MiRNA-495 inhibits cell proliferation and invasion abilities in gastric cancer cells by down-regulation of FGFRL1

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Abstract: Gastric cancer (GC) is the leading cause of human mortality worldwide. MicroRNAs (miRNAs) are a potentially useful diagnostic and therapeutic tool for a variety of types of human cancer, including GC. In the present study, miRNA-495 was found to be significantly down-regulated in GC tissues and cells as indicated by RT-qPCR analysis. MTT and BrdU assays demonstrated that miRNA-495 suppressed the proliferative abilities of the GC cells, and the Transwell chamber assay suggested that miRNA-495 inhibited the metastatic and invasion abilities of the GC cells. Furthermore, fibroblast growth factor receptor-like 1 (FGFRL1), a key modulator in the differentiation and development of skeletal tissues, kidney and embryos, is a direct target gene of miRNA-495. RT-qPCR analysis found that the expression of FGFRL1 is up-regulated in GC tissues and cells, and MTT, BrdU and invasion assays demonstrated that FGFRL1 promoted the proliferation and invasion abilities of GC cells. In conclusion, the results of the present study suggested that miRNA-495 inhibits the proliferative, metastatic and invasion abilities of GC cells by directly targeting to FGFRL1. These results contribute to further understanding of the tumorigenesis of GC, and the development of therapeutic strategies for its treatment.

Keywords: MicroRNA-495, fibroblast growth factor receptor-like 1, gastric cancer, cell proliferation, invasion

Introduction

Gastric cancer (GC) is the leading cause of human mortality worldwide [1]. Due to the high metastatic potential, significant rates of mortality are observed in patients with GC, particularly for patients with peritoneal metastasis [2]. Despite significantly progress in the treatment of GC in past decades, a lack effective treatment options remains for a substantial number of patients with metastatic GC. Therefore, development of novel therapeutic methods for GC remains urgent.

MicroRNAs (miRNAs) are a class of evolutionarily conserved, small non-coding RNAs, which functions in the post-transcriptional regulation of gene expression by binding to the 3’-untranslated region (UTR) of target mRNAs, subsequently leading to the degradation of the target mRNAs and repression of translation [3]. Almost 500 miRNAs in human have been identified, and it has been suggested that the human genome may encode up to 1000 miRNAs [4]. Accumulating data have provided support for the involvement of miRNAs as key regulators in the regulation of almost all crucial cellular events, including differentiation, development, proliferation, apoptosis and metabolism [5, 6]. Therefore, the dysregulation of miRNA biogenesis or expression levels is likely to alter cellular differentiation, cell cycle control and the apoptosis of normal cells, finally lead to the initiation and progression of malignant tumor growth [4]. It is well documented that aberrant expression of miRNAs is closely associated with carcinogenesis, and several miRNAs have increasingly been recognized as oncogenes or tumor suppressors [7]. Therefore, miRNAs are a potentially useful diagnostic and therapeutic tool for a variety of malignant tumors [4].

miRNA-495 was originally demonstrated to regulate the expression of brain-derived neurotrophic factor in the brain [8], however, increas-
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Material and methods
Tumor samples

Paired high-grad GC tumors and adjacent normal tissues were obtained from 20 patients who underwent primary surgical resection at the Second Xiangya Hospital of Central South University (Changsha, China). These samples were snap-frozen in liquid nitrogen, and then stored at -80°C. The present study was approved by the ethic committee of the second Xiangya Hospital of Central South University (Changsha, China). All patients were required to provide written informed consent.

Cell culture and transfection

HFE-145 and GES-1 human gastric cell lines and SGC7901, MKN28 and BGC823 human GC cell lines were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in Dulbecco’s Modified Eagle’s medium (DMEM; Gibco, Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) and maintained at 37°C in a humidified atmosphere with 5% CO₂. For functional analysis, negative control miRNA (scrambled miRNA), miRNA-495 mimics and miRNA-495 inhibitor (Ambion; Thermo Fisher Scientific, Inc.) were transfected into the MKN28 cells with 70% confluence using X-tremeGENE transfection reagent (Roche Diagnostics), according to the manufacturer’s protocol. pCMV-Myc, pcDNA3.1-his/myc-FGFRL1 (Myc-FGFRL1) (GeneCopoeia, Inc., Guangzhou, China), control siRNA (scrambled siRNA) and FGFRL1 siRNA (Invitrogen Life Technologies, Inc.) were transfected into the MKN28 cells at 70% confluence using Lipofectamine 2000™ transfection reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer’s protocol.

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis

Total RNA was extracted from the GES-1, HFE-145, SGC7901, MKN28 and BGC823 cells using the mirVana™ miRNA isolation kit (Ambion; Thermo Fisher Scientific, Inc.). cDNA was synthesized from 10 ng of the total RNA using a TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA, USA) with

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Figure 1. miRNA-495 is down-regulated in GC tissues and cells. A. Expression levels of miRNA-495 were determined in 20 pairs of human GC tissues and their adjacent normal tissues using RT-qPCR. *P<0.05, compared with normal tissues. B. Expression levels of miRNA-495 were determined in GES-1, HFE-145, SGC7901, MKN28 and BGC823 cell lines using RT-qPCR. *P<0.05, compared with GES-1 cells. Data are expressed as the mean ± standard deviation.

Dual luciferase reporter assay

The 3'-UTR of FGFRL1 mRNA from nucleotide 1332 to nucleotide 1551 (GenBank accession number, NM_001004356.2) was amplified by PCR using the forward primer (5'-GAAGGAA-GACTGGGTGCG-3') and the reverse primer (5'-TGGAGGAAAGGGATGCACTT-3') from total cDNA, which was isolated from the MKN28 cells. The PCR was performed with the conditions as follows: DNA initial denaturation step of 10 min at 95°C, followed by 33 cycles consisting of 40 sec at 95°C for denaturation, 40 sec at 60°C for annealing and 70 sec at 72°C for extension, and final extension step of 5 min at 72°C. Following amplification, the 3'-UTR of FGFRL1, containing the miRNA-495 binding sites and corresponding mutated sequence, were cloned into a pMir-Report vector (Ambion; Thermo Fisher Scientific, Inc.), which were termed pMir-FGFRL1 and pMir-MutFGFRL1, respectively. 70% confluence of the MKN28 cells were co-transfected with the reporter constructs and the miRNA-495 mimics, miRNA-495 inhibitors or scrambled miRNA using Lipofectamine 2000™ transfection reagent (Invitrogen; Thermo Fisher Scientific, Inc.). The luciferase activities were determined after 48 h using a Dual-Luciferase® Reporter assay system (Promega Corporation, Madison, WI, USA) and a Beckman Coulter LD400 luminometer (BD Biosciences, Franklin Lakes, NJ, USA). Activity
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was normalized and presented as the firefly/Renilla luciferase ratio.

Western blot analysis

The cultured cells were lysed in cell lysis buffer (Cell Signaling Technology, Inc., Danvers, MA, USA) containing 1% PMSF, followed by centrifugation at 16,000 g at 4°C for 10 min. The supernatants were quantified with v-1000 spectrophotometer (Qingdao Ziquan Instrument Co. Ltd, Qingdao, China) by using Bio-Rad protein assay (Bio-Rad laboratories, Inc., Hercules, California, USA). 20 μL of protein samples were separated by 10% SDS-PAGE (Invitrogen Life Technologies, Inc.), following which the samples were transferred onto nitrocellulose membranes (Invitrogen Life Technologies, Inc.). Following blocking with blocking buffer (Invitrogen Life Technologies, Inc.) overnight at 4°C, the membranes were immunoblotted with rabbit polyclonal FGFRL1 antibody (1:1,000, cat no. NBP1-31461, Novus Biological Inc. Littleton, CO, USA) or mouse monoclonal β-actin antibody (1:10,000, cat no. A3854, Sigma-Aldrich) at 4°C for 12 h, followed by incubation with a 1:10,000 dilution of horseradish peroxidase-conjugated secondary antibody (1:6,000; cat

Figure 2. Effect of miRNA-495 on MKN28 cell proliferation. A. Ectopic expression levels of miRNA-495 in MKN28 cells transfected with scramble negative control, miRNA-495 mimics or inhibitors were determined using RT-qPCR analysis. Data are expressed as the mean ± standard deviation. **P<0.01, compared with miRNA-SCR. B. Proliferation rates of the MKN28 cells were determined using an MTT assay. The MKN28 cells were transfected with scramble negative control, miRNA-495 mimics or inhibitors, and the MTT assay was performed every 24 h for 5 days. Data are expressed as the mean ± standard deviation. *P<0.05, compared with miRNA-SCR. C. Proliferation rates of the MKN28 cells were determined using BrdU assay. The MKN28 cells were transfected with scramble negative control, miRNA-495 mimics or inhibitors, and the BrdU assay was performed 24 and 48 h post-transfection for 24 h. Data are expressed as the mean ± standard deviation. *P<0.05, compared with miRNA-SCR.
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Figure 3. Effect of miRNA495 on MKN28 cell migration and invasion. A. Analysis using a Transwell assay revealed that the miRNA-495 mimics and inhibitors significantly decreased and increased the number of migrated MKN28 cells, respectively. B. Analysis using a Transwell assay revealed that the miRNA-495 mimics and inhibitors significantly attenuated and increased the number of invaded MKN28 cells, respectively. Data are expressed as the mean ± standard deviation. *P<0.05, compared with miRNA-SCR.

3-(4,5-dimethyldiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay

Cell viability was evaluated using an MTT assay. Briefly, $2 \times 10^3$ cells were cultured at 37°C for 24 h, followed by transfection with miRNA for another 24 h. At every 24 h within 5 days, 10 μl of 10 mg/ml MTT (Sigma-Aldrich, MO, USA) was added to the cells and incubated at 37°C for 2 h. Subsequently, the reaction was terminated by removal of the supernatant, followed by the addition of 100 μl DMSO to dissolve the formazan product. After 20 min, the optical density (OD) 570 was measured using a plate reader (EL×808; Bio-Tek Instruments, Inc., Winooski, VT, USA).

5-Bromo-2-deoxyUridine (BrdU) assay

A BrdU incorporation assay kit (Roche Diagnostics) was used for analyzing the incorporation of BrdU during DNA synthesis in the proliferating cells, according to the manufacturer’s protocols. Briefly, $2 \times 10^3$ MKN28 cells were cultured for 24 or 48 h, followed by incubation with a final concentration of 10 μM BrdU (BD Pharmingen, San Diego, CA, USA) for between 2 and 24 h. The absorbance values were measured at 450 nm using the plate reader.

Cell migration and invasion assays

The cell migration and invasion abilities were evaluated using extracellular matrix-coated invasion chambers (EMD Millipore) as described previously (11). Briefly, $3 \times 10^5$ MKN28 cells were plated into the upper compartment of the chambers, which were precoated with Matrigel (BD Biosciences) in serum-free medium (in triplicate). RPMI 1640 medium (Invitrogen Life Technologies, Inc.) with 10% FBS were added to the lower chambers. Following incubation at 37°C for 24 h, the adherent cells on the inner side of the upper chamber membrane were removed using cotton swabs, and the cells on the upper and lower chamber were respectively fixed and stained with 0.4% crystal violet (Sigma-Aldrich, Inc., St. Louis, MO, USA) at room temperature for 30 min. The cells in the upper and lower chamber were randomly count-
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ed under an upright light microscope (BX51; Olympus, Japan).

Statistical analysis

The data are expressed as the mean ± standard deviation from at least three repeated experiments. Statistical analyses were performed using one-way analysis of variance and SNK-Q test for comparison of multiple groups or Student’s t-test for comparison of two groups using SPSS 18.0 software (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to have statistically significant difference.

Results

miRNA-495 is down-regulated in GC tissues and cells

A previous study had found that the expression of miRNA-495 is reduced in GC cells [2]. To
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investigate whether the expression of miRNA-495 was down-regulated in GC tissues, RT-qPCR analysis was performed in the present study to examine the expression levels of miRNA-495 in 20 sets of GC tissue samples. Consistently, a significant reduction in the expression levels of miRNA-495 were observed in the GC tissues, compared with the adjacent normal tissues (Figure 1A). Subsequently, the expression levels of miRNA-495 were compared between GC cells and normal gastric cells. As expected, the expression levels of miRNA-495 were also significantly down-regulated in the GC cells, including the SGC7901, MKN28 and BGC823 cell lines (Figure 1B).

miRNA-495 inhibits the proliferation abilities of GC cells

As the expression of miRNA-495 was down-regulated in the GC cells, the present study investigated the effect of miRNA-495 on the proliferation of GC cells. It was observed that the expression levels of miRNA-495 were markedly increased and decreased in the MKN28 cells by transfection with the miRNA-495 mimics and miRNA-495 inhibitors, respectively, which was detected using RT-qPCR (Figure 2A). In the MTT assays, the growth rate of the miRNA-495 mimic-transfected GC cells was significantly lower than that of the miRNA-SCR-transfected GC cells, whereas the growth rate of the miRNA-495 inhibitor-transfected GC cells was significantly increased, compared with that of the miRNA-SCR-transfected cells (Figure 2B). Similar results were also observed in the BrdU assays, in which the GC cells transfected with the miRNA-495 mimics grew more slowly and those transfected with the miRNA-495 inhibitors grew more rapidly, compared with the control group (Figure 2C). These results indicated that miRNA-495 inhibited the proliferation of the GC cells.

miRNA-495 inhibits the migration and invasion abilities of GC cells

In addition, the present study investigated whether miRNA-495 was also responsible for inhibiting the migration and invasion of GC cells. As expected, the results showed that the transfection of the miRNA-495 mimics and miRNA-495 inhibitors into MKN28 cells resulted in a significant inhibition and increase in the number of migrated and invaded GC cells, respectively (Figure 3A, 3B). This suggested that miRNA-495 inhibited the migration and invasion abilities of the GC cells.

FGFRL1 is a direct target of miRNA-495

To identify the potential target of miRNA-495, a bioinformatic algorithms tool, TargetScan (http://www.targetscan.org), was used. As a result, a putative miRNA-495 binding site was
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identified in the 3'UTR of FGFRL1 (Figure 4A), which has been reported to regulate the proliferation and invasion of various cancer cells [17]. To investigate the effect of miRNA-495 on FGFRL1 mRNA, RT-qPCR was performed to detect the mRNA expression levels of FGFRL1 in MKN28 cells transfected with scramble negative control, miRNA-495 mimics or inhibitors. The results revealed that the mRNA expression of FGFRL1 was significantly repressed and increased in MKN28 cells transfected with miRNA-495 mimics or inhibitors, respectively (Figure 4B). Western blot analysis was then performed to examine the effect of miRNA-495 on the protein expression of FGFRL1. Similarly, the endogenous expression of FGFRL1 was significantly suppressed by the miRNA-495 mimics, and significantly enhanced by the miRNA-495 inhibitors (Figure 4C).

The present study subsequently examined the effect of miRNA-495 on the activities of two reporter constructs containing either the wild-type 3'UTR of FGFRL1 or a mutant FGFRL1 comprising a mutated miRNA-495 binding site. The results showed that the miRNA-495 mimics and miRNA-495 inhibitors significantly decreased and significantly increased the luciferase activities of the wild-type FGFRL1 reporter, respectively. No effect on the luciferase activities of the mutant reporters was observed (Figure 4D). Taken together, these results suggested that FGFRL1 was a direct target of miRNA-495.

FGFRL1 is up-regulated in GC tissues and cells

To determine whether the expression of FGFRL1 is up-regulated in GC tissues, RT-qPCR was performed to detect the mRNA expression levels of
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FGFRL1 in the same 20 sets of GC tissue samples. The quantitative analysis confirmed that the expression of FGFRL1 was up-regulated significantly in tumor samples, compared with the adjacent normal tissues (Figure 5A). The mRNA expression of FGFRL1 was also significantly up-regulated in the GC cells, compared with the normal cells (Figure 5B), which was also determined using RT-qPCR.

**FGFRL1 accelerates the proliferation of GC cells**

As the expression of FGFRL1 was inversely correlated with the expression of miRNA-495, which had an inhibitory effect on the proliferation and invasion of GC cells, the present study investigated whether FGFRL1 was involved in the proliferation of MKN28 cells. MTT and BrdU assays were performed to assess proliferation and invasion, respectively. The results showed that transfection with the FGFRL1-overexpression vector led to increased protein expression levels of FGFRL1, whereas FGFRL1 siRNA transfection led to decreased protein expression levels of FGFRL1 (Figure 6A). In the MTT assays, the ectopic expression of FGFRL1 significantly increased the growth rate of the MKN28 cells, whereas transfection with FGFRL1 siRNA led to a significant reduction in the growth rate of the MKN28 cells at day 5 (Figure 6B). Similarly, the BrdU assays demonstrated that cells transfected with the FGFRL1-overexpression vector grew more rapidly than the control cells. However, the specific suppression of endogenous FGFRL1 by transfection with FGFRL1 siRNA significantly attenuated the growth rate of the MKN28 cells at 48 h (Figure 6C).

**FGFRL1 promotes the migration and invasion of GC cells**

To investigate the role of FGFRL1 in the invasion of GC cells, the transwell assay was used. The results demonstrated that the forced expression of FGFRL1 enhanced the migration and invasion abilities of MKN28 cells, respectively, compared with the pCMV-Myc-transfected cells. By contrast, transfection with FGFRL1 siRNA significantly inhibited the number of migrated and invaded MKN28 cells, respectively (Figure 7A, 7B).

**Discussion**

In the present study, miRNA-495 was identified as an important tumor suppressor, which was down-regulated in GC tissues and cells and exerted an inhibitory effect on the proliferation and invasion of the GC cells. In addition, for the first time, to the best of our knowledge, FGFRL1 was identified as a novel target gene of miRNA-495, with the expression of the latter being negatively correlated with the expression of
miRNAs are involved in the regulation of crucial cellular events, including differentiation, development, proliferation, apoptosis and metabolism [5]. Therefore, the dysregulation of miRNAs is closely associated with the initiation and progression of human cancer. Furthermore, miRNA expression patterns in tumor tissues or body fluids have been shown as potential biomarkers for the classification, diagnosis and prognosis of cancer [19, 20]. Consistent with previous study [2], the results of the present study suggested that miRNA-495 was significantly down-regulated in GC tissues, indicating that miRNA-495 may serve as a novel biomarker for the detection of GC. miRNA-495 had been reported as either an oncogene and tumor suppressor [21, 22]. In the present study, miRNA-495 functioned as a tumor suppressing miRNA in GC, with transfection of miRNA-495 mimics significantly inhibiting the proliferation, migration and invasion abilities of MKN28 cells. This suggested the potential therapeutic applications of miRNA-495 for the treatment of GC. Future investigations are required to further validate whether miRNA-495 is a useful strategy to treat GC.

In the present study, FGFRL1 was identified as a direct target gene of miRNA-495, as miRNA-495 mimics and inhibitors respectively suppressed and increased FGFRL1 expression levels, and mutation of the miRNA-495 binding site in the 3'UTR of FGFRL1 significantly inhibited the effect of the miRNA-495 mimics on the luciferase activity of the pMir reporter vector controlled by the FGFRL1 3'UTR. Furthermore, FGFRL1 mRNA expression was increased in GC tissues and cells. FGFRL1 is the most recently identified modulator of the FGF receptor, which is preferentially expressed in cartilaginous tissues and is important in the differentiation and development of skeletal tissues [12, 23], and FGFRL1 protein is closely associated with Wolf-Hirschhorn syndrome [15]. In addition, emerging data have increasingly emphasized the potential role of FGFRL1 in oncogenesis, which may act as either a tumor suppressor or oncogene in a variety of types of human cancer. Schild et al found that FGFRL1 functions as a tumor suppressor to inhibit the proliferation of MG63 osteosarcoma cells [16]. Martino E et al found that inactivation of the protein expression of FGFRL1 in bladder tumors may result in bladder carcinogenesis, as FGFRL1 is a putative 4p16.3 deletion target in bladder cancer [18]. By contrast, Tsuchiya et al suggested that FGFRL1 accelerates cancer cell proliferation in human esophageal squamous cell carcinoma by preventing cell cycle arrest at G1/G0 [17]. Similarly, the results of the present study suggested that FGFRL1 mRNA expression levels were significantly higher in GC tissues and cells that in normal tissues and cells, and FGFRL1 promoted the proliferation and invasion abilities of GC cells. Although the mechanisms by which FGFRL1 is up-regulated in GC remains to be elucidated and the functions of FGFRL1 in cancer may be complex, the results of the present study provide support for the role of FGFRL1 in the regulation of proliferation and invasion of GC cells as a novel oncogene in GC.

In conclusion, miRNA-495 expression was significantly down-regulated and FGFRL1 was up-regulated in GC tissues and cells. Although miRNA-495 can regulate more than one target gene, the present study demonstrated, at least in part, that miRNA-495 inhibited the proliferation, migration and invasion abilities of GC cells by targeting FGFRL1. Future investigations are required to further investigate the mechanisms and roles of the miRNA-495/FGFRL1 axis in GC, which may offer potential in the development of therapeutic strategies for the treatment of GC.

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Disclosure of conflict of interest

None.

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