Abstract. Gastric cancer is the fourth most common and the second leading cause of cancer mortality worldwide. The dysregulation of microRNAs has been demonstrated to be significant in gastric cancer carcinogenesis and progression due to changes in expression of their target genes. In the current study, microRNA-455 (miR-455) was identified to be significantly downregulated in gastric cancer tissue samples and cell lines. A low expression level of miR-455 was correlated with the clinical stage, lymph node metastasis and tumor invasion in gastric cancer. Restoration of miR-455 expression inhibited cell proliferation, migration and invasion of gastric cancer cells in vitro. Bioinformatic analysis and luciferase reporter assay revealed that miR-455 directly targeted the 3'-untranslated region of insulin-like growth factor 1 receptor (IGF-1R). In addition, the IGF-1R mRNA expression level was increased in gastric cancer tissue samples and was inversely correlated with miR-455 expression levels. Restoration of miR-455 downregulated IGF-1R mRNA and protein expression levels in gastric cancer cells. Furthermore, silencing of IGF-1R significantly inhibited gastric cancer cell proliferation, migration and invasion, which was similar to the functions induced by miR-455 overexpression. Thus, these results indicate that miR-455 is involved in gastric cancer progression by directly targeting IGF-1R and may serve as a novel therapeutic target for the treatment of gastric cancer.

Introduction

Gastric cancer, one of the major malignant tumor types, is the fourth most common and the second leading cause of cancer mortality around the world, with ~1,000,000 new cases and 700,000 mortalities every year (1,2). In the past two decades, the morbidity and mortality of gastric cancer have gradually been declining around the world (2). However, in East Asia, it remains the second leading cause of cancer-associated mortality and the most common type of gastroenteric tumor (3). Increasing numbers of studies have demonstrated that many risk factors are involved in the occurrence and development of gastric cancer, such as genetic alterations, the Helicobacter pylori infection, smoking, alcohol drinking, habitual excessive intake of salt, and environmental factors (4). Although great effort has been made in various therapeutic strategies that combined surgery resection, radiotherapy and chemotherapy, the overall prognosis remains unsatisfactory (5). Therefore, an improved understanding of the mechanisms underlying the carcinogenesis and progression of gastric cancer would facilitate the development of novel therapeutic treatments for this disease.

MicroRNAs (miRNAs) are a large group of 19- to 25-nucleotide, endogenous, short RNA molecules (6). miRNAs negatively regulate the expression of their target genes at the post-transcriptional and/or translational level by directly binding to the 3'-untranslated regions (UTRs) of their target genes, resulting in degradation of the target genes or inhibition of protein translation (7,8). Numerous studies demonstrate that miRNAs exert a critical role in various types of biological...
processes, including cell proliferation, the cell cycle, invasion, migration, metastasis and differentiation (9,10).

Furthermore, the dysregulation of miRNAs has been reported in a diverse range of human cancers, such as colorectal (11) and cervical (12) cancers, hepatocellular (13) and renal cell (14) carcinoma, and gastric cancer (15) amongst others. Increasing evidence has demonstrated that miRNAs function as tumor suppressors or oncogenes in the carcinogenesis and progression of numerous types of cancer. For example, miR-524-5p inhibited gastric cancer proliferation and invasion by negative regulation of oncogenes, matrix metalloproteinase (MMP)-2 and MMP-9 (16). miR-3646 acted as an oncogene in breast cancer, through promoting cell proliferation, migration and invasion via directly targeting the p53/p21/cyclin-dependent kinase 1/cyclin B1 pathway (17).

In the current study, the expression level, biological functions and molecular mechanism of miR-455 were investigated in gastric cancer.

Materials and methods

Clinical samples. A total of 57 gastric cancer and matched, non-tumorous gastric tissue samples were collected from patients of the The Affiliated Huai’an Hospital of Xuzhou Medical University (Huaian, China) and The Second People’s Hospital of Huaian (Huaian, China) between 2011 and 2014. None of the gastric cancer patients received chemotherapy or radiotherapy treatments prior to surgery. All tissue samples were immediately frozen in liquid nitrogen and stored at -80°C. The seventh edition of UICC tumor node metastasis (TNM) classification system was used as clinical staging criteria (18). The present study was approved by the Ethics Committee of The Affiliated Huai’an Hospital of Xuzhou Medical University and The Second People’s Hospital of Huaian, and written informed consent was obtained from all gastric cancer patients.

Cell culture. Five gastric cancer cell lines (SGC-7901, BGC-823, AGS, MKN-1 and MKN-45) and a normal gastric epithelium cell line (GES-1) were purchased from the Shanghai Institute of Biochemistry and Cell Biology (Shanghai, China) and cultured in Gibco Dulbecco’s modified Eagle’s medium (DMEM; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with Gibco 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Inc.) at 37°C in an atmosphere consisting of 5% CO₂.

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was isolated from tissue samples and cells using Invitrogen TRIzol (Thermo Fisher Scientific, Inc.). A SYBR PrimeScript miRNA RT PCR kit (Takara Biotechnology Co., Ltd., Dalian, China) was used to determine the miR-455 expression level and U6 served as an internal control. To quantify IGF-1R mRNA expression, total RNA was used to synthesize cDNA using a PrimeScript RT reagent kit (Takara Biotechnology Co., Ltd.), followed by qPCR using SYBR Premix Ex Taq (Takara Biotechnology Co., Ltd.). The amplification reaction was performed using the following cycling conditions: An initial pre-denaturation step for 5 min at 95°C, followed by 40 cycles of denaturation at 95°C for 30 sec and annealing at 65°C for 45 sec. The primer sequences were as follows: Forward, 5′-ACACTCCAGCTGGGTATGTGCCTT-3′ and reverse, 5′-GTGCCAGGGTCCAGGT-3′ for miR-455; forward, 5′-CTCGTTTGGTCGACATTAT-3′ and reverse, 5′-ACGCTTCAGAAATTGCGTGTC-3′ for U6; forward, 5′-CCGGTCGACAAATGTTGCGCA-3′ and reverse, 5′-TGTTTTCGAGCGCGTCTG-3′ for IGF-1R; and forward, 5′-TGACTTCAAGCGGACACCCA-3′ and reverse, 5′-CAACCTGTTGGTCTGTAGCCCAA-3′ for GAPDH. RT-qPCR was performed in triplicate on an AB7300 thermo-recycler (Applied Biosystems; Thermo Fisher Scientific, Inc.). The relative expression of miR-455 and IGF-1R mRNA were determined using the 2^ΔΔCq cycle threshold method (19).

Oligonucleotides and cell transfection. miR-455 mimics and negative control miRNA mimics (NC) were purchased from Shanghai GenePharma Co., Ltd. (Shanghai, China). Small interfering RNAs (siRNAs) specifically for IGF-1R and the control siRNA (IGF-1R siRNA and NC siRNA) were obtained from the Chinese Academy of Sciences (Changchun, China). Cells were transfected with these oligonucleotides using Invitrogen Lipofectamine 2000 (Thermo Fisher Scientific, Inc.). Following transfection for 6-8 h, the culture medium containing Lipofectamine 2000 and oligonucleotides was discarded and replaced with fresh culture medium.

MTT assay. Following the 24-h transfection, the transfected cells were collected and re-seeded in 96-well plates at a density of 2,000 cells per well. Cells were then incubated at 37°C in 5% CO₂ atmosphere. At different time points (24, 48, 72 and 96 h), an MTT assay (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was performed according to the manufacturer’s instructions. Briefly, 20 µl MTT solution (5 mg/ml) was added to each well and subsequently incubated at 37°C for 4 h. The culture medium containing MTT solution was discarded and 200 µl DMSO solution (Sigma-Aldrich; Merck KGaA) was added to dissolve the formazan precipitates. The absorbance at a wavelength of 490 nm was measured using an automatic multi-well spectrophotometer (BioTek Instruments, Inc., Winooski, VT, USA).

Transwell migration and invasion assay. Following a 48-h transfection, the transfected cells were harvested, washed with Gibco phosphate-buffered saline (PBS; Thermo Fisher Scientific, Inc.) three times and resuspended in PBS-free culture medium. For the Transwell migration assay, 5x10⁴ cells were plated into the top side of polycarbonate Transwell chambers (8 µm; Costar; Thermo Fisher Scientific, Inc.). For the Transwell invasion assay, 5x10⁴ cells were plated into the upper chambers coated with Matrigel (BD Biosciences, San Jose, CA, USA). The lower chambers were filled with DMEM containing 20% FBS. The chambers were incubated at 37°C in an atmosphere of 5% CO₂ for 48 h. Migratory and invasive cells on the lower membranes were fixed, stained with 0.5% crystal violet, washed with PBS three times and counted in five random fields (magnification, x200) per Transwell chamber using an IX71 inverted microscope (Olympus Corporation, Tokyo, Japan).

Protein extraction and western blotting. Following transfection for 72 h, cells were washed with ice-cold PBS and lysed in RIPA buffer (Bioytime Institute of Biotechnology, Haimen, China) in
the presence of protease inhibitor cocktail. The concentration of total protein was quantified using the BCA Protein Assay kit (Beyotime Institute of Biotechnology). Proteins (30 µg) were separated on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene fluoride membrane at 100 V for 1.5 h. Subsequent to blocking in 5% non-fat milk in Tris-buffered saline containing Tween-20 for 1 h at room temperature, the membranes were probed with mouse anti-human monoclonal IGF-1R (1:1,000 dilution; cat. no. sc-81646; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and mouse anti-human monoclonal GAPDH (1:1,000 dilution; cat. no. sc-137179; Santa Cruz Biotechnology, Inc.) at 4°C overnight, followed by incubation with the goat anti-mouse horseradish peroxidase-conjugated secondary antibodies (1:5,000 dilution; cat. no. sc-2005; Santa Cruz Biotechnology, Inc.) for 1 h at room temperature. The signals were visualized using the ECL Plus Detection kit (Pierce; Thermo Fisher Scientific, Inc.).

Bioinformatic analysis and luciferase reporter assay. Bioinformatic analysis was performed to investigate the potential target genes of miR-455 using three algorithm databases: TargetScan (http://www.targetscan.org/), PicTar (http://pictar.bio.nyu.edu) and miRanda (http://www.sanger.ac.uk).

Luciferase reporter assay was performed to investigate whether IGF-1R was a direct target gene of miR-455. Luciferase report vectors, PGL3-IGF-1R-3’UTR Wt and PGL3-IGF-1R-3’UTR Mut, were synthesized by Shanghai GenePharma Co., Ltd. HEK293T cells, purchased from Shanghai Institute of Biochemistry and Cell Biology, were seeded into 24-well plates at a density of 30–40%, and transfected with PGL3-IGF-1R-3’UTR Wt or PGL3-IGF-1R-3’UTR Mut, along with miR-455 mimics or NC using Lipofectamine 2000. At 48 h post-transfection, transfected cells were collected, washed with PBS, and subjected to luciferase reporter assay using a Dual-Luciferase Reporter Assay System (Promega Corporation, Madison, WI, USA) according to the manufacturer’s instructions. All samples were run in triplicate.

Statistical analysis. Data are presented as the mean ± standard deviation, and were compared using a Student’s t-test or one-way analysis of variance followed by the Student-Newman-Keuls post hoc test using SPSS version 13.0 (SPSS, Inc., Chicago, IL, USA). Spearman's correlation analysis was used to analyze the association between miR-455 and IGF-1R mRNA expression levels. Two-tailed P<0.05 was considered to indicate a statistically significant difference.

**Results**

miR-455 was downregulated in gastric cancer tissue samples and cell lines. miR-455 expression was detected in gastric cancer tissue samples and their matched non-tumorous gastric tissue samples using RT-qPCR. The expression level of miR-455 in the gastric cancer tissue samples was significantly lower than that in the matched non-tumorous gastric tissue samples (Fig. 1A; P<0.05).

In addition, the association between miR-455 expression and the clinicopathological features of gastric cancer were evaluated. As shown in Table 1, reduced miR-455 expression was significantly associated with the clinical stage (P=0.003), lymph node metastasis (P=0.008) and tumor invasion (P=0.032). However, there was no correlation between miR-455 expression and age (P=0.962), sex (P=0.607), tumor size (P=0.314) or age (P=0.578).

Subsequently, miR-455 expression levels in the gastric cancer cell lines were compared with the normal gastric epithelium cell line. As presented in Fig. 1B, miR-455 was downregulated in all five gastric cancer cell lines (SGC-7901, BGC-823, AGS, MKN-1 and MKN-45) compared with that in GES-1 (P<0.05).

miR-455 inhibited gastric cancer cell proliferation, migration and invasion. Given the downregulation of miR-455 in gastric cancer, it was hypothesized that miR-455 may be involved in the progression of cervical cancer. Firstly, BGC-823 and MKN-45 cells were transfected with miR-455 mimics or NC. At 48 h
post-transfection, results of RT-qPCR indicated that miR-455 was markedly upregulated in miR-455 mimic-transfected BGC-823 and MKN-45 cells (Fig. 2A; P<0.05). Subsequently, MTT assay, and Transwell migration and invasion assays were used to analyzed the effects of miR-455 on cell proliferation, migration and invasion in gastric cancer. BGC‑823 and MKN-45 cells transfected with miR -455 mimics exhibited decreased proliferation (Fig. 2B; P<0.05), migration and invasion (Fig. 2C; P<0.05) compared with NC.

IGF-1R was a direct target of miR-455. Bioinformatic analysis with three algorithm databases indicated that IGF-1R was a potential target of miR-455 (Fig. 3A). A luciferase reporter assay was used to confirm whether the 3’-UTR of IGF-1R was directly targeted by miR-455. HEK293T cells were co‑transfected with luciferase reporter vectors and miR-455 mimics or NC. As presented in Fig. 3B, miR-455 overexpression significantly decreased the luciferase activities of the PGL3-IGF-1R-3’UTR Wt. However, there was no significant difference between the miR-455 mimics and NC groups co-transfected with PGL3-IGF-1R-3’UTR Mut.

The expression levels of IGF-1R mRNA in gastric cancer tissue samples and their matched non-tumorous gastric tissue samples were observed using qRT-PCR. As demonstrated in Fig. 3C, IGF-1R mRNA was expressed at higher levels in gastric cancer tissue samples when compared with the matched non-tumorous gastric tissue samples (P<0.05). Spearman's correlation analysis revealed a significant inverse correlation between miR-455 and IGF-1R mRNA (r=—0.8408) in gastric cancer tissue samples (Fig. 3D; P<0.001).

To investigate whether miR-455 regulates the IGF-1R mRNA and protein expression levels in gastric cancer cells, RT-qPCR and western blotting were performed. The results demonstrated that IGF-1R mRNA (Fig. 3E; P<0.05) and protein (Fig. 3F; P<0.05) expression levels were suppressed by upregulation of miR-455 in the BGC-823 and MKN-45 cells. These findings indicate that miR-455 may directly target IGF-1R in gastric cancer cells via interaction with the binding sites in the 3’UTR of IGF-1R.

Knockdown of IGF-1R inhibited cell proliferation, migration and invasion in cervical cancer. To evaluate the effects of IGF-1R on gastric cancer, IGF-1R siRNA or NC siRNA was injected into BGC‑823 and MKN‑45 cells. Following transfection, RT-qPCR and western blotting were performed. The results demonstrated that IGF-1R mRNA (Fig. 4A; P<0.05) and protein (Fig. 4B; P<0.05) expression levels were suppressed by downregulation of IGF-1R in the BGC-823 and MKN-45 cells. These findings indicate that miR-455 may directly target IGF-1R in gastric cancer cells via interaction with the binding sites in the 3’UTR of IGF-1R.

Table I. Association between miR-455 expression and clinicopathological features of gastric cancer.

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<th>High</th>
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</tr>
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<td>≥60</td>
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<td>46</td>
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miR-455, microRNA-455.
In the current study, the miR-455 expression level was measured in gastric cancer tissue samples and five gastric cancer cell lines, and the expression levels of miR-455 were found to be reduced in the gastric cancer tissue samples and cell lines when compared with the matched non-tumorous gastric tissue samples and normal gastric epithelium cell line, respectively. In addition, a low miR-455 expression level was identified to be significantly associated with the clinical stage, lymph node metastasis and tumor invasion in gastric cancer. These findings indicate that a low miR-455 expression level was contributed to the progression of this disease. In addition, rapid growth and metastasis are the leading cause of gastric cancer.

Discussion

In the current study, the miR-455 expression level was measured in gastric cancer tissue samples and five gastric cancer cell lines, and the expression levels of miR-455 were found to be reduced in the gastric cancer tissue samples and cell lines when compared with the matched non-tumorous gastric tissue samples and normal gastric epithelium cell line, respectively. In addition, a low miR-455 expression level was identified to be significantly associated with the clinical stage, lymph node metastasis and tumor invasion in gastric cancer. These findings indicate that a low miR-455 expression level was contributed to the progression of this disease. In addition, rapid growth and metastasis are the leading cause of gastric cancer.

Figure 2. miR-455 affected cell proliferation, migration and invasion of gastric cancer cells. (A) Reverse transcription-quantitative polymerase chain reaction analysis indicated that miR-455 was significantly increased in BGC-823 and MKN-45 cells following transfection with miR-455 mimics. Data are presented as the mean ± standard deviation. (B) MTT assays demonstrated that upregulation of miR-455 suppressed the proliferative ability of BGC-823 and MKN-45 cells (magnification, x200). Data are presented as the mean ± standard deviation. (C) Transwell migration and invasion assays indicated that overexpression of miR-455 decreased cell migration and invasion abilities of BGC-823 and MKN-45 cells (magnification, x200). Data are presented as the mean ± standard deviation. *P<0.05 vs. NC. miR-455, microRNA-455; NC, negative control miRNA mimics.
The roles of miR-455 on cell proliferation, migration and invasion of gastric cancer were evaluated in the present study. Functional experiments demonstrated that enforced miR-455 expression significantly suppressed gastric cancer cell proliferation, migration and invasion in vitro. Notably, IGF-1R was identified as a direct target of miR-455.
Figure 4. IGF-1R knockdown inhibited cell proliferation, migration and invasion of gastric cancer cells. (A) Reverse transcription-quantitative polymerase chain reaction analysis indicated that the expression level of IGF-1R mRNA was reduced in BGC-823 and MKN-45 cells following transfection with IGF-1R siRNA. Data are presented as the mean ± standard deviation. (B) IGF-1R protein was downregulated in IGF-1R siRNA-transfected BGC-823 and MKN-45 cells. (C) MTT assay revealed that downregulation of IGF-1R inhibited the proliferative ability of BGC-823 and MKN-45 cells. Data are presented as the mean ± standard deviation. (D) Transwell migration and invasion assays demonstrated that IGF-1R knockdown repressed the cell migration and invasion capacities of BGC-823 and MKN-45 cells. Data are presented as the mean ± standard deviation. *P<0.05 vs. NC siRNA. IGF-1R, insulin-like growth factor 1 receptor; siRNA, small interfering RNA; NC, negative control miRNA mimics.
and functional target of miR-455, which was confirmed by a series of experiments. These findings elucidated the decreased expression levels of miR-455 in gastric cancer and detailed roles of miR-455 in gastric cancer, and further contributed to establishing effective therapeutic targets for the treatment of gastric cancer.

The expression levels of miR-455 vary in different diseases. Li et al (20) reported that miR-455 was significantly downregulated in non-small cell lung cancer tissue samples and cell lines. A study by Chai et al (21) demonstrated that miR-455 expression was lower in colorectal cancer tissue samples when compared with matched normal tissue samples. Cheng et al (22) demonstrated that the expression level of miR-455 was greater in oral squamous cancer tissue samples than in the normal tissue samples. In addition, its expression was also upregulated in oral squamous cancer cell lines in comparison with that in normal keratinocyte cell lines (22). In addition to in tumors, miR-455 was identified to be upregulated in human cartilage from patients with osteoarthritis compared with that in healthy control subjects (23). These findings indicate that miR-455 expression is diverse and tissue-dependent.

The roles of miR-455 have primarily been evaluated in human cancers. For example, in non-small cell lung cancer, restoration of miR-455 expression repressed cell growth and motility by downregulation of zinc finger E-box binding homeobox 1 (20). In colorectal cancer, upregulation of miR-455 suppressed proliferation and invasion of colorectal cancer cells by directly targeting Raf-1 proto-oncogene, serine/threonine kinase (21). In oral squamous cancer, reduced expression of miR-455 decreased the cell anchorage-independent growth and proliferative ability by inhibition of ubiquitin conjugating enzyme E2 B (22). In addition, in cardiac hypertrophy, overexpression of miR-455 decreased myocardial fibrosis and inhibited apoptosis by targeting calreticulin, indicating a potential therapeutic strategy to reverse pressure-induced cardiac hypertrophy and prevent maladaptive cardiac remodeling (24). Furthermore, miR-455 was found to be involve in brown adipogenesis via regulation of differentiation and thermogenesis via hypoxia inducible factor 1a subunit, an AMP-activated protein kinase-PPAR coactivator 1a signaling pathway (25). These studies demonstrated that miR-455 significantly contributes to these diseases, and may therefore serve as a potential therapeutic target for their treatment.

miRNAs perform their biological roles by binding to the 3'-UTRs of their target genes and decreasing gene expression by degrading the target mRNA or repressing its translation. It is therefore important to investigate the potential target genes involved in miR-455-mediated tumor suppressive roles in gastric cancer. In the current study, IGF-1R was demonstrated to be a novel direct and functional downstream target of miR-455, and IGF-1R expression was negatively modulated by miR-455 by direct interaction with the 3'-UTR of IGF-1R. This finding was supported by a series of experiments. Firstly, bioinformatic analysis indicated that the 3'-UTR of IGF-1R contained potential binding sites of miR-455. Secondly, IGF-1R was identified as a direct target of miR-455 using a luciferase reporter assay. Thirdly, the expression and association between miR-455 and IGF-1R mRNA expression in gastric cancer were analyzed, and it was demonstrated that their expression levels were inversely correlated in tumor tissue samples. Additionally, the expression levels of IGF-1R mRNA and protein were downregulated by miR-455 overexpression in gastric cancer cells. Finally, IGF-1R knockdown imitated the effects of miR-455 overexpression on gastric cancer cell proliferation, migration and invasion. These findings indicate that IGF-1R was a novel direct target of miR-455 in gastric cancer, and, to the best of our knowledge, this is the first study to investigate the miR-455/IGF-1R interaction.

IGF-1R, a transmembrane tyrosine kinase receptor of the insulin receptor family, contains two extracellular α subunits with the ligand-binding site and two transmembrane β subunits with intracellular tyrosine kinase activity (26). In gastric cancer, IGF-1R was significantly upregulated in tumor tissue samples (27,28). The expression of IGF-1R was correlated with the TNM staging, lymph node metastasis and distant metastasis (29). Furthermore, gastric cancer patients exhibiting low IGF-1R expression levels demonstrated longer overall survival than those with high IGF-1R expression (30). Additionally, functional experiments have indicated that IGF-1R knockdown decreased gastric cancer cell growth, motility and invasion and enhanced cell apoptosis (31). All these studies demonstrated that IGF-1R is upregulated in gastric cancer, and act as an oncogene in tumorigenesis and tumor development. Combined with the current results, miR-455/IGF-1R based targeted therapy may present as an effective therapeutic treatment for gastric cancer.

In conclusion, the present study demonstrated that miR-455 acted as a tumor suppressor in gastric cancer by inhibiting cell proliferation, migration and invasion. Furthermore, it was identified that miR-455 exerted its functions by directly targeting IGF-1R, thus providing further insights into the molecular mechanisms of gastric cancer carcinogenesis and progression. However, the effects of miR-455 on growth and metastasis in gastric cancer cells in vivo were not examined. This will be investigated in future research.

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References


