (A) A schematic diagram for the predicted interaction between *miR-203* and the 3'-UTR of VEGFA mRNA. (B) pGL3-VEGFA was cotransfected with *miR-203* mimic or inhibitor into HEK293T cells for dual-luciferase assay. (C) The expression of *miR-203* in each group was detected by using qRT-PCR. (D) Mutation clone of four nucleotides was constructed to identify the binding site of *miR-203*. The results were analyzed by Student's *t*-test, and $P < 0.05$ was considered as statistically significant. * $P < 0.05$ and ** $P < 0.01$. (E) Hep-2 cells were transfected with *miR-203* mimic or inhibitor; 48 hours later, the endogenous VEGFA protein level was detected by Western blot.

Abbreviations: VEGFA, vascular endothelial growth factor A; UTR, untranslated region; mRNA, messenger RNA; qRT-PCR, quantitative reverse transcription-polymerase chain reaction.

Expression of VEGFA and Cox-2 is negatively correlated with *miR-203* level in vivo

To further examine the relationship between *miR-203* level and its target genes in vivo, we detected the expression of *miR-203* in every independent sample, and meanwhile, the protein levels of VEGFA and Cox-2 were also detected by Western blot. The relative band intensity was quantified by using ImageJ software (National Institutes of Health [NIH] Bethesda, MD, USA) with β -actin as the loading control. Analyzed with the expression of *miR-203*, VEGFA, and Cox-2, we found inverse correlations between the expression levels of *miR-203* and VEGFA or Cox-2 in 60 clinical samples of LSCC. Low levels of *miR-203* were associated

with high expression of VEGFA (Pearson's correlation, $r = -0.398$; $P < 0.01$; Figure 4A and B) and Cox-2 (Pearson's correlation, $r = -0.302$; $P < 0.05$; Figure 4C and D).

miR-203 suppresses proliferation, migration, and invasion of laryngeal cancer cells

To further test whether *miR-203* may execute tumor-suppressive functions by targeting VEGFA and Cox-2, we investigated the effect of *miR-203*-mediated cell proliferation by MTT assay in Hep-2 cells (Figure 5A). The cell proliferation was significantly reduced by *miR-203* mimic, ~54.8%. Meanwhile, the cell proliferation was significantly upregulated by *miR-203* inhibitor in Hep-2 cells, ~33.1%.

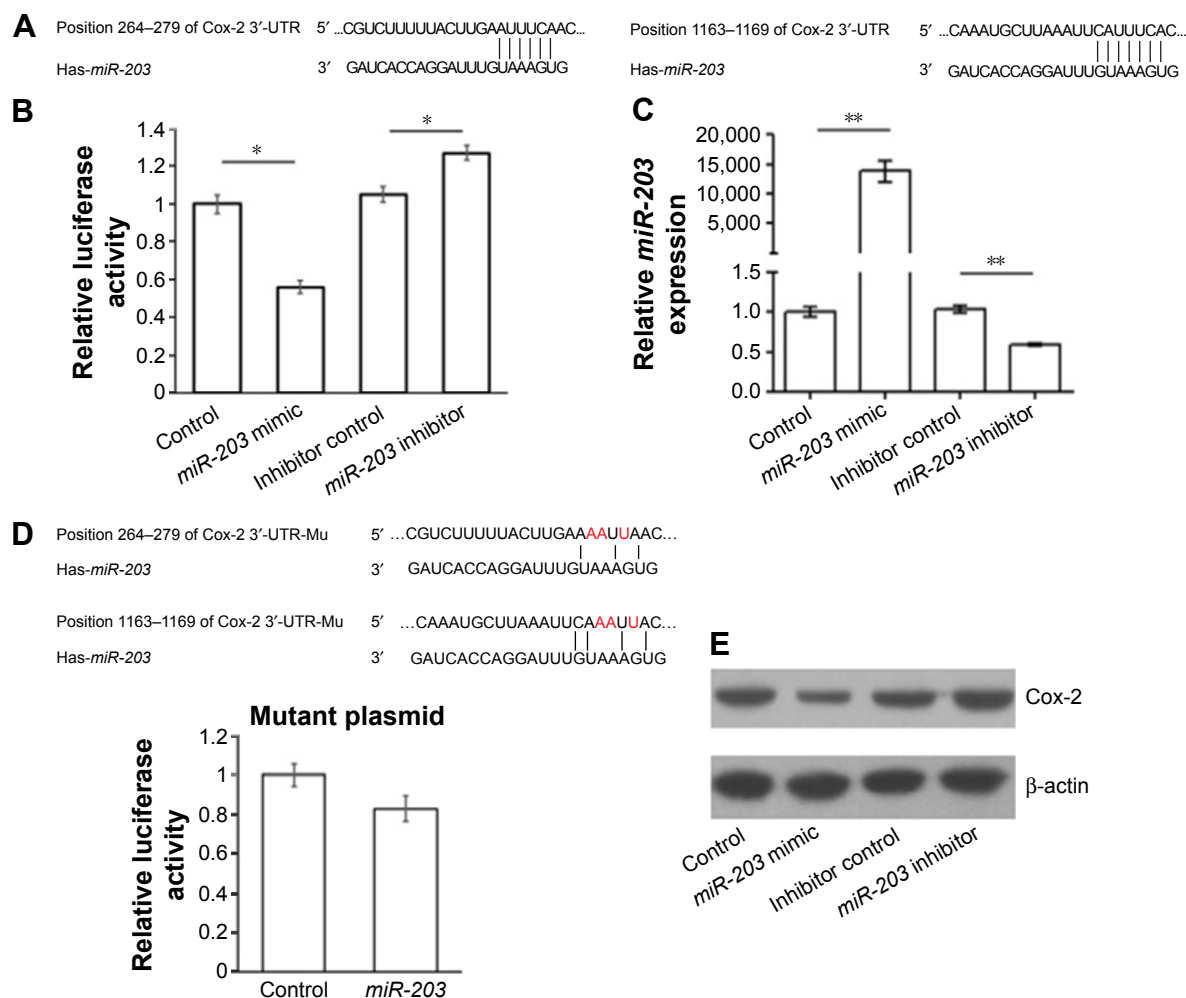


Figure 3 Cox-2 is a direct target of miR-203. **Notes:** (A) A schematic diagram for the predicted interaction between miR-203 and the 3'-UTR of Cox-2 mRNA. (B) pGL3-Cox-2 was cotransfected with miR-203 mimic or inhibitor into HEK293T cells for dual-luciferase assay. (C) The expression of miR-203 in each group was detected by using qRT-PCR. (D) The mutation clone of six nucleotides was constructed to identify the binding site of miR-203. The results were analyzed by Student's *t*-test, and $P < 0.05$ was considered as statistically significant. * $P < 0.05$ and ** $P < 0.01$. (E) Hep-2 cells were transfected with miR-203 mimic or inhibitor; 48 hours later, the endogenous Cox-2 protein level was detected by Western blot. **Abbreviations:** Cox-2, cyclooxygenase-2; UTR, untranslated region; mRNA, messenger RNA; qRT-PCR, quantitative reverse transcription-polymerase chain reaction.

To further confirm whether miR-203 represses cell proliferation by targeting VEGFA and Cox-2, we cotransfected VEGFA or Cox-2 expression vector with miR-203 mimic into Hep-2 cells. As shown in Figure 5B, VEGFA and Cox-2 expression vector can partially rescue the repressed cell proliferation caused by miR-203 overexpression, which means that miR-203 suppresses the ability of cell proliferation partially through targeting VEGFA and Cox-2.

In order to further research the roles of miR-203 in controlling the metastasis of laryngeal cancer, we analyzed the effects of miR-203 on the migratory and invasive behaviors of Hep-2 cells. As shown in Figure 5C and E, the migration and invasion capacities were significantly suppressed in cells transfected with miR-203 mimic compared with control ($P < 0.05$). In the contrary, the number of migrated

and invaded cells was significantly upregulated by the miR-203 inhibitor ($P < 0.05$). Meanwhile, VEGFA overexpression can partially rescue the repressed cell migration and invasion capacity caused by miR-203 ($P < 0.05$) Figure 5D and F. Cox-2 overexpression can also slightly rescue the cell migration and invasion capacity, but the differences were not significant ($P > 0.05$) Figure 5D and F. These results suggest that the level of miR-203 is closely associated with the metastasis of laryngeal cancer.

Discussion

The development of LSCC is a multistep process involving multiple factors. miRNAs, a group of important negative regulator of gene expression, have also been confirmed to be involved in the LSCC pathogenesis. In the present study,

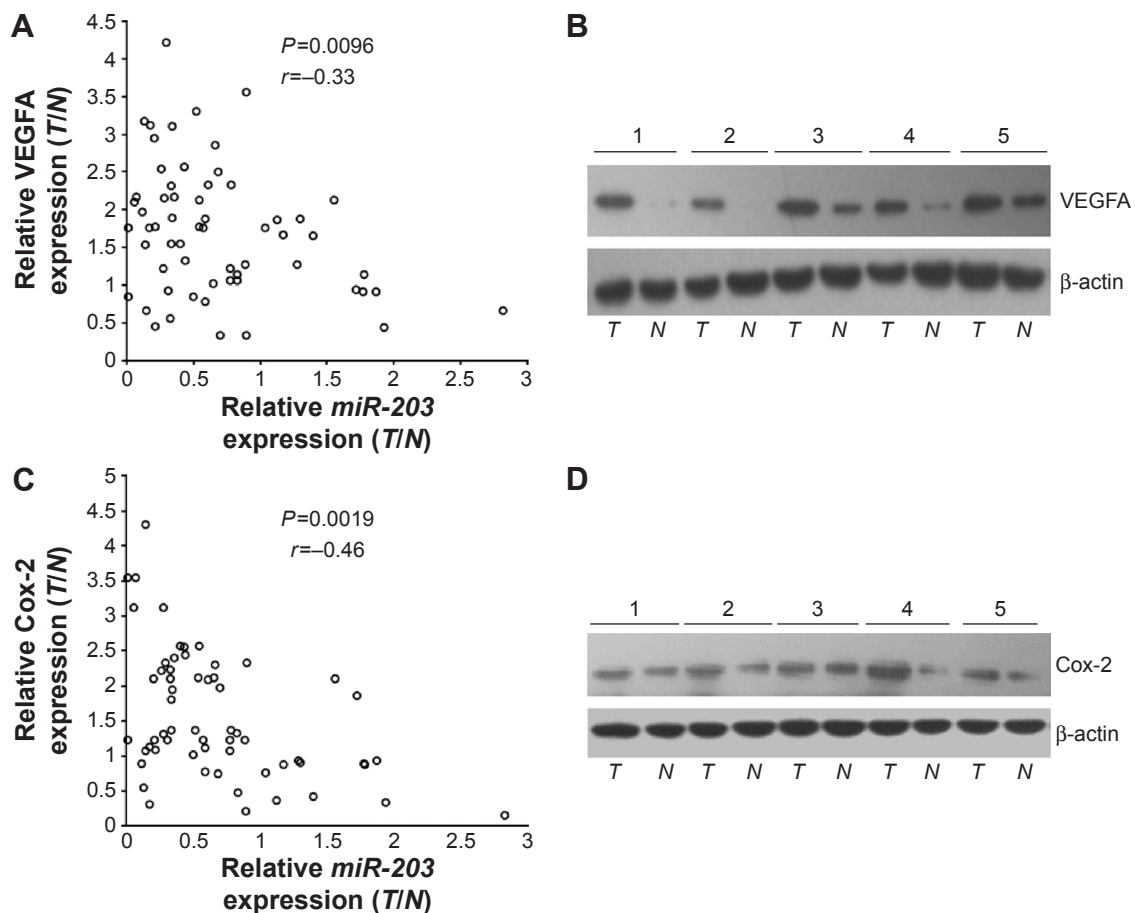


Figure 4 The correlation between the expression of *miR-203* and the levels of VEGFA or Cox-2 protein in LSCC patients.

Notes: (A) The expression of *miR-203* in every tumor and adjacent nontumor samples was detected using stem-loop qRT-PCR. The expression of VEGFA was detected by Western blot. To evaluate the expression of VEGFA, the band intensity of Western blot was semiquantitatively calculated. Correlation analysis was used to analyze the relationship between the expression of *miR-203* and VEGFA. (B) An example of the Western blot results detecting VEGFA expression in tissues. (C) Correlation analysis between the expression of *miR-203* and Cox-2. (D) An example of the Western blot results detecting Cox-2 expression in tissues.

Abbreviations: VEGFA, vascular endothelial growth factor A; Cox-2, cyclooxygenase-2; LSCC, laryngeal squamous cell carcinoma; qRT-PCR, quantitative reverse transcription-polymerase chain reaction.

we compared the expression of nine selected miRNAs in the LSCC tissues and adjacent nontumor tissues. We found that the expression of *miR-203* was significantly reduced in the LSCC tissues. Predicted by using bioinformatics tools, we found that VEGFA and Cox-2 might be the direct targets of *miR-203*. By subsequent determination through dual-luciferase assay and Western blot, we confirmed that *miR-203* suppresses the expression of VEGFA and Cox-2 by directly targeting 3'-UTR. Meanwhile, by analyzing the relationship between *miR-203* and VEGFA in clinical tissue samples, we found that a negative correlation existed in the expression of *miR-203* and VEGFA. Similarly, the expression of *miR-203* and Cox-2 also had a negative correlation. Subsequently, in vitro functional study indicated that *miR-203* played as a tumor suppressor by repressing proliferation, migration, and invasion of Hep-2 cells. The overexpression of VEGFA partially rescued the effect of overexpressed

miR-203. Overexpressed Cox-2 partially rescued the effect of *miR-203* on the proliferation of Hep-2 cell but not on the cell migration and invasion capacity. These results indicated that *miR-203* plays as a tumor suppressor in LSCC, partially by regulating VEGFA and Cox-2.

Although *miR-203* has been reported to be upregulated in some kinds of tumors,^{5,14} it is widely accepted that *miR-203* functions as a tumor suppressor and has been downregulated especially in head and neck cancer, prostate cancer, gastric cancer, and colon cancer.¹⁵⁻¹⁷ Attempts to validate *miR-203* target genes have increasingly been made to investigate the functional role of *miR-203* in carcinogenesis. In this study, we identified for the first time that VEGFA and Cox-2 are the direct targets of *miR-203*. The function of VEGFA is related to not only regulating vasculogenesis and angiogenesis but also affecting the tumor microenvironment. Cox-2 has prostaglandin (PG) hydroperoxidase activity to synthesize

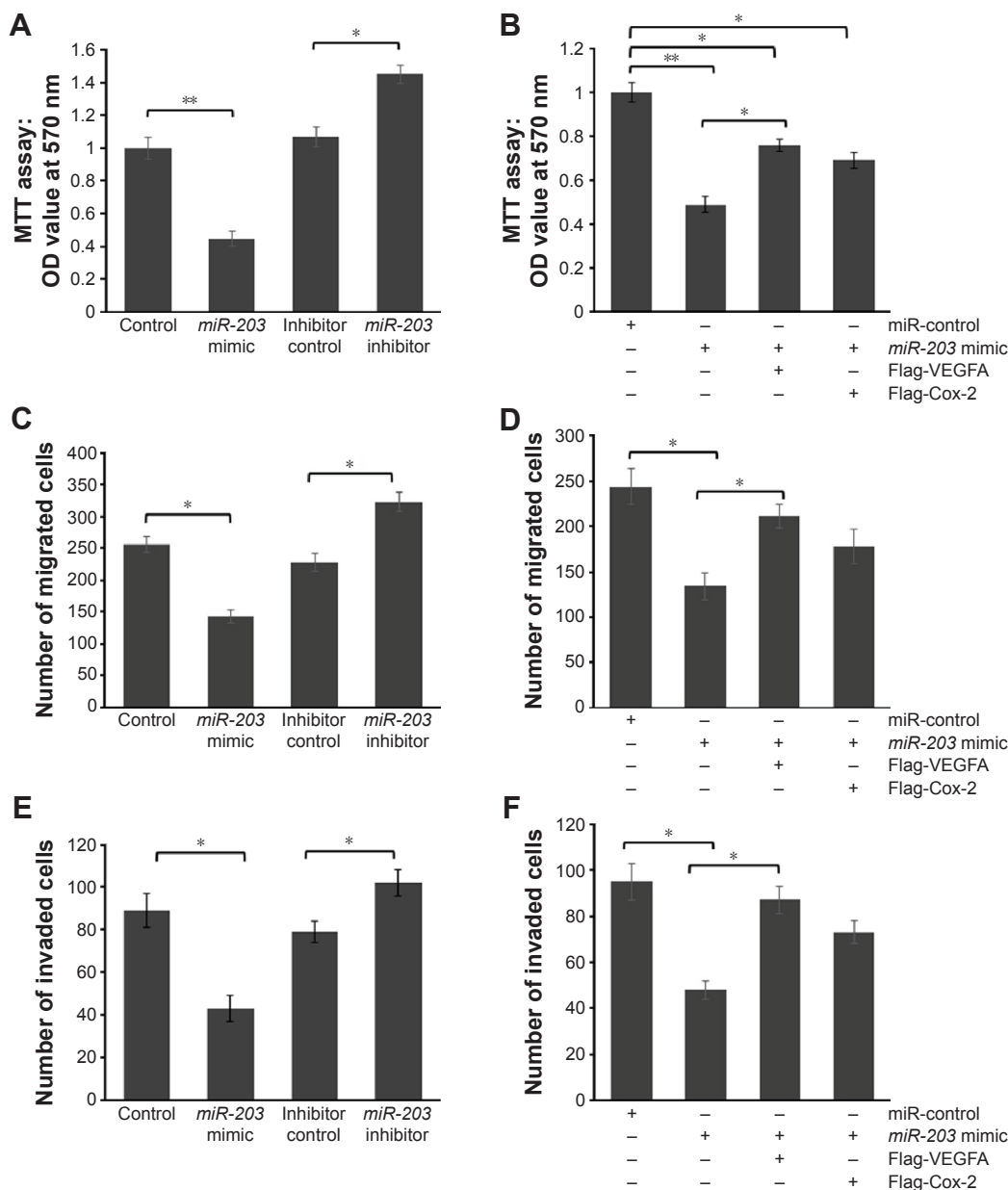


Figure 5 MiR-203 functions as a tumor suppressor via repression of cancer proliferation, migration, and invasion.

Notes: (A and B) Hep-2 cells were seeded in 96-well plates at a low density (5×10^3) in the DMEM culture and allowed to attach overnight. The cells were then transfected with *miR-203* mimic or inhibitor, with sequence scrambled single-strand or double-strand short RNA as control. MTT assay was employed for detecting the cell proliferation after 48 hours of transfection. (C and D) For migration assays, Hep-2 cells were transfected with the *miR-203* mimic or inhibitor with scramble RNA as control. The transfected cells were harvested and subjected to the following assays after 48 hours of transfection. The transfected cells were seeded in an 8.0 mm pore membrane chamber. Following a 12-hour incubation period, the cells that passed through the membrane to attach to the bottom of membrane were fixed and stained with hematoxylin and eosin. The cells were scraped and removed from the top of chamber. The membranes were mounted on cover slides, and the cells were counted. Cell migration was quantified by counting the amount of cells passing through the pores from five different fields per sample at 100 \times selected in a random manner. (E and F) Cell invasion assays were carried out using modified Boyden chambers in 24-well tissue culture plates at 5×10^4 cells/well. All experiments were performed in duplicate. The results were analyzed using Student's t-test, and $P < 0.05$ was considered statistically significant. * $P < 0.05$ and ** $P < 0.01$.

Abbreviations: DMEM, Dulbecco's Modified Eagle's Medium; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; OD, optical density; VEGFA, vascular endothelial growth factor A; Cox-2, cyclooxygenase-2.

PGH2 from PGG2, and the expression of Cox-2 is induced during inflammation, cell proliferation, and differentiation. COX-2 levels have been found to be elevated in various tumors.¹⁸⁻²⁰ In this study, we confirmed that negative correlations existed between the expression of *miR-203* and VEGFA or Cox-2, which contributes to understanding the

mechanism of the higher level of VEGFA and Cox-2 in the LSCC tissues.

Conclusion

miR-203 functions as a tumor suppressor via targeting VEGFA and Cox-2, and downregulated *miR-203* expression

contributes to LSCC carcinogenesis through enhanced tumor cell proliferation, migration, and invasion.

Disclosure

The authors report no conflicts of interest in this work.

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