

MicroRNA-195 inhibits cell proliferation, migration and invasion in laryngeal squamous cell carcinoma by targeting ROCK1

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Received July 19, 2016; Accepted July 20, 2017

DOI: 10.3892/mmr.2017.7460

Abstract. Laryngeal carcinoma is the second most common malignancy of the head and neck cancers. The most common type of laryngeal carcinoma comprises laryngeal squamous cell carcinoma (LSCC), which accounts for ~95% of laryngeal carcinoma cases. Despite great progress in diagnostic and therapeutic techniques over the last few decades, the prognosis for patients with LSCC remains poor. A number of studies reported that various miRNAs are dysregulated in LSCC and serve critical roles in LSCC tumorigenesis and tumor development. The present study aimed to evaluate the expression level of microRNA (miR)-195 and its possible roles in LSCC. Briefly, miR-195 was downregulated in LSCC tissues and cell lines. In addition, low miR-195 expression was significantly correlated with lymph node metastasis and TNM stage of LSCC patients. Further study has demonstrated that miR-195 overexpression suppressed cell proliferation, migration and invasion of LSCC. Moreover, rho-associated kinase 1 (ROCK1) was identified as a direct target gene of miR-195. Downregulation of ROCK1 exerted similar roles to that of miR-195 overexpression in LSCC, suggesting ROCK1 was a direct downstream target of miR-195. These findings elucidated a novel molecular mechanism for the pathogenic mechanism in LSCC carcinogenesis and progression, and may have a potential role in the treatment of patients with LSCC.

Introduction

Head and neck squamous cell carcinoma represents the sixth most common malignancy worldwide (1). Laryngeal carcinoma is the second most common malignancy of the head and neck cancers (2). In the United States, it is estimated that there would be 13,560 new cases and 3,640 mortalities due to laryngeal carcinoma in 2016 (3). The most common type of laryngeal carcinoma is comprised of laryngeal squamous cell

carcinoma (LSCC), which accounts for ~95% of laryngeal carcinoma cases (4). Until now, tobacco smoking, alcohol drinking, air pollution and unhealthy diet are the major risk factors for LSCC (5,6). Currently, the main therapeutic treatments for LSCC are surgery or total laryngectomy, followed by radiotherapy and chemotherapy (7). Despite great progress in diagnostic and therapeutic techniques over the last few decades, the prognosis for patients with LSCC remains poor with a 5-year survival rate of 64% (8). Most LSCC patients diagnosed with advanced-stage die of recurrence and/or metastasis (9). Therefore, fully understanding the molecular mechanism underlying LSCC would provide effective therapeutic targets to improve outcomes for patients with this disease.

MicroRNAs (miRNAs/miRs), ~22-25 nucleotides in length, are the most characterized of the non coding RNAs and endogenously expressed in animal and plant cells (10,11). They regulate the expression of protein-coding genes at the translational level and post-translational level through interaction with the 3'-untranslated region of their target genes in sequence-specific base pairing manner, modulating mRNA stability and/or translation inhibition (12,13). A number of studies have demonstrated that miRNAs serve critical roles in many physiological and pathological processes, including cell proliferation, differentiation metabolism, apoptosis, cell cycle, invasion, migration and death (14-16). The dysregulation of miRNAs are significantly correlated with many diverse diseases, such as neuronal disorders (17), inflammation (18) and cancer (19). Accumulated studies reported that a large number of miRNAs are dysregulated in LSCC. For example, miR-153 was downregulated in LSCC and functioned as a tumor suppressor through inhibiting cell proliferation and invasion via targeting KLF5 (20). miR-365a-3p was upregulated in LSCC and promoted cell growth and metastasis through regulating the PI3K/AKT pathway (21). Therefore, miRNAs may be molecular therapeutic targets for cancer diagnosis and treatments.

In the present study, the authors measured miR-195 expression in LSCC tissues and cell lines. In addition, they explored the functional roles of miR-195 in LSCC and its underlying molecular mechanism. The purpose of the present study was to validate the anticancer effects of miR-195 in LSCC.

Materials and methods

Tissue samples. A total of 51 pairs of LSCC tissues and adjacent normal epithelial tissues were obtained from

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Key words: laryngeal squamous cell carcinoma, microRNA-195, rho-associated kinase 1, therapeutic treatment

patients who received primary surgical resection of LSCC between September 2012 and July 2015 in the Department of Otolaryngology, Head and Neck Surgery, Tianjin Union Medical Center (Tianjin, China). None of the LSCC patients were treated with radiotherapy or chemotherapy prior to surgery. Tissue samples were snap-frozen in liquid nitrogen immediately following resection and stored at -80°C until use. The present study was approved by the Ethics Committee of Tianjin Union Medical Center (Tianjin, China), and all patients gave their informed written consent.

Cell lines, culture condition and transfection. Three human LSCC cell lines (Hep-2, AMC-HN-8 and Tu-177), a normal human keratinocyte cell line (HaCaT) and 293T cell line were purchased from American Type Culture Collection (Manassas, VA, USA). Cells were cultured in RPMI-1640 or Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) and were grown in a humidified atmosphere at 37°C with 5% CO₂.

miR-195 mimics and miRNA mimics negative control (miR-NC) were obtained from Shanghai GenePharma Co., Ltd. (Shanghai, China). Small interfering (si)RNA targeting Rho-associated kinase (ROCK)1 (si-ROCK1) and its control siRNA (si-NC) were chemical synthesized by Guangzhou RiboBio Co., Ltd. (Guangzhou, China). For cell transfection, cells (8x10⁵ cells/well) were seeded in 6-well plates. Following overnight incubation at 37°C with 5% CO₂, cells were transfected with miRNA mimics or siRNA by using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) following to the manufacturer's instructions.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The concentration and purity of total RNA was measured using a NanoDrop® ND-1000 spectrophotometer (NanoDrop; Thermo Fisher Scientific, Inc., Wilmington, DE, USA). For miR-195 expression, the One Step PrimeScript miRNA cDNA Synthesis kit (Takara Bio, Inc., Otsu, Japan) was used to perform reverse transcription, followed by qPCR with SYBR® Premix Ex Taq™ II (Takara Bio, Inc.). The temperature protocol for reverse transcription was as follows: 37°C for 15 min and 85°C for 5 sec. qPCR was performed with the following thermo cycling conditions: 5 min at 95°C, followed by 40 cycles of 95°C for 30 sec and 65°C for 45 sec.

For ROCK1 mRNA expression, cDNA was synthesized from RNA by using cDNA Synthesis kit (Takara Bio, Inc.). qPCR was carried out using SYBR® Premix Ex Taq™ II (Takara Bio, Inc.). The temperature protocol for reverse transcription was as follows: 37°C for 60 min and 85°C for 5 sec. The thermocycling conditions for qPCR was as follows: 5 min at 95°C, followed by 40 cycles of 95°C for 30 sec and 65°C for 45 sec. U6 and GAPDH were used as control for miR-195 and ROCK1 mRNA expression, respectively. The primers were designed as follows: miR-195, 5'-ACATCCAGCTGGGT AGCAGCACAGAAAT-3' (forward) and 5'-TGGTGTCGT GGAGTCG-3' (reverse); U6, 5'-GCTTCGGCAGCATATA

CTAAAAT-3' (forward) and 5'-CGCTTCACGAATTTGCGT GTCAT-3' (reverse); ROCK1, 5'-AGGAAGGCGGACATA TTGATCCCT-3' (forward) and 5'-AGACGATAGTTGGT CCCGGC-3' (reverse); and GAPDH, 5'-CCCCTTCATTGA CCTCAACT-3' (forward) and 5'-ATGAGTCCTTCCACG ATACC-3' (reverse). The data were calculated using the 2^{-ΔΔCq} method (22).

MTT assay. At 24 h post-transfection, cells were collected and seeded into 96-well plates at a density of 2,000 cells/well. Cells were then cultured for 24, 48, 72 and 96 h. At each time point, MTT (5 mg/ml; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) assay was carried out. A total volume of 20 μl MTT solution was added to each well and incubated at 37°C with 5% CO₂ for another 4 h. The culture medium was then removed and 150 μl DMSO was added to each well. Following incubation at 37°C for 10 min with a constant shaking, the absorbance at 490 nm was determined by using a microtiter plate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Cell migration and invasion assay. Cell migration and invasion assays were performed using Trans well chambers (Corning Life Sciences, Corning, NY, USA) and Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) coated Trans well chambers, respectively. At 48 h post-transfection, cells were collected and suspended in FBS-free culture medium. A total of 1x10⁵ cells were seeded into the upper chamber, whereas 500 μl culture medium containing 20% FBS was added in the lower chamber. After incubation at 37°C with 5% CO₂ for 48 h, those cells left on the upper chamber were removed with cotton swabs. The migrated or invaded cells were fixed with 95% ethanol for 20 min and stained with 0.1% crystal violet for 10 min. After washing with PBS (Gibco; Thermo Fisher Scientific, Inc.), the migrated and invaded cells were photographed and quantified using an inverted microscope.

Luciferase reporter assay. The putative target genes of miR-195 were predicted using the TargetScan (www.targetscan.org) and miRanda (www.microrna.org/microrna/). Based on bioinformatics analysis, ROCK1 was identified as a potential target of miR-195. The wild type (Wt) or mutant (Mut) 3'-untranslated region (UTR) of ROCK1 harboring the miR-195 binding site was cloned into pGL3 control vector. For luciferase reporter assay, pGL3-ROCK1-3'UTR Wt or pGL3-ROCK1-3'UTR Mut together with miR-195 mimics or miR-NC were injected into 293T cells by using Lipofectamine 2000, according to the manufacturer's instructions. Luciferase activities were determined 48 h post-transfection using the Dual-Luciferase Reporter Assay system (Promega Corporation, Madison, WI, USA). The results were expressed as relative luciferase activities (firefly luciferase/Renilla luciferase).

Western blot analysis. At 72 h after transfection, total protein was extracted from transfected cells using radioimmunoprecipitation assay buffer (Beyotime Institute of Biotechnology, Haimen, China). The protein concentration was determined by using the bicinchoninic acid assay (Thermo Fisher Scientific,

Inc.). Equal amounts of protein (20 μ g) were separated by electrophoresis on a 10% SDS-PAGE and then transferred onto polyvinylidene fluoride membrane (EMD Millipore Corporation, Billerica, MA, USA). The membrane was blocked with 5% non-fat milk in 0.1% TBS and 0.05% Tween-20 (TBST; Beyotime Institute of Biotechnology) for 2 h and incubated with primary antibodies at 4°C overnight, including mouse anti-human monoclonal ROCK1 antibody (1:1,000 dilution; sc-365628; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and mouse anti-human monoclonal GAPDH antibody (1:1,000 dilution, sc-365062; Santa Cruz Biotechnology, Inc.). After washing three times with TBST, the membrane was incubated with corresponding horseradish peroxidase-conjugated secondary antibody (1:5,000 dilution; sc-2005; Santa Cruz Biotechnology, Inc.) at room temperature for 1 h. The protein bands were visualized with enhanced chemiluminescence detection system (Amersham; GE Healthcare Life Sciences, Chalfont, UK). ROCK1 protein expression was normalized to total GAPDH.

Statistical analysis. The SPSS software (version, 17.0; SPSS Inc., Chicago, IL, USA) was used for statistical analysis. All data were expressed as mean \pm standard deviation, and were compared by a Student's t test to determine the statistical significance. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

miR-195 was downregulated in LSCC and was correlated with cancer progression. The expression levels of miR-195 in LSCC tissues and adjacent normal epithelial tissues were determined using RT-qPCR. The results indicated that miR-195 was lower in LSCC tissues than adjacent normal epithelial tissues (Fig. 1A, $P < 0.05$). Furthermore, the authors analyzed miR-195 expression in LSCC cell lines. The data indicated that miR-195 was significantly downregulated in LSCC cell lines compared with normal human keratinocyte cell line (Fig. 1B, $P < 0.05$).

To further investigate whether there was an association between miR-195 expression and LSCC prognosis, statistical analysis was performed to analyze the correlation between miR-195 expression and the clinicopathological factors of LSCC. As presented in Table I, there were significantly association of miR-195 expression with lymph node metastasis ($P = 0.011$) and TNM stage ($P = 0.015$). However, there were no statistically correlation between miR-195 expression and other clinicopathological features, including sex distribution, age, location, alcohol history, pathological differentiation and T classification (all $P > 0.05$).

miR-195 suppressed proliferation, migration and invasion of LSCC cells. To investigate the biological roles of miR-195 on LSCC cancer cells, Hep-2 and AMC-HN-8 cells were transfected with miR-195 mimics or miR-NC. RT-qPCR showed that miR-195 was markedly upregulated in Hep-2 and AMC-HN-8 cells transfected with miR-195 mimics (Fig. 2A, $P < 0.05$). The effect of miR-195 on proliferation of LSCC cells was assessed using MTT assay. As demonstrated in Fig. 2B, upregulation of miR-195 suppressed Hep-2 and AMC-HN-8 cell proliferation ($P < 0.05$). Then, the

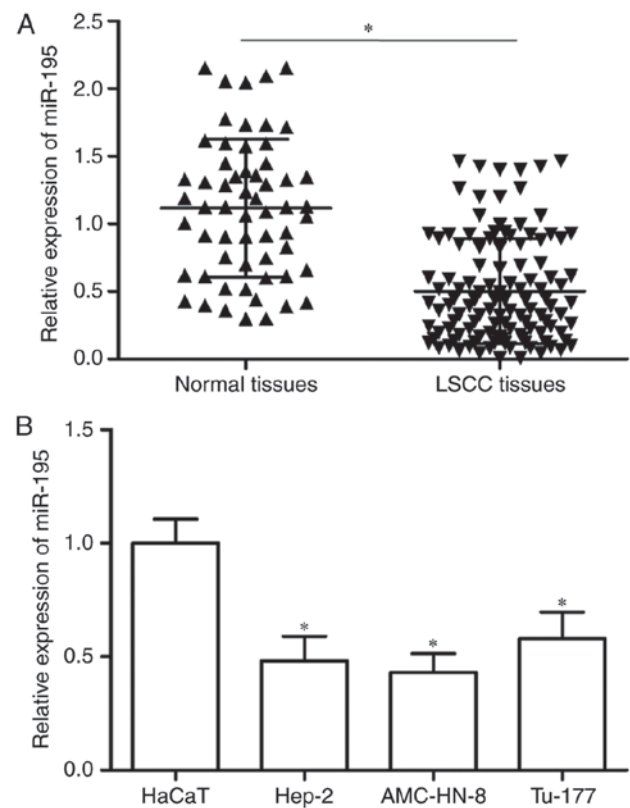


Figure 1. miR-195 expression levels in LSCC tissues and cell lines. (A) miR-195 expression was significantly lower in LSCC tissues than the level in adjacent normal epithelial tissues. * $P < 0.05$, as indicated (B) Levels of miR-195 were reduced in LSCC cell lines (Hep-2, AMC-HN-8, Tu-177) than that in HaCaT. Data are presented as mean \pm standard deviation. * $P < 0.05$ vs. HaCaT. miR, microRNA; LSCC, laryngeal squamous cell carcinoma.

author sex amined the effects of miR-195 on the migration and invasion capacities of LSCC cells by using cell migration and invasion assays. The results revealed that restoration of miR-195 obviously decreased the migration and invasion abilities of Hep-2 and AMC-HN-8 cells compared with miR-NC groups (Fig. 2C, $P < 0.05$). These data suggested that overexpression of miR-195 suppressed growth and metastasis of LSCC cells.

miR-195 directly targeted ROCK1. The potential molecular mechanism on how miR-195 suppressed cell growth and metastasis of LSCC was analyzed by exploring its direct target genes. Based on bioinformatics analysis with public databases, ROCK1 was identified as a potential target of miR-195 (Fig. 3A).

To verify whether ROCK1 was a direct target gene of miR-195, luciferase reporter assay was performed. 293T cells were transfected with miR-195 mimics or miR-NC as well as pGL3-ROCK1-3'UTR Wt or pGL3-ROCK1-3'UTR Mut. The results indicated that miR-195 overexpression reduced luciferase activities of vector containing wild type ROCK1 3'UTR (Fig. 3B, $P < 0.05$), while miR-195 had no regulation effect on mutant type of ROCK1 3'UTR, suggesting that this binding site in ROCK1 3'UTR was essential for the regulation by miR-195.

Moreover, the authors assessed the effects of miR-195 overexpression on the expression of ROCK1. RT-qPCR and

Table I. Relationship between miR-195 expression level and clinicopathological factors in laryngeal squamous cell carcinoma.

Clinical features	Case number	miR-195 expression		P-value
		Low	High	
Sex distribution				
Male	25	13	12	0.447
Female	26	15	11	
Age (year)				
<65	25	16	9	0.264
≥65	26	12	14	
Location				0.253
Supraglottic	30	14	16	
Glottic	21	14	7	
Alcohol history				0.404
Negative	25	12	13	
Positive	26	16	10	
Pathological differentiation				0.779
Moderately and highly differentiated	32	17	15	
Poorly differentiated	19	11	8	
T classification				0.264
T1-2	26	12	14	
T3-4	25	16	9	
Lymph node metastasis				0.011
Positive	24	18	6	
Negative	27	10	17	
TNM stage				0.015
I-II	28	11	17	
III-IV	23	17	6	

miR-195, microRNA-195.

western blotting indicated that ectopic of miR-195 expression suppressed ROCK1 mRNA (Fig. 3C, $P<0.05$) and protein (Fig. 3D, $P<0.05$) expression level in Hep-2 and AMC-HN-8 cells. Taken together, miR-195 can directly decrease ROCK1 expression through targeting the binding site in the 3'UTR of ROCK1.

Inhibition of ROCK1 exerted similar roles to that of miR-195 overexpression in LSCC. To study the effects of ROCK1 on LSCC, Hep-2 and AMC-HN-8 cells were transfected with si-ROCK1 or si-NC. After transfection, RT-qPCR and western blotting were used to evaluate its transfection efficiency. The results demonstrated that si-ROCK1 significantly decreased ROCK1 expression in Hep-2 and AMC-HN-8 cells at both mRNA (Fig. 4A, $P<0.05$) and protein (Fig. 4B, $P<0.05$) levels. Moreover, MTT assay, cell migration and invasion assays were used to investigate the effects of ROCK1 underexpression on LSCC cell proliferation, migration and invasion, respectively. The data revealed that inhibition of ROCK1 has similar effects to that of miR-195 overexpression, since it obviously suppressed growth (Fig. 4C, $P<0.05$) and metastasis (Fig. 4D, $P<0.05$) of Hep-2 and AMC-HN-8 cells. The results suggested

that miR-195 overexpression suppressed proliferation, migration and invasion of LSCC cells through downregulation of ROCK1.

Discussion

miR-195, one of the miR-16/15/195/424/497 family members, has been reported to be downregulated in various kinds of human cancer. For example, in hepatocellular carcinoma, miR-195 expression was markedly impaired in tumor tissues (23-25). Wang *et al* (26) found that miR-195 was downregulated in colorectal cancer. Its low expression was significantly associated with lymph node metastasis and advanced tumor stage. Kaplan-Meier survival analysis indicated that colorectal cancer patients with reduced miR-195 had a poor overall survival. Song *et al* (27) showed that expression level of miR-195 was reduced in breast cancer and obviously correlated with histological grade, tumor size, lymph nodal involvement and vessel invasion. In addition, Kaplan-Meier survival analysis indicated that breast cancer patients with high miR-195 level showed a positive association towards a longer survival. Downregulation of

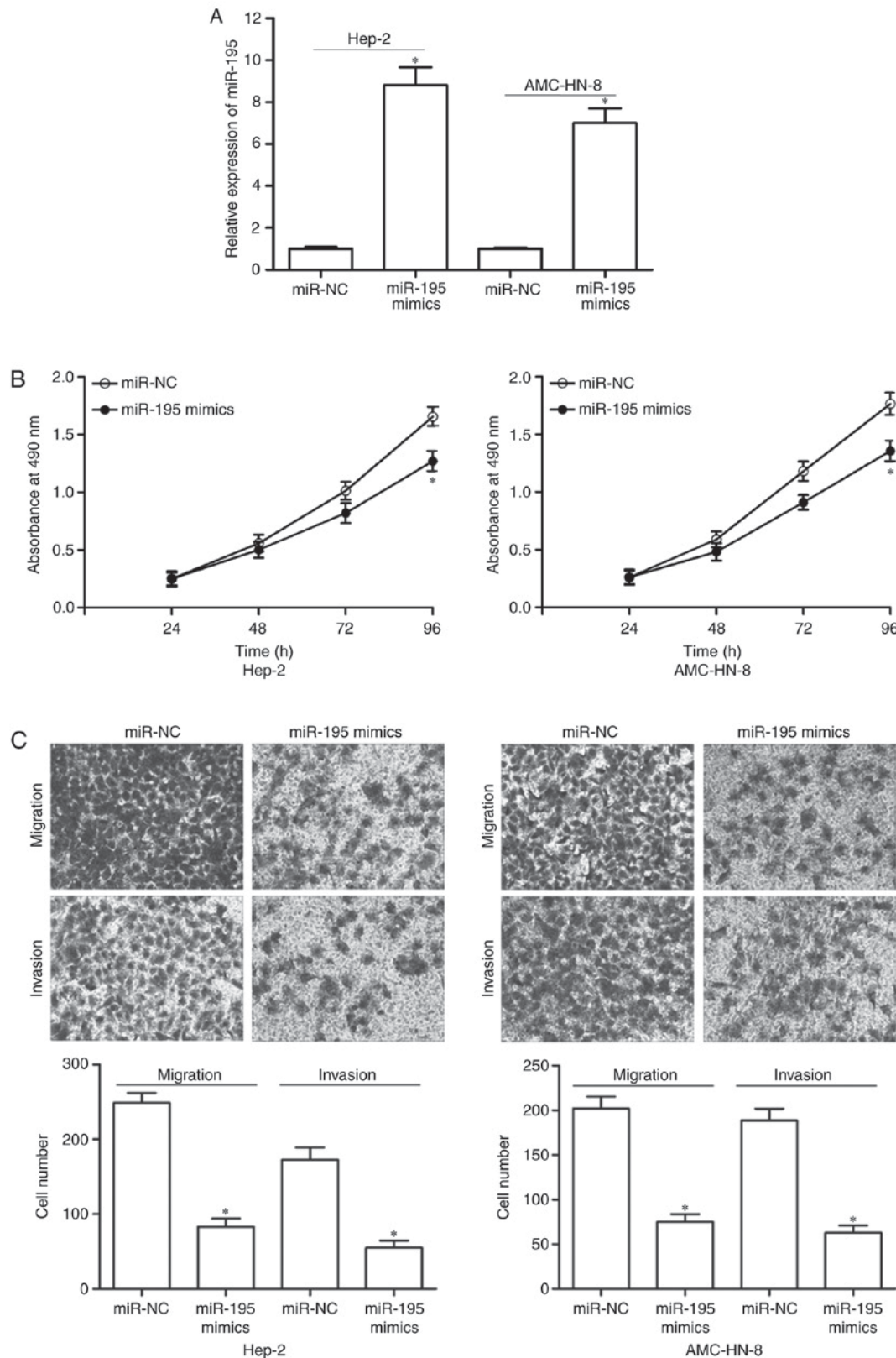


Figure 2. miR-195 suppressed Hep-2 and AMC-HN-8 cells proliferation, migration and invasion *in vitro*. (A) miR-195 was markedly increased in miR-195 mimics-transfected Hep-2 and AMC-HN-8 cells. (B) MTT assay revealed the suppressive effects of miR-195 on Hep-2 and AMC-HN-8 cells proliferation. (C) Cell migration and invasion assays showed the adverse effects of miR-195 on Hep-2 and AMC-HN-8 cells migration and invasion abilities (magnification, x200). *P<0.05 vs. miR-NC. Data are presented as mean \pm standard deviation. miR, microRNA; NC, negative control.

miR-195 was also observed in bladder cancer (28), glioblastoma (29), gastric cancer (30), glioma (31), tongue squamous

cell carcinoma (32) and non-small cell lung cancer (33). Consistent with these results, it was identified that miR-195

was downregulated in LSCC and correlated with lymph node metastasis and TNM stage. These results suggested that miR-195 serves important roles in these cancer types, and may therefore serve as a potential diagnostic and prognosis marker for these cancers.

To date, numerous studies have provided sufficient evidences to demonstrate that miR-195 functions as a tumor suppressor in human cancer. For example, in hepatocellular carcinoma, restoration of miR-195 dramatically suppressed cell migration, invasion, proliferation, angiogenesis, enhanced apoptosis and decreased tumor growth *in vivo* (23-25,34,35). Zhou *et al* (36) reported that ectopic of miR-195 decreased metastasis of cervical cancer. In colorectal cancer, introduction of miR-195 suppressed cell viability, colony formation, invasion induced apoptosis and increased the chemosensitivity of cells to the chemotherapeutic drug doxorubicin (37-39). Zhang *et al* (40) determined that miR-195 overexpression inhibited cell proliferation, cell cycle progression, migration, invasion, EMT and tumorigenesis in prostate cancer (41,42). In breast cancer, upregulation of miR-195 repressed breast cancer cells proliferation, cell colony formation, migration, invasion, enhanced apoptosis, radiosensitivity and chemosensitivity of cells to adriamycin (43-47). Liu *et al* (48) revealed that miR-195 inhibited growth and metastasis of non-small cell lung cancer. In present study, it was found that enforced miR-195 expression inhibited proliferation, migration and invasion of LSCC cells. These findings suggested that miR-195 could be a potential candidate therapeutic target for cancer treatments.

The present study further elucidated the molecular mechanism on how miR-195 regulated cell biological functions during the development of LSCC. Bioinformatics analysis predicted that ROCK1 is the potential target gene of miR-195. Luciferase reporter assays then confirmed that miR-195 decreased luciferase activities of vector containing wild type ROCK1 3'UTR, while miR-195 had no regulation effect on mutant type of ROCK1 3'UTR, suggesting that this binding site in ROCK1 3'UTR was essential for the regulation by miR-195. RT-qPCR and western blotting were performed to evaluate the regulation effect of miR-195 on ROCK1 expression. Results confirmed that miR-195 reduced ROCK1 expression at both mRNA and protein level. Finally, downregulation of ROCK1 had similar effects to that of miR-195 overexpression, since it obviously suppressed growth and metastasis. These results validated that ROCK1 was a direct functional downstream target of miR-195 in LSCC.

ROCK, an essential downstream effect or of the Rho small GTPase, acts as a molecular switch that binds GTP (active) and GDP (inactive) to regulate cell survival, proliferation and cytoskeleton organization, inducing alterations in cell shape/morphology, invasion and movement (49-51). ROCK1, located at chromosome 18 (18q11.1) (52), is frequently highly expressed in human cancers (53). A study by Zhang *et al* (54) found that ROCK1 expression was increased in LSCC tissues. Its expression was correlated with tumor size and lymph node metastasis. Functional study revealed that downregulation of ROCK1 inhibited cell proliferation, migration and invasion in LSCC. Combined with these findings, the authors speculated that the miR-195/ROCK1 axis could be developed as

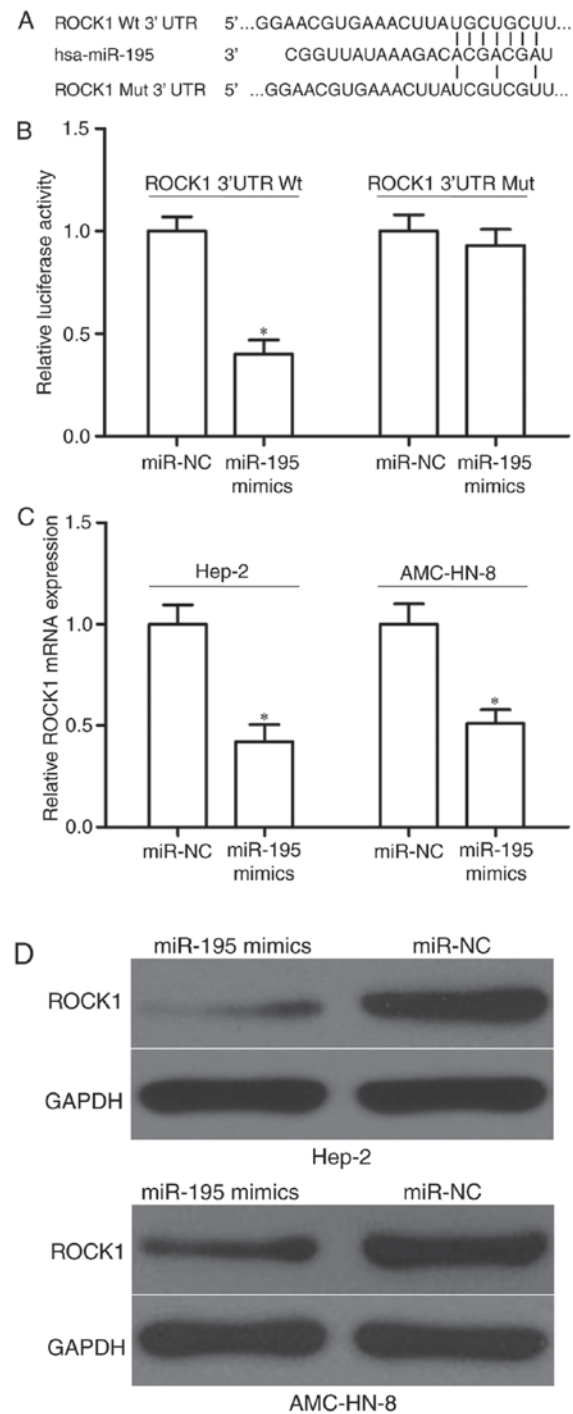


Figure 3. ROCK1 was a novel direct target of miR-195. (A) The wild type and mutation binding sites for miR-195 in the 3'UTR of ROCK1 are shown. (B) The luciferase activities of the pGL3-ROCK1-3'UTR Wt and pGL3-ROCK1-3'UTR Mut co-transfected with miR-195 or miR-NC in 293T cells were measured. (C) ROCK1 mRNA expression in Hep-2 and AMC-HN-8 cells transfected with miR-195 mimics or miR-NC was determined by using reverse transcription-quantitative polymerase chain reaction. (D) ROCK1 protein expression in Hep-2 and AMC-HN-8 cells transfected with miR-195 mimics or miR-NC was detected by using western blotting. * $P < 0.05$ vs. miR-NC. ROCK1, rho-associated kinase 1; miR, microRNA; UTR, untranslated region; Wt, wild-type; Mut, mutant; NC, negative control.

a therapeutic target for suppression of human LSCC rapidly growth and metastasis.

In summary, a downregulation of miR-195 was observed in LSCC tissues and cell lines. In addition, reduced miR-195

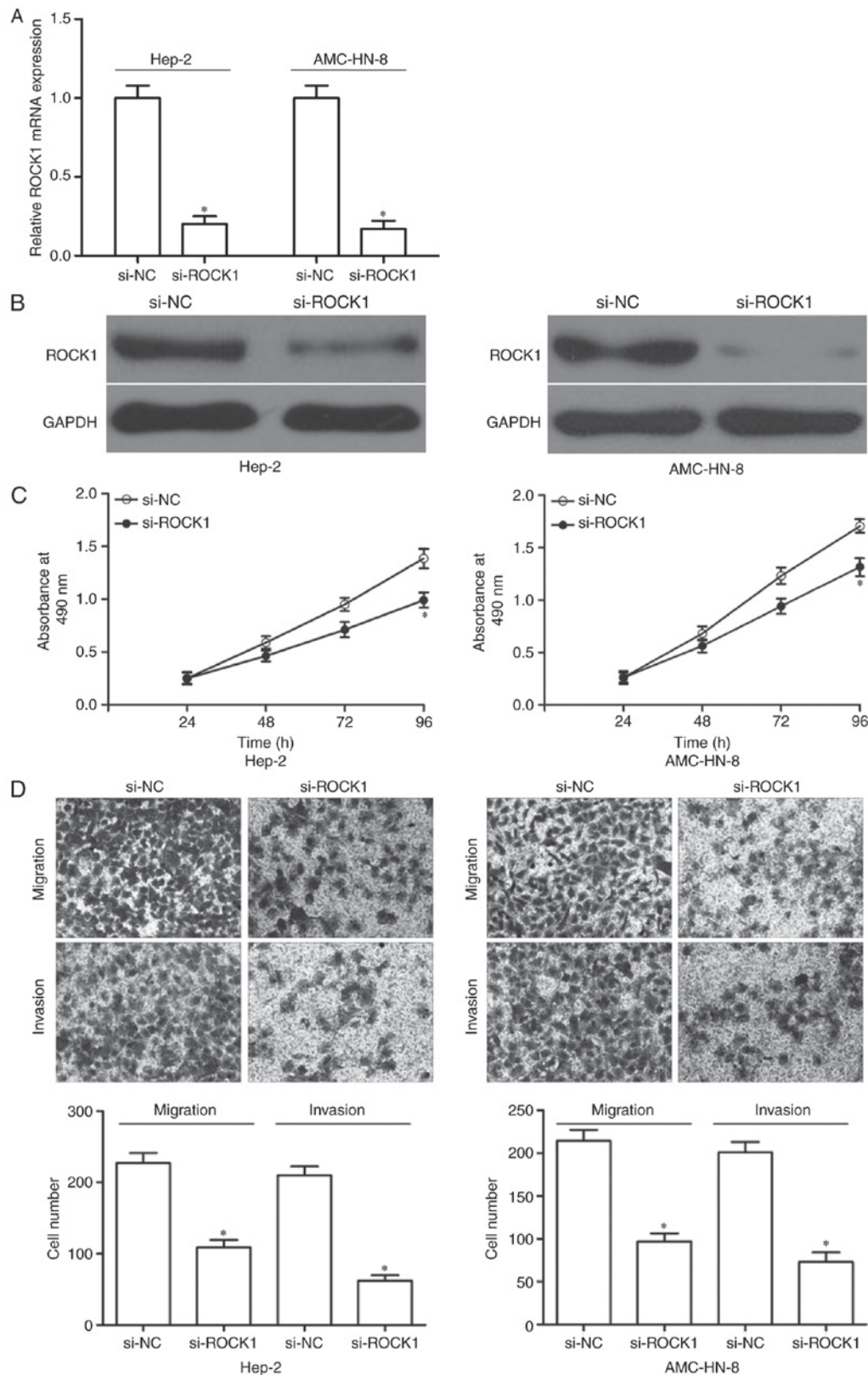


Figure 4. ROCK1 underexpression exerted similar roles to that of miR-195 overexpression in LSCC. (A and B) ROCK1 expression on mRNA and protein levels were determined in Hep-2 and AMC-HN-8 cells transfected with si-ROCK1 or si-NC. (C) MTT assay indicated the suppressive effects of si-ROCK1 on Hep-2 and AMC-HN-8 cells proliferation. (D) Cell migration and invasion assays revealed the adverse effects of si-ROCK1 on Hep-2 and AMC-HN-8 cells migration and invasion abilities (magnification, x200). * $P < 0.05$ vs. si-NC. Data are presented as mean \pm standard deviation. ROCK1, rho-associated kinase 1; NC, negative control.

expression was significantly correlated with lymph node metastasis and TNM stage. Moreover, it was demonstrated

that miR-195 may act as a tumor suppressor in LSCC tumorigenesis and tumor development through directly targeting

ROCK1, suggesting that miR-195 could potentially serve as a therapeutic target for the treatment of LSCC.

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