MicroRNA-195 inhibits the behavior of cervical cancer tumors by directly targeting HDGF

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Abstract. MicroRNAs (miRNAs/miRs) are a class of conserved non-coding endogenous small regulatory RNAs that regulate target gene expression by binding to the 3'-untranslated region of target mRNAs in a base-pairing manner, resulting in repression of transcription or degradation of target mRNAs. It has been demonstrated previously that the abnormal expression of miRNAs is involved in the carcinogenesis and progression of cervical cancer. The aim of the present study was to investigate the expression, biological functions and underlying molecular mechanisms of miR-195 in cervical cancer. The reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was used to detect the expression level of miR-195 in cervical cancer tissues and cell lines. Following transfection, an MTT assay, cell migration and invasion assays, western blot analysis and a dual-luciferase reporter assay were performed in human cervical cancer cells. In the present study, it was identified that miR-195 was downregulated in cervical cancer tissues and cell lines. Additionally, upregulation of miR-195 and knockdown of hepatoma-derived growth factor (HDGF) inhibited proliferation, migration and invasion of cervical cancer cells. Furthermore, a dual-luciferase reporter assay identified that HDGF was a direct target gene of miR-195. RT-qPCR and western blot analysis demonstrated that miR-195 mimic inhibited HDGF expression at the mRNA and protein levels, whereas miR-195 inhibitor enhanced HDGF expression at the mRNA and protein levels. These results indicated that miR-195 targeted HDGF to inhibit the behavior of tumors in cervical cancer. These results also suggested that miR-195 was a potential therapeutic biomarker of cervical cancer.

Introduction

Cervical cancer is the third most diagnosed gynecological cancer and the fourth leading cause of cancer-associated mortality in women worldwide (1). In the USA, there were an estimated 12,900 novel diagnosed cases and 4,100 mortalities caused by cervical cancer in 2015 (2). Developing countries account for >80% of all patients with cervical cancer, primarily due to the lack of widespread screening using cervical cytology (3). Increasing evidence has demonstrated that infection by high-risk variants of human papillomavirus (HPV) is the primary cause of cervical cancer, and in addition, genetic and epigenetic alterations of host cellular genes have important functions in the progression from a precancerous disease to an invasive cancer (4-6). Currently, the primary standard therapeutic method for treating cervical cancer is surgery, radiotherapy and concurrent platinum-based chemotherapy (7). In locally advanced cervical cancer cases, combined therapy is able to improve overall survival and recurrence rates (8-10). However, the overall survival rates for stage III and stage IV cervical cancer are 60 and 15%, respectively (11). Furthermore, ~30% of patients presented with cancer recurrence, lymph node recurrence and distant metastasis, and ultimately received an unfavorable prognosis (12). Therefore, it is essential to understand the underlying molecular mechanisms of initiation and progression of cervical cancer, and develop more effective therapies for patients with cervical cancer.

Previous studies have demonstrated the abnormal expression of multiple microRNAs (miRNAs/miRs) in cervical cancer (13-15). miRNAs are a class of conserved non-coding endogenous small regulatory RNAs of 18-25 nucleotides in length that regulate target gene expression at the post-transcriptional level (16). miRNAs are predicted to regulate >67% of genes (17). miRNAs bind to the 3'-untranslated region (UTR) of target mRNAs in a base-pairing manner and result in transcription repression or degradation of target mRNAs (18-20). miRNAs perform vital functions in a number of physiological and pathological processes, including cell proliferation, differentiation, cell cycle regulation, apoptosis, migration and invasion (21). Previous studies have demonstrated that miRNAs are involved in the initiation and progression of cancer, and...
they function as tumor suppressors or oncogenes, depending on their targets (22-24). Therefore, identification of the targets of miRNAs is essential for understanding the roles of miRNAs in carcinogenesis and development of cancer. Furthermore, miRNAs may be investigated as a targeted therapy for cancer.

In the present study, miR-195 was identified to be significantly downregulated in cervical cancer tissues and cell lines. Functional analysis demonstrated that miR-195 inhibited the proliferation, migration and invasion of cervical cancer cells. In addition, hepatoma-derived growth factor (HDGF) was identified as a direct target of miR-195 in vitro. The results of the present study indicate important functions of miR-195 in the carcinogenesis and progression of cervical cancer.

Materials and methods

Clinical specimens and cell lines. Human cervical cancer tissues were collected from patients at The First Affiliated Hospital of Anhui Medical University (Hefei, China). A total of 36 paired cervical cancer tissues and matched non-cancerous adjacent tissues (NATs) were obtained from patients diagnosed with cervical cancer who had undergone primary surgery. All the patients with cervical cancer did not receive chemotherapy, radiotherapy or other treatment prior to surgery. Cervical cancer tissues and NATs were immediately frozen in liquid nitrogen and stored at -80°C. The present study was approved by the Research Ethics Committee of The First Affiliated Hospital of Anhui Medical University, and written informed consent was also obtained from each patient involved.

A total of 4 cervical cancer cell lines (HeLa, CaSki, C33A and SiHa) and human embryonic kidney (HEK)-293T cells were purchased from the Shanghai Institute of Biochemistry and Cell Biology (Shanghai, China). HaCaT, an immortalized HPV-negative skin keratinocyte cell line, was obtained from the American Type Culture Collection (Manassas, VA, USA). All cell lines were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.; Gibco; Thermo Fisher Scientific, Inc.; and 100 units/ml penicillin or 100 mg/ml streptomycin. All cell lines were grown at 37°C in a humidified atmosphere of 5% CO₂.

Cell transfection. miR-195 mimic, miR-195 inhibitor, negative control (NC), NC inhibitor, and luciferase reporter plasmid were synthesized by Shanghai GenePharma Co., Ltd. (Shanghai, China). The following miRNA sequences were used: miR-195 forward, 5'-UAG CAG CAC AGA AAU GGC-3'; miR-195 inhibitor, 5'-GGC AAU AUU UCU GUG CUG CUA-3'; and NC inhibitor, 5'-GCC AAU AUU UCU GUG CUG CUA-3'; and NC, 5'-CAGUACUUUGUGUAGUCAA-3'. HDGF siRNA and NC siRNA were obtained from Ambion (Thermo Fisher Scientific, Inc.). The sequence of the HDGF siRNA was 5'-CAAGGAGAGAAGCAGAAA-3' and the sequence of the NC siRNA was 5'-AACAGGCACAGTCCGAG GT-3'. Cells in the exponential phase of growth were seeded in a 6-well plate and cultured in DMEM without antibiotics. Cell transfection was performed using Lipofectamine™ 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) when the cell density reached 50-60%, according to the manufacturer's protocol.

RNA isolation, reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from tissues or cells using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. cDNA was synthesized from 1 µg total RNA using a PrimeScript™ RT Reagent kit (Takara Biotechnology Co., Ltd., Dalian China). The cycling conditions were as follows: 42°C for 5 min; 95°C for 10 sec; and 40 cycles of 95°C for 5 sec, 55°C for 30 sec and 70°C for 30 sec. To determine the expression of miR-195 and HDGF mRNA, qPCR was performed using SYBR Premix Ex Taq Master mix (Takara Biotechnology Co., Ltd.) in an Applied Biosystems 7500 Real-Time PCR system (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. The cycling conditions for qPCR were as follows: 95°C for 30 sec; 40 cycles of 95°C for 5 sec; and 60°C for 30 sec. U6 small nuclear RNA (U6) and GAPDH were used as internal controls for miR-195 and HDGF mRNA expression, respectively. The primer sequences were as follows: miR-195 forward, 5'-ACATCCGAGCTGGGAGAGACA GAAAT-3' and reverse, 5'-TGGTGTCTGGGATCC-3'; U6 forward, 5'-CTCGTTGCGGAGACA-3' and reverse, 5'-AACGCTTACAGAATTGCGAT-3'; and HDGF forward, 5'-GAGGGTACCGGATGAAAGAG-3' and reverse, 5'-GAA ACATTGTGGCTACAGG-3'; and GAPDH forward, 5'-TGC ACCACCAACTGCTAGC-3' and reverse, 5'-GCGATGAG TGTTGTCATGAG-3'. Data were calculated using the 2-ΔΔCq method (25). All samples were amplified in triplicate.

MTT assay. An MTT assay was performed to analyze the function of miR-195 and HDGF on cell proliferation. Following transfection, 3,000 cells were seeded into each well of the 96-well plates. Following incubation at 37°C for various times (24, 48, 72 or 96 h), MTT assays (Sigma; Merck KGaA, Darmstadt, Germany) were performed. A total of 20 µl MTT solution (5 mg/ml) was added to each well prior to incubation for a further 4 h at 37°C. The formazan precipitate that formed was dissolved in 200 µl dimethylsulfoxide (Beyotime Institute of Biotechnology, Haimen, China) and the absorbance of each well at 490 nm was detected using an ELISA plate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA). All samples were amplified in triplicate.

Cell migration and invasion assays. Cell migration and invasion assays were performed to assess the cell migratory and invasive abilities using Transwell chambers (8 µm pore size; EMD Millipore, Billerica, MA, USA). For the cell invasion assay, the Transwell chamber was coated with Matrigel (BD Biosciences, San Jose, CA, USA). Following transfection of cells at room temperature with miR-195 mimic, NC, miR-195 inhibitor or NC inhibitor for 48 h, 1x10⁵ cells in 200 µl serum-free DMEM were added to the upper Transwell chamber. A 500 µl volume of DMEM containing 20% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) was added to the lower chamber.

Following incubation at 37°C for 24 h, cells remaining on the upper surface of the Transwell chamber membranes were scraped off using cotton swabs. Cells were fixed with 95% ethanol and stained with 0.5% crystal violet (Beyotime Institute of Biotechnology) for 20 min. Following washing in PBS three times, the migrated or invaded cells were counted under an inverted microscope (IX31; Olympus Corporation, Tokyo, Japan).
Japan) and images were captured at x200 magnification. Each experiment was repeated at least three times. For bioinformatics analysis, the potential target genes of miR-195 were predicted using TargetScan (www.targetscan.org) and miRanda (www.microrna.org/microrna).

Western blot analysis. The primary antibodies used were mouse anti-human monoclonal HDGF antibody (1:500 dilution; cat. no. sc-271344; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and anti-human β-actin antibody (1:1,000 dilution; cat. no. sc-1616-R; Santa Cruz Biotechnology, Inc.). Total protein from cells was extracted using radioimmunoprecipitation lysis buffer (Beyotime Institute of Biotechnology). Total protein concentration was determined using a bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Inc.). Equal amounts of protein (20 µg) from each group were separated by SDS-PAGE (10% gels; Beyotime Institute of Biotechnology) and electro-transferred onto polyvinylidene fluoride membranes (EMD Millipore). The membranes were blocked at room temperature with 5% non-fat dried milk in TBS/0.1%-Tween-20 (TBST) for 1 h and incubated overnight at 4°C with the aforementioned primary antibodies. The membranes were washed 3 times with Tris-buffered saline containing Tween 20 prior to incubation with corresponding horseradish peroxidase-conjugated secondary antibody (dilution, 1:300; cat. no. A0192; Beyotime Institute of Biotechnology) for 1 h at room temperature. Protein bands were visualized using an enhanced chemiluminescence kit (Pierce; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. The protein bands were analyzed with a FluorChem imaging system (version 4.1.0; Alpha Innotec, San Leandro, CA, USA).

Dual-luciferase reporter assay. HEK-293T cells were plated in a 12-well plate. Transfection was performed when the cell density reached ~90% confluence. HEK-293T cells were transfected with miR-195 or NC, and pGL3-HDGF-3’-UTR wild-type (Wt) or pGL3-HDGF-3’-UTR mutant (Mut) using Lipofectamine™ 2000. Following incubation at 37°C for 48 h, activities of firefly and Renilla luciferase were determined using the Dual-Luciferase Reporter assay system (Promega Corporation, Madison, WI, USA), according to the manufacturer's protocol. The firefly luciferase activities were used as an internal control. Each experiment was repeated at least three times.

Statistical analysis. Data are presented as the mean ± standard deviation, and were compared using SPSS software (version 13.0; SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

miR-195 is downregulated in cervical cancer tissues and cell lines. To investigate the functions of miR-195 in cervical cancer, miR-195 expression was determined in cervical cancer tissues and NATs. It was identified that miR-195 was significantly downregulated in cervical cancer tissues compared with NATs (P<0.05; Fig. 1A and B).

The expression level of miR-195 in cervical cancer cell lines and an immortalized HPV-negative skin keratinocyte line HaCaT was also determined. miR-195 was significantly downregulated in each of the four cervical cancer cell lines compared with HaCaT cells (P<0.05; Fig. 1C). The expression of miR-195 was decreased in HeLa and C33A cells compared with CaSki and SiHa cells. Therefore, HeLa and C33A cells were selected for the functional study.

miR-195 inhibits cell proliferation in HeLa and C33A cells. To investigate the roles of miR-195 in cervical cancer, miR-195 mimic, NC, miR-195 inhibitor and NC inhibitor were each transfected into HeLa and C33A cells. Following transfection, RT-qPCR was used to measure transfection efficiency. miR-195 was significantly upregulated in HeLa and C33A cells transfected with miR-195 mimic, whereas miR-195 was downregulated in HeLa and C33A cells transfected with miR-195 inhibitor (P<0.05; Fig. 2A).

To assess whether miR-195 affects cell proliferation, an MTT assay was performed. Cell proliferation was significantly
inhibited by overexpression of miR-195 and knockdown of miR-195 had the opposite effect on cell proliferation (P<0.05; Fig. 2B).

**miR-195 inhibits cell migration and invasion in HeLa and C33A cells.** To investigate the role of miR-195 in metastasis, cell migration and invasion assays were performed using Transwell chambers. Ectopic expression of miR-195 significantly decreased HeLa and C33A cell migration, whereas inhibiting miR-195 expression increased HeLa and C33A cell migration (P<0.05; Fig. 3A). In the cell invasion assay, overexpression of miR-195 decreased HeLa and C33A cell invasion, whereas inhibition of miR-195 expression enhanced HeLa and C33A cell invasion (P<0.05; Fig. 3B). These results indicated that miR-195 inhibits the migratory and invasive ability of cervical cancer cells.

**HDGF is a direct target of miR-195 in vitro.** To investigate the carcinogenic roles of miRNA-195 in cervical cancer, bioinformatics analysis was used to identify potential target genes of miR-195. Bioinformatic analysis predicted that HDGF was a direct target gene of miR-195 (Fig. 4A). HDGF contained a miR-195 seed match at position 37-43 of the HDGF 3’-UTR. In addition, HDGF was significantly upregulated in cervical cancer cells, as determined using western blot analysis (P<0.05; Fig. 4B). Furthermore, a dual-luciferase reporter assay demonstrated that miR-195 mimic significantly inhibited the luciferase activity of HDGF-3'-UTR Wt, but not of HDGF-3'-UTR Mut in HEK-293T cells (P<0.05; Fig. 4C). A slight, but not significant, decrease in HDGF mRNA expression was identified in HeLa and C33A cells following transfection with miR-195 mimic compared with transfection with NC, and following transfection with miR-195 inhibitor compared with transfection with NC inhibitor (Fig. 4D). HDGF protein expression was markedly downregulated in HeLa and C33A cells following transfection with miR-195 mimic compared with transfection with NC, and HDGF protein expression was markedly upregulated in HeLa and C33A cells following transfection with miR-195 inhibitor compared with transfection with NC inhibitor (Fig. 4E). The results for HDGF protein expression were identified to be significant following quantification (P<0.05; Fig. 4F). These results indicated that HDGF was a direct target gene of miR-195.

**HDGF is involved in miR-195-induced functions in HeLa and C33A cells.** To investigate whether HDGF functions as an important mediator of the roles of miR-195 in cervical cancer cells, HDGF siRNA and NC siRNA were transfected into HeLa and C33A cells. Following transfection for 48 h, western blot analysis was used to determine transfection efficiency. HDGF was markedly downregulated in HeLa and C33A cells following transfection with HDGF siRNA, compared with transfection with NC siRNA (Fig. 5A).
miR-195, a member of the miR-15/16 family, is located between 6881953 and 6862065 bp on chromosome 17p13.1 (26). A number of previous studies have demonstrated that miR-195 was downregulated, relative to non-malignant tissue, in various tumors, including breast cancer (27), prostate cancer (28), non-small cell lung cancer (NSCLC) (29), esophageal squamous cell carcinoma (30), hepatocellular carcinoma (31) and tongue squamous cell carcinoma (32). However, to the best of our knowledge, there are no studies on the miR-195 expression level in cervical cancer. In the present study, the expression level of miR-195 was identified to be decreased in cervical tissues and cell lines. These results suggested that miR-195 has a tumor-suppressive function in the initiation and progression of cervical cancer.

Functionally, it has been demonstrated that abnormal expression of miR-195 was hypothesized to contribute to the malignant phenotype of several tumors. For example, in breast cancer, ectopic expression of miR-195 inhibited cell proliferation, cell colony formation, migration and invasion, and led to an accumulation of cells in the G1 phase of the cell cycle by targeting cyclin E1 (33). In addition, upregulation of miR-195 significantly increased breast cancer cell radiosensitivity through downregulation of apoptosis regulator B cell lymphoma-2 (27). Furthermore, miR-195 increased breast cancer cell chemosensitivity to Adriamycin via inhibition of Raf-1 proto-oncogene (34). In prostate cancer, re-expression of miR-195 increased cell proliferation, migration and invasion in vitro, and decreased tumor xenograft growth, angiogenesis and invasion in vivo by directly targeting ribosomal protein S6 kinase β1 (28). In lung cancer, Guo et al (35) reported that miR-195 suppressed cell proliferation and invasion through regulation of HDGF (35). Liu et al (29) identified that miR-195 was downregulated in NSCLC and that the decreased expression level of miR-195 was associated with poor overall survival rates. Enforced miR-195 expression decreased cell proliferation and motility by directly targeting checkpoint kinase 1 (29). Furthermore, Wang et al (36) reported that miR-195 inhibited the proliferation and metastasis of NSCLC cells by directly targeting insulin-like growth factor 1 receptor, a novel discovered target in lung cancer (36). These results suggested that miR-195 have important functions in these types of cancer, and may be investigated as a potential therapeutic gene for the treatment of these types of cancer.

In the present study, it was identified that miR-195 inhibited the proliferation, migration and invasion in vitro of cervical cancer cells. miRNAs are involved in major physiological and pathological processes by regulating target mRNA expression. Therefore, it is important to identify the underlying molecular mechanism involved in miR-195-induced inhibition of mRNA expression. Hence, it is important to identify the underlying molecular mechanism involved in miR-195-induced inhibition of proliferation, migration and invasion of cervical cancer cells. In the present study, HDGF was identified as a direct target mRNA of miR-195. HDGF, a heparin-binding growth factor, was originally purified from conditioned culture medium from the hepatoma HuH7 cell line (37). The HDGF gene is located on chromosome 1, region q21–q23 (38). Previous studies have demonstrated that knockdown of HDGF decreased neoplastic transformation and proliferation (39-41). It has been confirmed that HDGF is involved in the regulation of cell apoptosis, angiogenesis, invasion and metastasis (42). A number of studies have demonstrated that HDGF was upregulated in various types of human tumor, including gastric cancer, hepatocellular carcinoma, NSCLC, pancreatic cancer and esophageal carcinoma, and that HDGF was associated with poor prognosis (43-46). HDGF was also identified to be
Figure 4. HDGF is a direct target gene of miR-195 in vitro. (A) The miR-195-binding site in the 3'-UTR of HDGF and the HDGF 3'-UTR mutant sequence. (B) Western blot analysis demonstrated that HDGF was upregulated in all four cervical cancer cell lines compared with HaCaT. β-Actin was used as a loading control. *P<0.05 vs. HaCaT cells. (C) A dual-luciferase reporter assay demonstrated that miR-195 decreased the luciferase activity of pGL3-HDGF-3'-UTR Wt but not pGL3-HDGF-3'-UTR Mut in human embryonic kidney HEK-293T cells. *P<0.05 vs. NC. (D) A slight, but not significant, decrease in HDGF mRNA expression in HeLa and C33A cells was identified following transfection with miR-195 mimic compared with transfection with NC, or transfection with miR-195 inhibitor compared with transfection with NC inhibitor. (E) HDGF protein expression was markedly downregulated in HeLa and C33A cells following transfection with miR-195 mimic compared with transfection with NC. HDGF protein expression was markedly upregulated in HeLa and C33A cells following transfection with miR-195 inhibitor compared with transfection with NC inhibitor. β-Actin was used as a loading control. (F) Quantification of HDGF protein expression. *P<0.05 vs. NC; #P<0.05 vs. NC inhibitor. HDGF, hepatoma-derived growth factor; miR-195, microRNA-195; UTR, untranslated region; Wt, wild-type; Mut, mutant.
HDGF has been identified to be regulated by multiple miRNAs in many types of cancer. For example, in lung cancer, HDGF has been identified to be regulated by multiple miRNAs in many types of cancer. For example, in lung cancer,
miR-16 and miR-497 negatively regulated HDGF expression to inhibit cell proliferation, invasion and angiogenesis (43.48). In gastric cancer, miR-141 suppressed cell proliferation, migration and invasion by directly targeting HDGF (49). In hepatocellular carcinoma, miR-214 is involved in tumor angiogenesis through the regulation of HDGF. In the present study, HDGF was identified to be regulated by miRNAs in cervical cancer. miR-195 targeted HDGF to inhibit proliferation, migration and invasion of cervical cancer cells. miR-195 may be investigated as a therapy to target HDGF, and inhibit cervical cancer proliferation and metastasis.

To the best of our knowledge, the present study is the first to demonstrate that miR-195 was downregulated in cervical cancer. In addition, it was also demonstrated that miR-195 decreases cell proliferation, migration and invasion. Furthermore, HDGF was identified as a direct target of miR-195 in vitro. These results may assist in our understanding of the underlying molecular mechanism of carcinogenesis and progression of cervical cancer, and also provide a theoretical basis for investigating miR-195 as a target for the treatment of cervical cancer.

References


