

MicroRNA-195 is associated with regulating the pathophysiologic process of human laryngeal squamous cell carcinoma

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Received January 13, 2016; Accepted December 20, 2017

DOI: 10.3892/mmr.2018.8523

Abstract. MicroRNAs (miRNAs) have been reported to be associated with the modulation of tumor development, including alterations associated with the development of human laryngeal squamous cell carcinoma (LSCC). The present study was designed to investigate whether miRNA-195 was associated with the pathophysiologic process of human LSCC and to identify its potential roles and underlying molecular mechanisms. To determine whether miRNA-195 serves a role in LSCC, reverse transcription-quantitative polymerase chain reaction was used to detect miRNA-195 expression in LSCC tissues. The tumor-suppressive effect of miRNA-195 was determined by *in vitro* assays. Gain-of-function studies using miRNA-195 mimics were performed to investigate cell viability, migration and invasion, and apoptosis in the AMC-HN-8 cell line. Western blotting was performed to reveal the molecular mechanisms of miRNA-195 and its downstream signaling pathways in the LSCC AMC-HN-8 cell line. The present study demonstrated that miRNA-195 is downregulated in primary LSCC tumors. Upregulating miRNA-195 *in vitro* suppressed cell viability, migration and invasion in AMC-HN-8 cells. Overexpression of miRNA-195 alone in AMC-HN-8 cells was sufficient to induce cell apoptosis, as identified by terminal deoxynucleotidyl transferase dUTP nick end labeling assay. Compared with the high expression of miRNA-195 in AMC-HN-8 cells, the expression levels of vascular endothelial growth factor receptor-II protein and downstream signaling pathway proteins, which were associated with cell viability, migration, invasion and apoptosis, were markedly decreased compared with control or miRNA-195 negative control treatment group. Together, these data suggest the therapeutic potential of miRNA-195

in modulating cell growth, migration and apoptosis during the pathophysiological progression of LSCC and that miRNA-195 may serve as a potential therapeutic target in human LSCC.

Introduction

Laryngeal squamous cell carcinoma (LSCC) is the second most common malignancy in the head and neck region and is reported to account for ~2.4% of all new malignancies worldwide each year (1). The incidence of LSCC has been rising gradually, particularly in Northeast China (2). Although rapid progress has recently been made in treatment, the therapeutic outcomes and the overall 5-year survival rate remain unsatisfactory (3). Together with invasive and metastatic behaviors, resistance to chemotherapy has been a major obstacle in improving the 5-year survival rate of LSCC patients (4). Furthermore, total laryngectomy, the recommended treatment for advanced patients, leads to serious lung infection and the loss of speech (5). Therefore, more effective diagnostic techniques for early stage LSCC have long been warranted. The improved detection of useful biological and molecular markers in the diagnosis and therapy of LSCC, and a new strategy for the treatment of LSCC, are urgently required.

It has been suggested that conventional pathological prognostic parameters are insufficient to accurately evaluate the clinical prognosis of patients with LSCC (6). However, biomarkers are considered to be beneficial not only in evaluating the prognosis but also in guiding personalized therapy for patients with cancer (7), which further indicates the importance of identifying potential biomarkers for human LSCC. MicroRNAs (miRNAs) are a family of endogenous small single-strand RNAs with a length between 21 and 25 nucleotides, which have emerged as an important class of gene regulators (8). It has been demonstrated that miRNAs serve critical roles in tumor cell viability, differentiation, metastasis and apoptosis *in vitro* and *in vivo* (9). In addition, miRNAs have been functionally classified as tumor suppressors or proto-oncogenes and are aberrantly expressed in different cancers, including lung (10), liver (11), stomach (12), leukemia (13), lymphoma (14), breast (15), colorectal (16), and head and neck cancer (17). It has been suggested that miRNAs may act as the molecular targets for human cancer diagnosis

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Key words: laryngeal squamous cell carcinoma, microRNA-195, vascular endothelial growth factor receptor 2, viability, migration, invasion, apoptosis

and personalized therapy (18,19). miRNA-195 has been identified to be downregulated in a variety of human solid tumors, but the potential biological roles of miRNA-195 in LSCC remain to be elucidated.

The present study initially examined the expression of miRNA-195 in LSCC and identified downregulation of miRNA-195 in LSCC cancer tissue. Subsequently, the role of miRNA-195 in cell viability, migration and apoptosis in AMC-HN-8 cells was demonstrated. It was identified that miRNA-195 modulated vascular endothelial growth factor receptor 2 (VEGFR2) expression, and was thereby associated with the VEGFR2 signaling network. These findings demonstrated that miRNA-195 serves a key role in the pathologic progression of LSCC and revealed the potential molecular mechanisms of miRNA-195 in human LSCC.

Materials and methods

Patient samples. A total of 23 cases of laryngeal squamous cell carcinoma tissue samples and adjacent healthy tissue samples between February 2016 and March 2017 were obtained from the Department of Otolaryngology in the Fourth Affiliated Hospital of Harbin Medical University (Harbin, China). There were 10 males and 13 females in the disease group, with a median age of 61 years. The present study was performed in accordance with the ethical codes of the World Medical Association (20) and was approved by the Ethics Committee for Use of Human Samples of Harbin Medical University. All human participants provided informed written consent and all clinical investigations were conducted according to the principles expressed in the Declaration of Helsinki. All specimens were coded and identified. The specimens comprised a panel of 23 LSCC patient cases obtained during surgical procedures, which were immediately stored in liquid nitrogen or fixed in formalin.

Cell culture. AMC-HN-8 cells used in the present study were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). AMC-HN-8 cells were cultured in RPMI-1640 (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37°C with 5% CO₂ in a humidified incubator. The cultures were supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) and 100 µg/ml penicillin/streptomycin.

Oligo transfection, miRNA-195 knockdown, and miRNA-195 overexpression in AMC-HN-8 cells. Cells were seeded in antibiotic-free RPMI-1640 medium for 24 h prior to transfection. For upregulation of miRNA-195, the cells (1×10⁵) in a six-well plate were transfected with miRNA-195 mimic (Shanghai GenePharma Co., Ltd., Shanghai, China) using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) in serum-free Opti-MEM (Gibco; Thermo Fisher Scientific, Inc.) medium according to the manufacturer's instructions. For knockdown of miRNA-195, the cells (1×10⁵) in a six-well plate were transfected with miRNA-195 inhibitor (Shanghai GenePharma Co., Ltd.) using Lipofectamine® 2000 (Thermo Fisher Scientific, Inc.). Transfection complexes were added to medium at a final oligonucleotide concentration of 50 nM. miRNA-195 mimic negative control (NC) and

miRNA-195 inhibitor NC were also purchased from Shanghai GenePharma Co., Ltd. and were transfected at a final concentration of 50 nM using Lipofectamine® 2000 according to the manufacturer's instructions. Opti-MEM medium was replaced at 4 h post-transfection with regular culture medium and incubated at 37°C for a further 48 h. The sequences of miRNA-195 mimic and mimic-NC were: 5'-UAGCAGCACAGAAU AUUGGC-3' and 5'-UUCUCCGAACGUGUCACGUTT-3', respectively. The sequences of miRNA-195 inhibitor and inhibitor-NC were: 5'-GCCAAUAAUUCUGUGCUGCUA-3' and 5'-CAAUAAUUCUGUGCUGCUAAU-3', respectively. Following transfection, the subsequent experiments were performed within 8 h.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA from human LSCCs was isolated using TRIzol reagent (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Total RNA (0.5 µg) was then reverse transcribed using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) to obtain cDNA according to the manufacturer's instructions. The RNA levels of miRNA-195 were determined using Power SYBR™ Green PCR Master Mix (Thermo Fisher Scientific, Inc.) incorporation method on an ABI 7500 fast Real Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.) at 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec, 60°C for 30 sec and 72°C for 30 sec, with U6 as an internal control. The relative quantitative expression of gene was calculated using method 2^{ΔΔC_q} (21). The sequences of primers were as follows: hsa-miRNA-195 forward, 5'-GGG GTAGCAGCACAGATT-3' and reverse, 5'-TCCAGTGCG TGTCGTGGA-3'; and U6 forward, 5'-GCTTCGGCACAT ATACTAAAT-3' and reverse, 5'-CGCTTCACGAATTG CGTGTCAT-3'.

Evaluation of cell viability via MTT assay. AMC-HN-8 cells were plated in 96-well plates and transfected with miRNA-195 mimics, miRNA-195 mimics-NC, miRNA-195 inhibitor and miRNA-195 inhibitor-NC respectively for 4 h. Following transfection, the serum-free medium was removed and cells were cultured at 37°C with regular culture medium for a further 48 h. To monitor cell survival, AMC-HN-8 cells in each well were incubated at 37°C for 4 h with 0.5 mg/ml MTT (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). Subsequently, the MTT solution was removed and the formazan crystals in the cells dissolved in 150 µl sterile dimethyl sulfoxide (Sigma-Aldrich; Merck KGaA) by incubating at 37°C for 15 min. Absorbance was recorded at 490 nm using an Easy Reader 340 AT (SLT-Lab Instruments, Salzburg, Austria). Relative cell survival was calculated by setting control absorbance from untreated cells at 100%. Experiments were performed in triplicate.

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay. Apoptotic AMC-HN-8 cells in the different groups were detected using a TUNEL assay as previously described (22). TUNEL staining was detected using the *In Situ* Cell Death Detection kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions. Sections were also counterstained at 37°C for 5 min with

DAPI (1:5 dilution; Invitrogen; Thermo Fisher Scientific, Inc.) for nuclei. The number of the TUNEL-positive nuclei ratio in ≥ 10 representative microscopic fields (magnification, $\times 200$) was calculated by fluorescence microscopy (Nikon Corporation, Tokyo, Japan) to compare the apoptosis ratio within the different groups.

Invasion assays. Invasion assays were conducted using 8 μM polyethylene terphthalate filters (BioCoat Matrigel Invasion Chambers; BD Pharmingen; BD Biosciences, Franklin Lakes, NJ, USA), as previously described (23). AMC-HN-8 cells (5×10^4 /well) transfected with miRNA-195 mimics, inhibitor or NC were allowed to invade through matrigel-coated filters for 16 h in a Transwell plate. The volume of medium plated in the upper chamber was 200 μl . Cells invaded to the lower chamber of the Transwell plate and were fixed using 4% (w/v) paraformaldehyde at room temperature for 30 min and stained using 0.05% crystal violet at room temperature for 20 min; the cell numbers were counted (magnification, $\times 200$) by a fluorescence microscopy (Nikon, Tokyo, Japan) as previously described (23).

Measurement of cell migration with Transwell migration assay. A Transwell migration assay was used to perform cell migration. Nuclepore filters with 8-nm pores (Corning, Inc., Corning, NY, USA) were coated with type IV collagen (Sigma-Aldrich; Merck KGaA) overnight at 37°C prior to the assay. AMC-HN-8 cells (5×10^4) in 200 μl medium (Gibco; Thermo Fisher Scientific, Inc.) from different treatment groups were added to the upper chambers, and the lower chambers were filled with normal RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.). Following 48 h of incubation at 37°C , the cells on the upper side were removed and discarded, and the cells that migrated to the lower side of the membrane were fixed with 4% paraformaldehyde at room temperature for 30 min, stained with 0.1% crystal violet for 30 min at room temperature and washed three times with PBS. The migrated cells in the lower side of the membrane were observed and imaged under an inverted microscope (magnification, $\times 200$). Images were captured from three randomly selected fields and the migrated cells counted.

Western blot analysis. The total amount of protein was extracted by RIPA buffer (Beyotime Biotechnology, Shanghai, China) from AMC-HN-8 cells for immunoblotting analysis. Briefly, the protein concentrations were determined with a bicinchoninic acid protein assay kit using bovine serum albumin (Beyotime Institute of Biotechnology) as the standard. Equal amounts of protein (100 μg) were separated by 10% SDS-PAGE and blotted onto PVDF membranes (EMD Millipore, Billerica, MA, USA). The blots were blocked at room temperature with 5% non-fat milk dissolved in PBS for 2 h, then probed overnight at 4°C in 5% milk tris-buffered saline/Tween-20 with the following primary antibodies: VEGFR2 (9698S, 1:1,000; Cell Signaling Technology, Inc., Danvers, MA, USA), Raf (ab33899, 1:1,000; Abcam, Cambridge, UK), extracellular signal-regulated kinase (ERK, sc-514302, 1:1,000), phosphorylated (p)-ERK

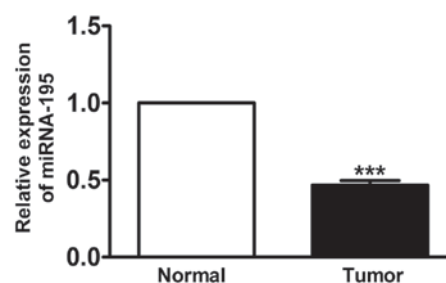


Figure 1. Expression levels of miRNA-195 in LSCC samples. Expression level of miRNA-195 in normal and LSCC samples. Data are expressed as mean \pm standard error of the mean, $n=23$. *** $P<0.01$ vs. normal control. miRNA, microRNA; LSCC, laryngeal squamous cell carcinoma.

(sc-81492, 1:1,000) (both from Santa Cruz Biotechnology, Inc., Dallas, TX, USA), total mitogen activated protein kinase kinase (MEK; 601121, 1:200), and p-MEK (558375, 1:1,000) (both from BD Biosciences), p-SRC (12432; 1:1,000), focal adhesion kinase (FAK; 3285; 1:1,000), p-FAK (8556; 1:1,000) (all from Cell Signaling Technology, Inc.), protein kinase B (AKT, 610860; 1:1,000) and p-AKT (550747; 1:1,000) (both from BD Biosciences), B-cell lymphoma 2 (Bcl-2; 15071; 1:1,000; Cell Signaling Technology, Inc.), Bcl-2-like protein 4 (Bax; 5023; 1:1,000), caspase-3 (9662; 1:500) and GAPDH (5174; 1:500) (all from Cell Signaling Technology, Inc.). Membranes were washed three times, 15 min each, with PBS containing 0.5% Tween-20 and incubated with the following secondary antibodies: Alexa Fluor[®] 700 goat anti-mouse immunoglobulin G (IgG) [heavy and light chains (H+L); A-11029; 1:8,000] or Alexa Fluor[®] 800 goat anti-rabbit IgG (H+L; A32730; 1:8,000; Invitrogen; Thermo Fisher Scientific, Inc.) in PBS at room temperature for 1 h. Images of western blot bands were captured using the Odyssey Infrared Imaging System and quantified with Odyssey software, version 1.2 (both from LI-COR Biosciences, Lincoln, NE, USA) by measuring the band intensity (area \times optical density) in each group and normalizing to GAPDH as an internal control. Unless otherwise stated, western blot analyses were repeated four times.

Statistical analysis. All quantitative data are expressed as the mean \pm standard error of the mean and analyzed using SPSS software version 13.0 (SPSS, Inc., Chicago, IL, USA). Two-tailed unpaired Student's *t*-tests were used between two groups and one-way analysis of variance were used for statistical evaluation of the data between multiple groups with post hoc contrasts by Student-Newman-Keuls test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

miRNA-195 is downregulated in human LSCC in vivo. RT-qPCR was used to determine the expression of miRNA-195 for the LSCC and the matched normal tissue samples obtained from 23 patients diagnosed with LSCC. For the LSCC samples, the mean miRNA level for miRNA-195 was significantly decreased by 55% compared with the corresponding matched samples (Fig. 1; $P<0.01$).

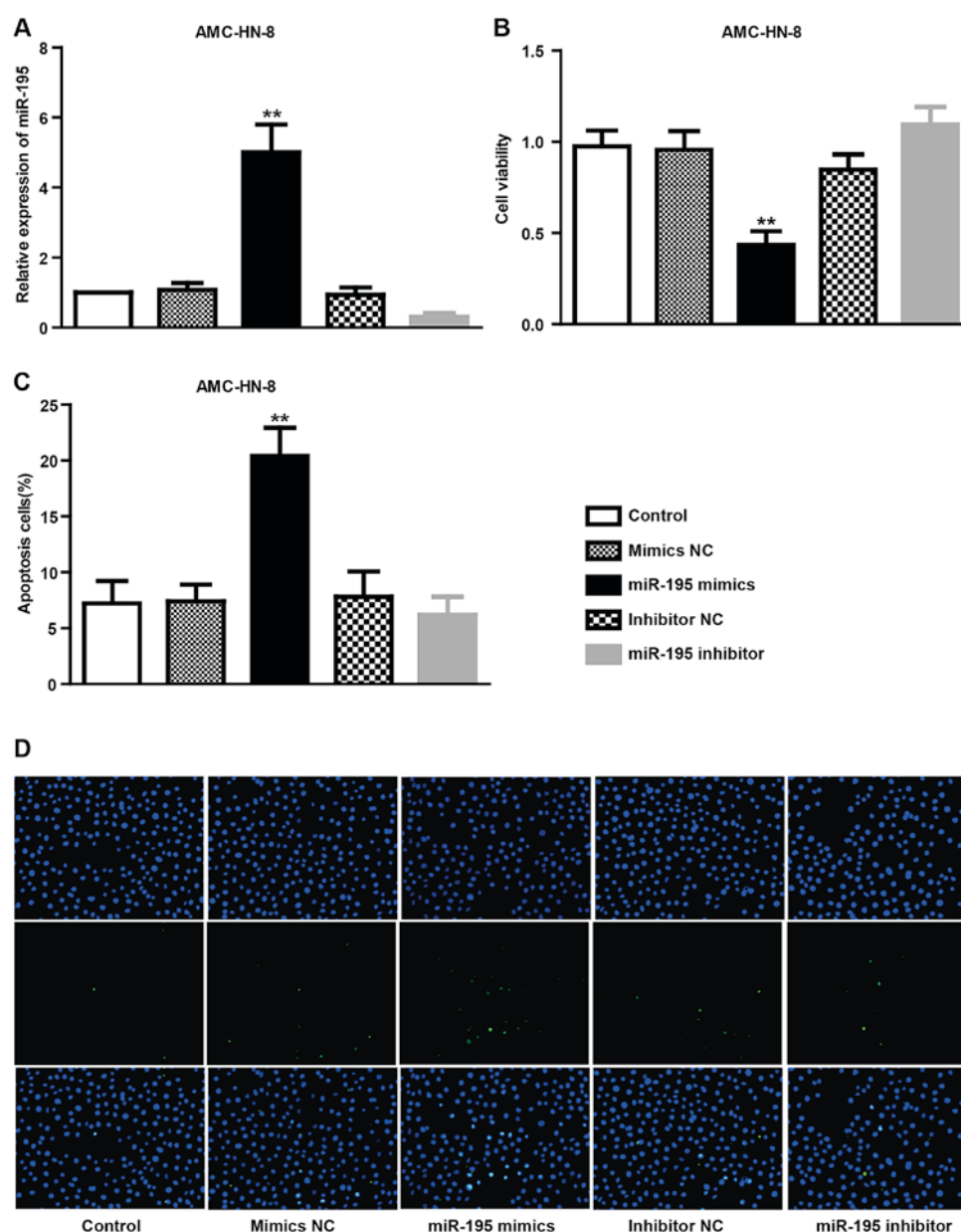


Figure 2. Effects of miRNA-195 on viability and apoptosis in AMC-HN-8 cells. (A) Expression levels of miRNA-195 in AMC-HN-8 cells in different treatment groups. Data are expressed as mean \pm standard error of the mean, $n=4$. (B) MTT assay for AMC-HN-8 cell viability. Data are expressed as mean \pm standard error of the mean, $n=3$. (C) The number of apoptotic AMC-HN-8 cells as determined by TUNEL assay. Data are expressed as mean \pm standard error of the mean, $n=10$. ** $P<0.01$ vs. control group. (D) Apoptotic AMC-HN-8 cells determined via TUNEL staining (original magnification, $\times 200$). Green, TUNEL-stained apoptotic cell; blue, cell nucleus stained by DAPI. miRNA/miR, microRNA; NC, negative control; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling.

Overexpression of miRNA-195 decreases cell viability and induces apoptosis in AMC-HN-8 cells. The present study evaluated whether miRNA-195 contributes to the survival rates of AMC-HN-8 cells by overexpression with specific miRNA-195 mimics (Fig. 2). The results demonstrated that the expression level of miRNA-195 in AMC-HN-8 cells from the miRNA-195 mimics treatment group was markedly higher compared with those from the control or miRNA-195 NC treatment groups, whereas the miRNA-195 expression from inhibitor treatment was markedly lower compared with the control or NC treatment groups (Fig. 2A). In comparison with control cells, the viability of AMC-HN-8 cells as determined by MTT analysis was reduced significantly in cells treated with miRNA-195 mimics ($P<0.01$), but not with the

miRNA-195 inhibitor and scramble, which did not significantly affect the viability of AMC-HN-8 cells (Fig. 2B). In addition, TUNEL staining results demonstrated that transfection with miRNA-195 mimics, but not with the inhibitor, increased the percentage of TUNEL-positive cells in the AMC-HN-8 cell population, suggesting that miRNA-195 may provide an anti-apoptotic effect in AMC-HN-8 cells ($P<0.01$; Fig. 2C and D). Together, these data suggest that upregulation of miRNA-195 may serve anti-proliferative and pro-apoptotic roles in AMC-HN-8 cells.

Effects of miRNA-195 on migration and invasion of AMC-HN-8 cells. The potential effects of miRNA-195 on cell migration and invasion were assessed using Transwell

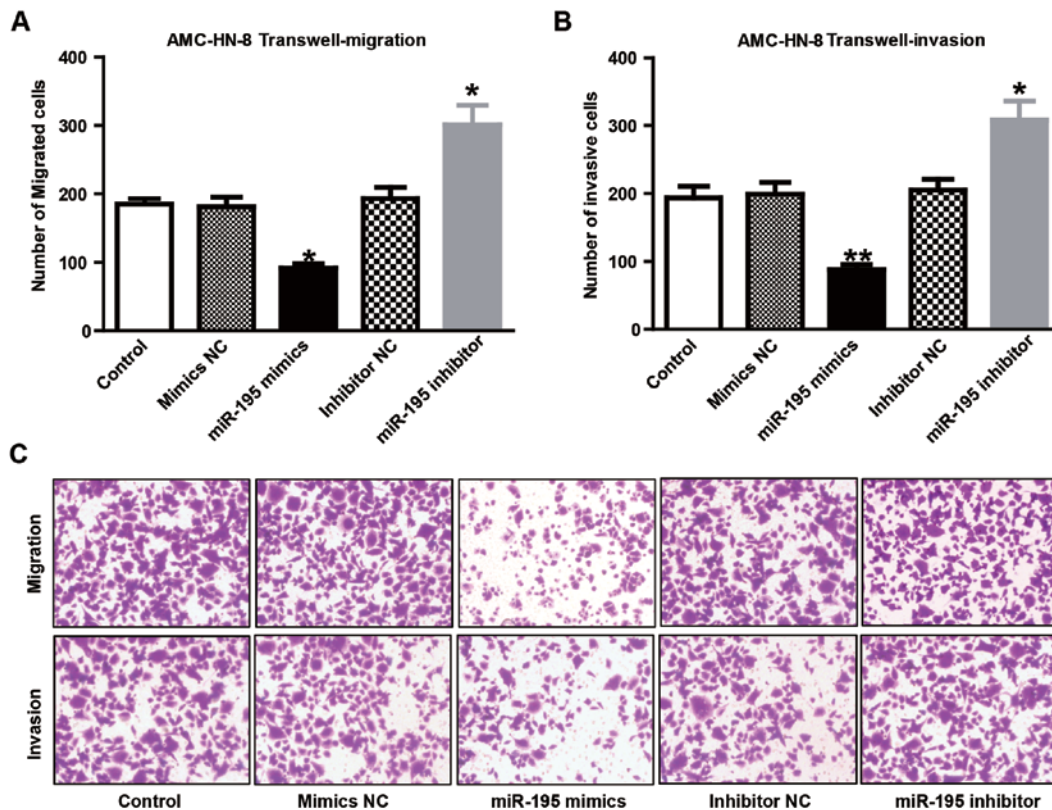


Figure 3. Overexpression of miRNA-195 inhibits the migration and invasion of cultured AMC-HN-8 cells. (A) Number of migrated AMC-HN-8 cells in different treatment groups. (B) Number of invaded AMC-HN-8 cells in different treatment groups. Data are expressed as mean \pm standard error of the mean, $n=3$. * $P<0.05$, ** $P<0.01$ vs. control group. (C) The AMC-HN-8 cells observed in the lower chambers following migration and invasion assays (original magnification, $\times 200$). miRNA/miR, microRNA; NC, negative control.

migration and invasion assays. AMC-HN-8 cells were selected for overexpression and knockdown of miRNA-195 using transient transfection, and Transwell migration and Matrigel invasion assays demonstrated that miRNA-195 overexpression resulted in a significant reduction of AMC-HN-8 cell migration (Fig. 3A; $P<0.05$) and invasion rate (Fig. 3B; $P<0.01$) compared with the NC group. By contrast, miRNA-195 knockdown resulted in an increase of AMC-HN-8 cell migration (Fig. 3A; $P<0.05$) and invasion rate (Fig. 3B; $P<0.05$) compared with the NC group. Fig. 3C shows the representative pictures of invasion and migration of AMC-HN-8 cells.

Association of VEGFR2/PI3K/AKT signal pathways with the miRNA-195-induced pro-apoptotic effect on AMC-HN-8 cells. The present study investigated whether miRNA-195 produced a pro-apoptotic effect by targeting VEGFR2 and downstream signaling pathway proteins in AMC-HN-8 cells (Fig. 4). As was hypothesized, negative regulation was identified between miRNA-195 and VEGFR2 in AMC-HN-8 cells (Fig. 4D). To evaluate the effect on cell apoptotic protein expression, AMC-HN-8 cells were transiently transfected with miRNA-195 mimics, miRNA-195 inhibitor or their NC. Lower levels of Bcl-2 (Fig. 4B) were detected in the miR-195 mimics-transfected cells compared with cells transfected with the NC or untransfected cells. In addition, higher levels of Bax (Fig. 4A) and caspase-3 (Fig. 4C) were detected compared with cells transfected with NC or

untransfected cells. Additionally, since the AKT/p-AKT is the upstream of Bax, Bcl-2 and caspase-3, the expression levels of AKT/p-AKT were also detected. As shown in Fig. 4E and F, the expression level of p-AKT, but not AKT, was significantly decreased in the miR-195 mimics-transfected cells compared with cells transfected with the NC or untransfected cells.

Overexpression of miRNA-195 inhibits viability, migration and invasion via regulating Raf/MEK/ERK and SRC/FAK signal pathways respectively in AMC-HN-8 cells. To evaluate whether overexpression of miRNA-195 serves a role in the regulation of the viability, migration and invasion of AMC-HN-8 cells, AMC-HN-8 cells were transiently transfected with miRNA-195 mimic, miRNA-195 inhibitor or their NC. Among various signaling molecules downstream of VEGFR2, the Raf/MEK/ERK pathway mainly promotes cell growth and differentiation. The western blot analysis data from the present study demonstrated that the RAF, p-MEK and p-ERK genes were significantly inhibited by upregulation of miRNA-195 at the protein level in AMC-HN-8 cells (Fig. 5A; $P<0.05$) compared with the control group. In addition, it was also identified that miRNA-195 overexpression resulted in lower expression of p-SRC and p-FAK proteins in AMC-HN-8 cells (Fig. 5B; $P<0.05$) compared with the control group. Together, these data reveal that overexpression of miRNA-195 is able to inhibit VEGFR2 and downstream signaling pathways, including Raf/MEK/ERK and SRC/FAK,

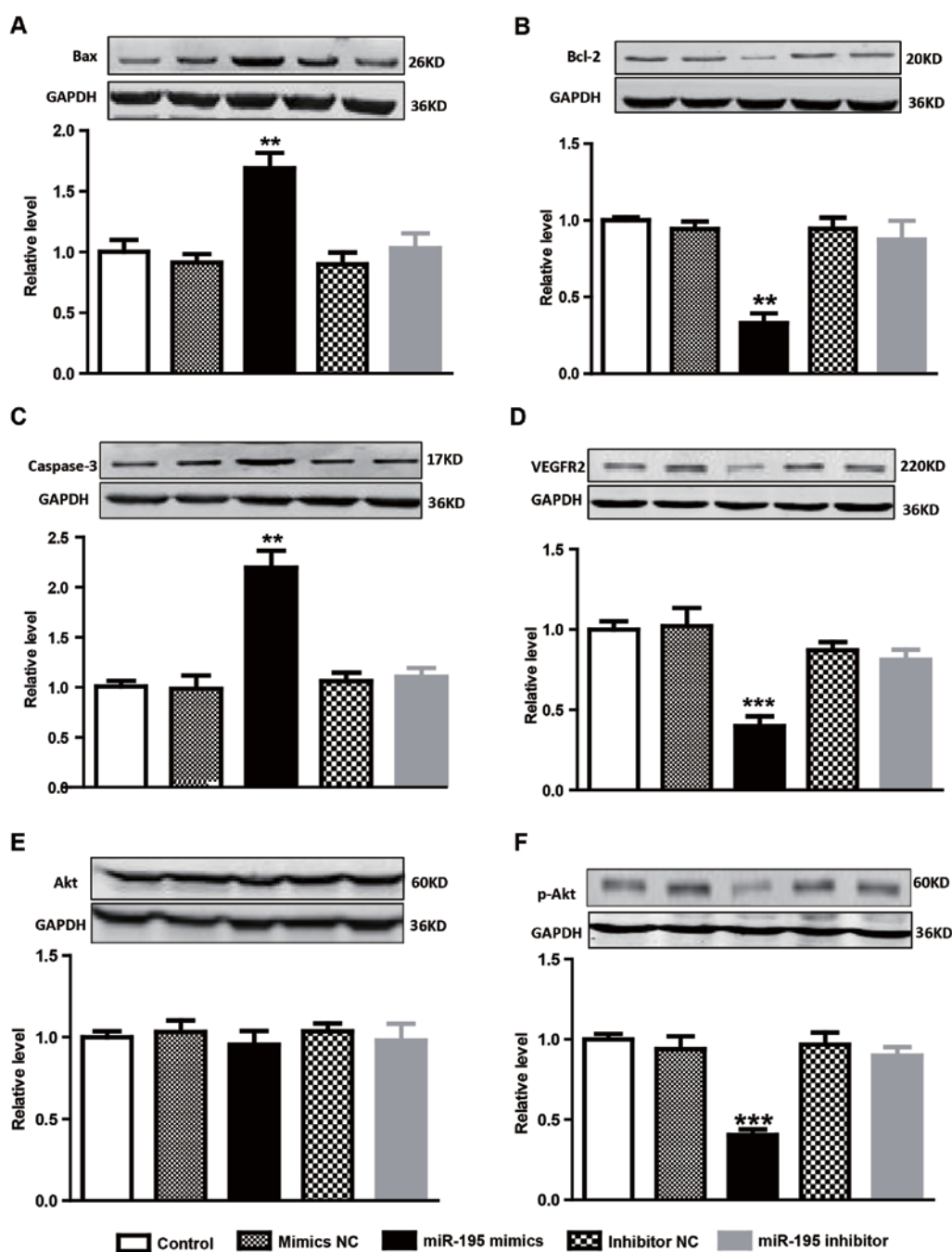


Figure 4. Protein levels in differently treated AMC-HN-8 cells were determined by western blotting. Protein levels of (A) Bax, (B) Bcl-2, (C) caspase-3, (D) VEGFR2, (E) AKT and (F) p-AKT were detected. Data are expressed as mean \pm standard error of the mean, $n=4$. ** $P<0.01$ or *** $P<0.001$ vs. control group. VEGFR2, vascular endothelial growth factor receptor 2; AKT, protein kinase B; p, phosphorylated; Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-like protein 4.

in AMC-HN-8 cells, which inhibit LSCC cell viability, migration and invasion, respectively.

Discussion

The present study identified downregulation of miRNA-195 in LSCC tissue samples compared with matched normal tissue samples and that overexpression of miRNA-195 in AMC-HN-8 cells was able to suppress cell viability, migration and invasion, and induce cell apoptosis. In addition, the data suggest that suppression of VEGFR2 and its downstream signal proteins, including Raf/MEK/ERK1/2, SRC/FAK and

apoptosis-related proteins, mediate the antitumor effect of miRNA-195 (Fig. 6). The results of the present study aid the understanding of the mechanisms of miRNA-195 in regulating the viability, migration and apoptosis in LSCC cell lines and support the suggestion that miRNAs may serve as potential therapeutic and drug targets.

With the development of RT-qPCR and microarrays, a number of recent studies (24,25) have identified that dysregulation of miRNAs is closely associated with the development and progression of various types of cancer, via cell viability, cell invasion and apoptosis, by acting as oncogenes or tumor suppressor genes. However, the mechanism of miRNAs in

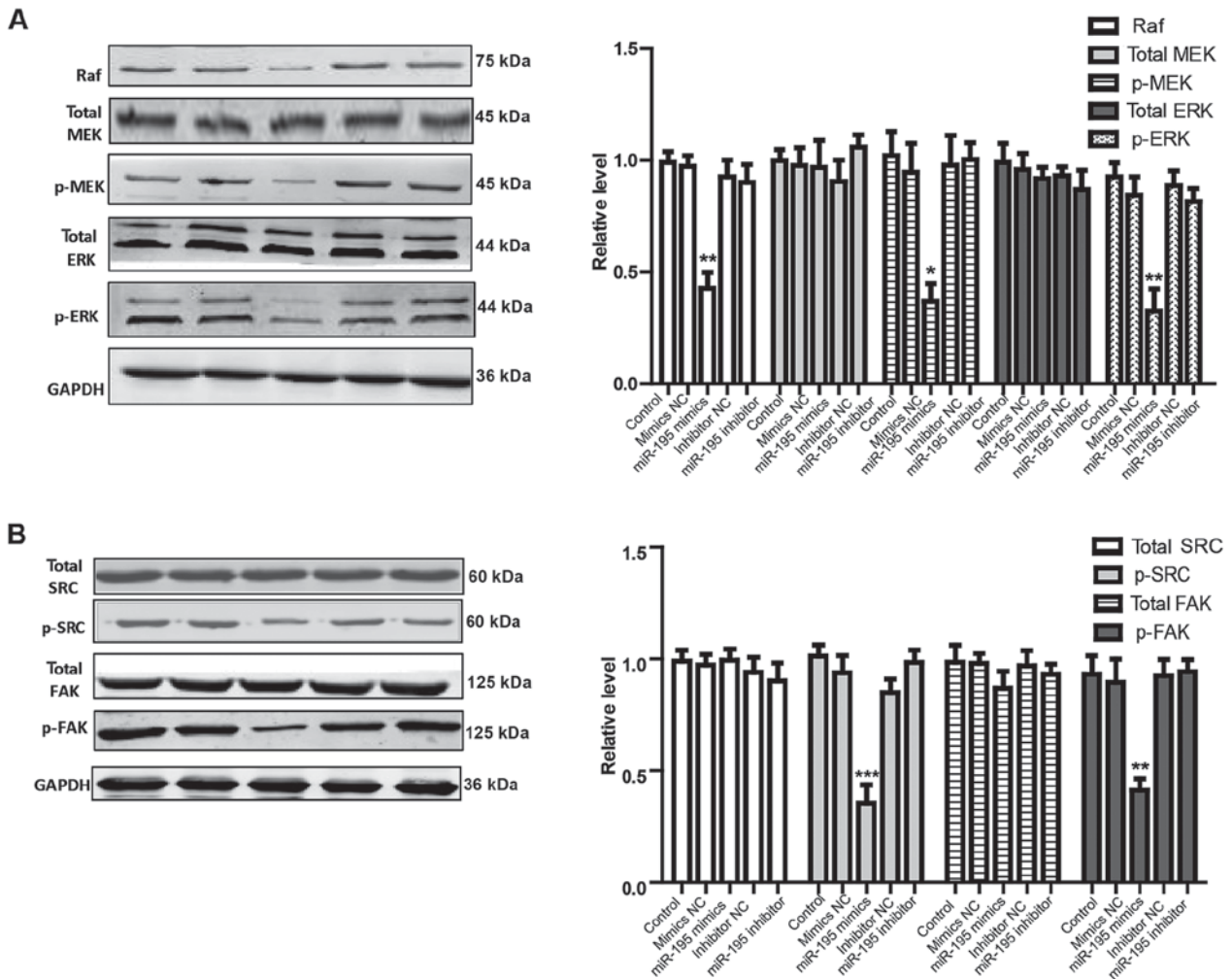


Figure 5. Effects of miRNA-195 overexpression on Raf/MEK/ERK and SRC/FAK signaling pathways in cultured AMC-HN-8 cells. Proteins expression of (A) Raf, total-MEK, p-MEK, total-ERK and p-ERK, and (B) total SRC, p-SRC, total FAK, and p-FAK were determined by western blotting. Data are expressed as mean \pm standard error of the mean, n=4. * P <0.05, ** P <0.01 or *** P <0.001 vs. control group. miRNA/miR, microRNA; NC, negative control; MEK, mitogen activated protein kinase kinase; p, phosphorylated; ERK, extracellular signal-regulated kinase; FAK, focal adhesion kinase.

LSCC remains to be elucidated. miRNA-195 downregulation has been reported in various types of cancer, including human hepatocellular carcinoma (26), breast cancer (18), esophageal squamous cell carcinoma (27), adrenocortical cancer (28), colorectal cancer (29) and human tongue squamous cell carcinoma (30). The present study examined the expression level of miRNA-195 in human LSCC tissues using RT-qPCR, which demonstrated that miRNA-195 was downregulated in primary LSCC tissues compared with matched adjacent non-cancerous tissues. These data suggest that downregulation of miRNA-195 in human LSCC tissues may be one of the molecular events causing its development. In a previous study (31), miRNA-195 was observed to regulate a number of target proteins, which are associated with the cell cycle, apoptosis and viability in multiple diseases, such as cancer, schizophrenia and heart failure (31-33). However, to the best of our knowledge, the expression level of miR-195 in human LSCC, its clinical role and its prognostic value have not been fully investigated thus far. The present study demonstrated the role of miRNA-195 in regulating cell biological functions during the development of LSCC. It was identified that overexpression of miRNA-195 significantly inhibited cell viability, migration and invasion,

and promoted apoptosis in AMC-HN-8 LSCC cells. The identification of miRNA-195 target genes is significant for the improved understanding of the role of miRNA-195 in tumorigenesis. At present, certain genes such as WEE1 (34), cyclin dependent kinase 6 (35), and Bcl-2 have been confirmed as targets of the miRNA-195 gene. According to the expression level of miRNA-195 in LSCC, the present study selected the presumed tumor-related gene VEGFR2 as a potential target of miRNA-195 among the predicted genes. The current study identified that overexpression of miRNA-195 represses the expression level of VEGFR2 in AMC-HN-8 cells. VEGFR2 mediates a variety of important molecular signaling pathways via its downstream signal proteins, including Raf/MEK/ERK1/2, SRC/FAK and apoptosis-related proteins, which serve a key role in regulating tumor development (36). Data from the present study further support the hypothesis that inhibition of VEGFR2/Raf/MEK/ERK and VEGFR2/SRC/FAK pathways regulates cancer cell growth and migration, respectively, mediated by miRNA-195 upregulation *in vitro*. It is noteworthy that not only was the expression of VEGFR2 in AMC-HN-8 cells treated with miRNA-195 mimics significantly lower than the control group, but also that

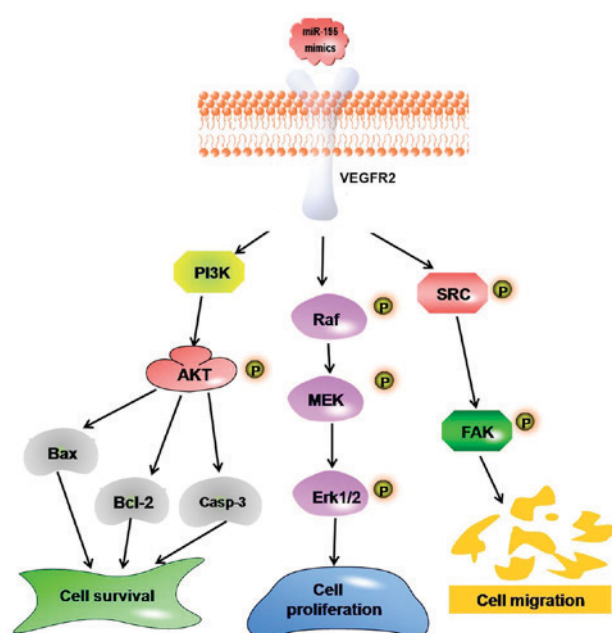


Figure 6. Schematic illustration of the possible targeting and signaling mechanisms by which miRNA-195 produces antitumor effects in cultured AMC-HN-8 cells. Increased miRNA-195 represses cells viability, migration and invasion by targeting VEGFR2, which leads to decreases in the activation of the Raf/MEK/ERK and SRC/FAK signaling pathways in cultured AMC-HN-8 cells. In addition, miRNA-195 may induce AMC-HN-8 cell apoptosis by downregulating the PI3K/AKT signaling pathway. miRNA/miR, microRNA; VEGFR2, vascular endothelial growth factor receptor 2; PI3K, phosphoinositide 3-kinase; AKT, protein kinase B; Bcl-2, B-cell lymphoma 2; Casp-3, caspase-3; MEK, mitogen activated protein kinase; ERK, extracellular signal-regulated kinase; FAK, focal adhesion kinase; p, phosphorylated.

the levels of p-AKT and Bcl-2 in LSCC cells with miRNA-195 overexpression were significantly decreased. These results suggest that the miRNA-195/VEGFR2 signaling pathway may serve an important role in the development of LSCC. However, it should be noted that the present study was performed in cell lines and the findings may not be capable of being extrapolated directly to animal models or to humans. There is a need for caution when applying the results of the present study to animals or patients.

The present study identified a novel antitumor miRNA, miRNA-195, in human LSCC tissue. The data suggest that the therapeutic potential of miRNA-195 in modulating cell growth, migration and apoptosis during the pathophysiological progression of LSCC, and the underlying mechanism, is associated with the inhibition of VEGFR2 and downstream signaling pathways, including Raf/MEK/ERK, SRC/FAK and PI3K/AKT. The present study indicates that exogenous application of miRNA-195 may be a promising intervention in the management of human LSCC and the associated pathological processes. The findings provide rationale for additional studies to investigate whether the miR-195 mechanisms associated with modulating cell growth, migration and apoptosis also operate in the clinical setting.

Acknowledgements

The authors thank all members of their laboratory for helpful discussions and comments on the present study.

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