MicroRNA-200c represses migration and invasion of gastric cancer SGC-7901 cells by inhibiting expression of fibronectin 1

H.-B. CHEN, H.-T. ZHENG

Department of Gastrointestinal Surgery, The Affiliated Yantai Yuhuangding Hospital of Qingdao University Medical College, Yantai, China

Hongbing Chen and Haitao Zheng contributed equally to this work

Abstract. – OBJECTIVE: Gastric cancer remains a worldwide burden as a leading cause of cancer-related death. Drug resistance of chemotherapy looms as a major clinical challenge to effective treatment. Recent research data has suggested that microRNAs could be a potential gastric cancer treatment strategy. To further evaluate the role of microRNAs on gastric cancer cells and its underlying possible mechanism, we transfected human gastric cancer SGC-7901 cells with microRNA-200c.

MATERIALS AND METHODS: The cell proliferation, migration and invasion of SGC-7901 were determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay, Transwell assay and cell invasion assay. The expression of FN1 was detected by the reverse transcriptase-polymerase chain reaction (RT-PCR) assay.

RESULTS: The cell proliferation, migration and invasion were all significantly decreased after microRNA-200c transfection. Moreover, Fibronectin 1 (FN1) expression was significantly inhibited by microRNA-200c transfection. These results indicated that the mechanism by which microRNA-200c impresses human gastric cancer SGC-7901 cells may be mediated by its inhibition on FN1 expression.

CONCLUSIONS: This study highlighted the potential of using microRNA-200c as a new treatment strategy for human gastric cancer.

Key Words:
Fibronectin 1, MicroRNA-200c, Gastric Cancer, SGC-7901 cells.

Introduction

Gastric cancer is the major health problem worldwide due to its frequency, poor prognosis and limited treatment options, which is the leading cause of cancer-associated mortality in the world, accounting for 8% of all newly-diagnosed cancer cases and 10% of cancer deaths worldwide. Generally, the disease is diagnosed at advanced stage, and the available therapeutic methods in most patients are limited. Although, operation and chemotherapy are remaining the priority choices, drug resistance looms as a major clinical obstacle to successful treatment. The prognosis with a reported 5-year survival rate still remains poor. New technologies have been explored to treat gastric cancer, including targeting microRNAs (miRNAs). MiRNAs are 19-25 nucleotides of non-coding RNA molecules that mediate post-transcriptional gene expression through the 3'-UTR of their target mRNAs. MiRNAs play a crucial role in many complex biological processes, including cancer development, proliferation, differentiation and apoptosis. MicroRNA-200c (miRNA-200c) is a member of the miRNA-200 family that plays an important role in the epithelial mesenchymal transition (EMT). Roybal et al indicated that miRNA-200c inhibits lung adenocarcinoma cell invasion and metastasis. Yu et al reported that miRNA-200c is an independent prognostic factor in pancreatic cancer and its up-regulation inhibits pancreatic cancer invasion. Also, miRNA-200c was found to restore the sensitivity of NSCLC cells to cisplatin and cetuximab. Chen et al showed that miRNA-200c may act as a promising therapeutic target for improvement of responsiveness to chemotherapy in breast cancer. Notably, it was reported to regulate the sensitivity of chemotherapy in gastric cancer SGC-7901/DDP cells by directly targeting RhoE, which encouraged more researches on the mechanism of miRNA-200c on gastric cancer. Fibronectin (FN) is a high-molecular weight protein involved in cell adhesion and migration.

Corresponding Author: Hongbing Chen, MD; e-mail: chb2087@sina.com
(~440kDa) glycoprotein of the extracellular matrix that binds to membrane-spanning receptor proteins called integrins. FN plays a crucial role in several cellular processes including cell adhesion, migration, growth, differentiation, and clot formation.

The malignant level of gastric cancer is inevitably connected with the integral basement membrane structure around the gastric glands. The infiltration and metastasis of cancer cells firstly have to break this membranous structure. On the one hand, the hyperplastic fibrotic tissue of extracellular matrix can provide a support for the infiltrative growth of cancer cells; on the other hand, this can also reflect a tendency of resisting neoplastic metastasis in human body. Thus, it is inferred that the fibronectin can be viewed as a valuable parameter for evaluating malignant level and lymphangial metastatic tendency of the gastric cancer. FN1, a member of the FN family, exerts different functions during a variety of biological processes such as cell adhesion, cell migration and cytoskeleton organization in diseases such as cancer, atherosclerosis and arthritis. It was found that FN1 is a potential biomarker for radio-resistance in head and neck squamous cell carcinoma and is differently expressed in ovarian cancer platinum resistance. FN1 was also shown to activate specific matrix metallo-proteinases to promote breast cancer invasion and metastasis. FN1 was also reported as a target of SOX2 to promote cell migration and invasion in ovarian cancer. Therefore, FN1 expression in a good indicator of cancer cells status. Thus, the present study aimed to investigate the role of miRNA-200c in cell proliferation, migration and invasion of human gastric cancer SGC-7901 cells, and its possible mechanism relating the expression of FN1.

Materials and Methods

Cell Culture and Transfection

The SGC-7901 cells were obtained from the China General Microbiological Culture Collection Center (Beijing, China). The cells were seeded at a density of 5×10⁴ cells/well into 6-well dishes and cultured in Dulbecco’s modified Eagle’s medium (DMEM), Invitrogen (Carlsbad, CA, USA) overnight at 37°C with 5% CO₂ until the cells reached 70-80% confluency. Then, the cells were divided into an untreated blank control group, a negative control (NC) group treated with an independent sequence siRNA, and a miRNA-200c group treated with miRNA-200c siRNA (Sangon Biotech, Shanghai, China). The cells were washed twice with serum free Dulbecco’s Modified Eagle Medium (DMEM) Gibco (Rockville, MD, USA) before transfection, which was performed using Invitrogen (Carlsbad, CA, USA) Lipofectamine 2000 reagent (Thermo Fisher Scientific, Waltham, MA, USA), according to the manufacturers protocol. Briefly, solution A was prepared by slowly diluting 10 μL Lipofectamine 2000 into 250 μL serum-free medium at room temperature; solution B was prepared by diluting 10 μL of independent sequence siRNA and 10 μL of Lipofectamine 2000 into 250 μL serum-free medium; solution C was prepared by diluting 10 μL of MiRNA-200c (200 nmol/L) and 10 μL of Lipofectamine 2000 Invitrogen (Carlsbad, CA, USA) into 250 μL serum-free medium. The mixed solution were kept at room temperature for 20 min before being transferred to the rinsed SGC-7901 cells in the three groups. The cells were then cultured for 6 h, and then the medium was replaced with Dulbecco’s Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS) Gibco (Grand Island, NY, USA). The cells were harvested after being cultured for 24 h.

MTT Assay on Cell Proliferation

3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl-triazolium bromide (MTT), Sigma-Aldrich (St. Louis, MO, USA) was used for the measurement of cell proliferation in each group. Briefly, SGC-7901 cells were inoculated at 3×10³ cells/well in 96-well plates and 20 μl MTT at 5 mg/ml was added to each well for subsequent 4 h incubation at 37°C. Then, the absorbance of the cell cultures was recorded at 490 nm by using a microplate reader. Result from pure culture medium wells without cell inoculation was used as blank control. Each sample was assayed in quintuplicate through at least three independent experiments.

Transwell Assay to Measure Cell Migration

For migration assays, SGC-7901 cells were suspended in 100 μL of serum-free medium were placed in a transwell insert (pore size, 8 μm; BD Biosciences, San Jose, CA, USA) following a published protocol. The lower chamber was filled with 600 μL medium containing 10% fetal bovine serum (FBS). After incubating cells for 24 h at 37°C and gently removing the cells in the up-
per chamber with a cotton swab, the cells on the underside of the membrane were fixed with 4% paraformaldehyde for 15 min, stained with 0.1% crystal violet in 20% ethanol, and counted in five randomly selected fields using phase contrast microscopy. Cells were imaged at 200× magnification using an Olympus microscope (Tokyo, Japan). Five independent fields per well were imaged. Each assay was performed in triplicate.

In vitro invasion assay

The cell invasion assay was conducted by using BD BioCoat Matrigel invasion chambers (BD Biosciences, Franklin Lakes, NJ, USA). Following the rehydration of the chambers, SGC-7901 cells (2.5×10^4) were suspended in serum-free media that contained FN1 (20 µg/ml) or independent sequence or vehicle and were plated into the Matrigel-coated inserts. Media containing 10% fetal bovine serum (FBS) were added to the lower chamber. After incubation for 36 h, the non-invading cells were removed from the upper surface of the membrane, whereas the cells that invaded through and adhered to the bottom of the membrane were fixed and stained with 0.1% crystal violet. Four random fields (×100 magnification) were captured for each membrane, and the invasive cells were counted and averaged.

Real Time Quantitative PCR to Measure FN1 Expression

Total RNA was isolated from SGC-7901 cells by using Trizol Reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer’s instructions. About 5 µg of total RNA was converted to cDNA by M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA, USA), supplement with Oligo (dT18) RT primers. Samples for Real-time PCR analysis were prepared with SYBR Premix Ex Taq (TaKaRa, Dalian, Liaoning, China) and the specific primers are as follows: human FN1 sense, GGAGTTTCTGAGGTTT and antisense GCAGAAGTTTTGGGTGA; GAPDH sense, AACCGATTGGTCTATGTTGG and antisense TCGCTCCTGGAAGATGGTGAT. All reactions were performed on the Bio-Rad CFX96 Real-time PCR System (Bio-Rad, Hercules, CA, USA) with each sample assayed in triplicate. The level of FN1 expression in each group was calculated as relative ratio to GAPDH expression level.

Statistical Analysis

Statistical analysis was performed with the program SPSS 19.0 statistical software (IBM Corp., Armonk, NY, USA). Data were expressed as the mean ± standard deviation (SD). Changes between samples were compared by Student’s t-test, and differences of groups were compared by the method of one-way ANOVA. To validate ANOVA, LSD test was used. *p<0.05 was considered statistically significant.

Results

MiRNA-200c Decreased Cell Proliferation

The MTT assay was used to measure the cell proliferation at 24, 48 and 72 h post transfection. The results were summarized in Figure 1. The cell proliferation rate increased over time, but there was no significant difference between the blank control group and the independent sequence control group at any time point. However, the MiRNA-200c transfected group showed significantly lower cell proliferation at 24 h compared to the other control groups (p<0.05), and more significantly lower at 48 h (p<0.01) and 72 h (p<0.01). Therefore, these results demonstrated the inhibition role of MiRNA-200c on proliferation of human gastric cancer SGC-7901 cells.

MiRNA-200c Repressed Cell Migration

The Transwell assay (Figure 2) demonstrated that the cell migration in the independent sequence control group was not significantly different from the blank control group (p>0.05). The

Figure 1. Cell proliferation assay. Proliferation rate of SGC-7901 cells was evaluated with the MTT assay at 24, 48 and 72 h post-transfection. *indicates the cell proliferation rate in C group (miRNA-200c transfected) was significantly lower (p<0.05) compared to the A group (blank control) and B group (independent sequence control group). #indicates the cell proliferation rate in C group was very significantly lower (p<0.01) compared to the A group and B group.
cell migration in the MiRNA-200c transfected group was significantly lower than that in both the blank control group and the independent sequence control group (p<0.01), suggesting that MiRNA-200c could repress the migration of human gastric cancer SGC-7901 cells.

**MiRNA-200c Repressed Cell Invasion**

The cell invasion assay using BD BioCoat Matrigel invasion chambers showed that the cell invasion in the independent sequence control group was not significantly different from the blank control group (p>0.05). The cell invasion in the MiRNA-200c transfected group was significantly lower than that in both the blank control group and the independent sequence control group (p<0.01) (Figure 3). This indicated that MiRNA-200c could repress the cell invasion of human gastric cancer SGC-7901 cells.

**MiRNA-200c inhibited FN 1 Expression**

Expression of FN 1 was examined by Real-time quantitative polymerase chain reaction (PCR) (Figure 4). It showed that the FN1 expression in the independent sequence control group was not significantly different from the blank control group (p>0.05). Also, FN1 expression in the MiRNA-200c transfected group was very significantly lower than that in both the blank control group and the independent sequence control group (p<0.01). This implies that MiRNA-200c could inhibit the FN1 expression in human gastric cancer SGC-7901 cells.

**Discussion**

Over the years, it has been well established that miRNA-200c plays a critical role in regulating multiple biological processes and signaling cascades varying from mitogen-activated protein kinases signaling, to Notch signaling, to Toll-like receptor signaling, etc. In the context of developmental program, miRNA-200c has been found to accumulate in epithelial buds in developing submandibular glands and control fibroblast growth factor receptor (FGFR)-mediated epithelial proliferation by targeting very low density lipoprotein receptor (VLDLR) and its ligand Reelin, which regulate FGFR signaling in these cells22. Furthermore, miRNA-200c-mediated activation of BMP signaling via upregulation of amelogenin and E-cadherin and via downregulation of noggin triggers tooth development and renewal through dental epithelial cell differentiation in mice23. In this study, we demonstrated that miRNA-200c inhibited the expression of FN1, which led to significantly decreased cell proliferation, migration and invasion. This data revealed a new mechanism by which miRNA-200c impresses human gastric cancer. With the advances in the field of non-coding RNAs over the last decade, researchers have focused on the interactions among various kinds of non-coding RNAs to understand the developmental and disease-associated mechanisms. For example, a long non-coding RNA (lncRNA) named ATB was shown to activate downstream of transforming growth factor receptor (TGF-β) signaling and thus promote cancer cell proliferation and migration.

![Figure 2. Decreased cell migration in miRNA-200c transfected SGC-7901 cells. Group A is the blank control group, group B is the independent sequence control group, and group C is the miRNA-200c transfected group. *indicates the cell migration in group C was significantly lower (p<0.01) compared to the A group and B group.](image1)

![Figure 3. Decreased cell invasion in miRNA-200c transfected SGC-7901 cells. Group A is the blank control group, group B is the independent sequence control group, and group C is the miRNA-200c transfected group. *indicates the cell invasion in group C was significantly lower (p<0.01) compared to the A group and B group.](image2)
factor-β (TGF-β), which competitively binds to miRNA-200 family in hepatocellular carcinoma (HCC) cells, upregulates ZEB1/2 transcription factors, and induces EMT24. In order to have a better understanding of its mechanism of action, further research on miRNA-200c function can also involve lncRNAs and relating proteins24. As a tumor suppressor, miRNA-200c has the potential to be used as therapeutic agents, but their delivery and stability in body fluids is of great concern25. Major challenges are poor penetration of miRNAs in tumor tissues, miRNA degradation in body fluids, miRNA-associated immunotoxicity and neurotoxicity, inefficient gene silencing due to poor intracellular delivery of miRNAs, off-target effects, and unavailability of sufficient miRNA-processing enzymes25. However, multiple miRNA modification techniques have been described to better overcome these barriers26. For example, targeted delivery of aptamer-conjugated let-7g in a xenograft model of lung adenocarcinoma has shown promising results in reducing tumor growth27. In addition, combining miRNAs with other therapy agents is another option. For instance, miRNA-200c has been found to increase the radio-sensitivity by directly regulating oxidative stress response genes PRDX2, GAPB/Nrf2, and SESN1, which led to the inhibition of DNA double-strand break repair, increased the levels of reactive oxygen species, and upregulated p2128. Therefore, as a great potential to be used as cancer therapy sensitizer, miRNA-200c is worthy to be further investigated.

**Conclusions**

The present study demonstrated that miRNA-200c transfection could repress the proliferation, migration and invasion of human gastric cancer SGC-7901 cells. MiRNA-200c also inhibited the expression of FN1, suggesting the possible mechanism of miRNA-200c by which it represses human gastric cancer may be mediated by the inhibition of the expression of FN1.

**Ethical Approval**

The research was conducted in accordance with the Declaration of Helsinki and the United National Institutes of Health.

**Conflict of interest**

The authors declare no conflicts of interest.

**References**


