Original Article

miR-485-5p acts as a negative regulator in gastric cancer progression by targeting flotillin-1

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Abstract: MicroRNAs (miRNAs) play important roles in cancer progression including gastric cancer. miR-485-5p is reported as a potential suppressor in breast cancer, but its expression, cellular function and clinical features in gastric cancer is not known. In our study, we found that miR-485-5p expression was down-regulated in gastric cancer cell lines. miR-485-5p could inhibit gastric cancer cell growth in vitro and in vivo. We also found that miR-485-5p suppressed gastric cancer cell metastasis and sphere formation. It was confirmed flotillin-1 (Flot1) as a direct target of miR-485-5p, and up-regulation of miR-485-5p could decrease expression of Flot1 in gastric cancer cells. Further investigation showed that ectopic expression of Flot1 partially reversed the inhibition effect of enforced miR-485-5p expression on the malignant phenotypes of gastric cancer cells. The low expression of miR-485-5p in gastric cancer tissues was related to advanced clinical features and poorer prognosis. Our study suggested that miR-485-5p could be a potential prognostic marker and functions as a tumor suppressor in human gastric cancer by post-transcriptionally targeting Flot1.

Keywords: Gastric cancer, miR-485-5p, Flot1

Introduction

Gastric cancer is one of the most common malignancy in the world and it is a frequent cause of death from cancer. Although the development of early diagnosis and treatment methods for gastric cancer, long-term survival for gastric cancer needs to improve and there are other problems like drug resistance, metastasis [1, 2]. All of the problems needs to further explore the molecular mechanism of gastric cancer.

MicroRNAs (miRNAs) are a family of small non-coding mRNA molecules with around 22-nucleotides, which act as important regulators of gene expression involving in various physiological and pathophysiological process such as development, cell proliferation, differentiation, apoptosis and others [1, 2]. miRNAs down-regulate gene expression by cleaving mRNAs or inhibiting their translation. miRNAs play critical roles in cancer progression as tumor suppressors or promoters, which are commonly dys-regulated in cancers [3]. There are many miRNAs which have been shown to regulate various cancer-associated genes and oncogenic functions in gastric cancer. Recent advances demonstrated that a number of miRNAs have been reported to be aberrantly over-expressed or down-regulated during gastric cancer progression, including miR-15a [4], miR-100 [5], miR-874 [6], miR-101 [7], miR-335 [8] and the others. miR-485-5p acts as a tumor suppressor in breast cancer, ovarian cancer and sarcoma [9-11]. However, there has no reports showing the role of miR-485-5p in gastric cancer.

To this study, we investigated the potential involvement of miR-485-5p in gastric cancer. We examined the expression of miR-485-5p in human gastric cancer cells and tested its effects on cell growth, migration, invasion and stem cell-like properties. Flot1 was predicted as a potential target gene of miR-485-5p. Further research demonstrated that miR-485-5p expression was down-regulated in gastric cancer tissues and was negatively correlated to Flot1 expression in gastric cancer.
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Material and methods

Cell culture

Human gastric cancer cell line MKN45 was obtained from the American Type Culture Collection (Manassas, VA, USA). BGC823 and SGC7901 gastric cancer cell lines were from Shanghai Cancer Institute (Shanghai, China). The immortalized gastric mucosal epithelial cell line GES-1 was obtained from Beijing ComWin Biotech Co. Ltd. (Beijing, China). The cells were maintained in RPMI 1640 culture medium (Invitrogen, USA) supplemented with 10% fetal bovine serum (Invitrogen, USA) in a humidified cell incubator with an atmosphere of 5% CO₂ at 37°C.

miR-485-5p and Flot siRNA transfection

Lentiviral vectors mediated miR-485-5p or the controls were constructed according to the instruction from Invitrogen (Carlsbad, CA, USA). Lentivirus was packaged in 293T cells with miR-485-5p and its packaged plasmids using Lipofectamine 2000 reagent (Invitrogen) based on the manufacturer’s instructions. Lentivirus-mediated silencing of miR-485-5p was examined by qRT-PCR. Flot1 siRNAs were ordered from Sigma (Sigma-Aldrich, Saint Louis, MO, USA). siRNA transfection was carried out in cancer cells using Lipofectamine 2000 reagent.

Quantitative real-time RT-PCR

According to the protocol to Trizol reagent (Invitrogen, Carlsbad, CA, USA), total RNA was isolated from the cells with miRNA or siRNA transfection using following the manufacturer’s instructions. To quantitate the miRNA or mRNA expression, the expression of small nuclear U6 or GAPDH was used as the internal controls. Quantitative real-time RT-PCR was performed using a sequence detector (ABI-Prism, Applied Biosystems, USA). Primers were ordered from Sangon Biotech (Shanghai, China). The relative expression levels were calculated by comparing Ct values of the samples with those of the reference.

MTT assay

First, cells were seeded into 6-well plates (2×10⁵ cells/well) and transfected with miRNAs or siRNAs. After transfection for 24 h, Cells were seeded in 96-well plates with 5000 cells per well. At 0, 24, 48, 72 and 96 h, 20 ul MTT solution (5 mg/ml) was added to each well. After the cells were cultured for another 4 h, the supernatant was discarded and 150 ul Dimethyl sulfoxide was used to dissolve the crystal completely. Finally, the absorbance at 570 nm was measured by a ELISA reader.

Cell apoptosis

Cells (2×10⁵) were seeded in 6-well plates, cultured and then harvested. The cells were washed with PBS and then stained with 5 ul of Annexin V and 5 ul of propidium iodide (PI) for 15 min at room temperature in the dark according to the manufacturer’s instructions (BD Biosciences, San Jose, CA, USA). The apoptosis rate (%) of the stained cells was analyzed using a Beckman Coulter Epics Altra II cytometer (Beckman Coulter, CA, USA).

Colony forming assay

Cells were seeded into 6-cm plates after the cells were transfected with miRNAs or siRNAs and then the cells were digested with trypsin for preparation of single cell suspension. These cells were then seeded into 6-well plates. The cells were changed growth medium after 3 days. When a colony consisted more than 50 cells, the number of colonies were recorded under a microscope.

Western blot analysis

Total protein was extracted from the gastric cancer cells with transfection and quantified using the method of Bradford assay (Bio-Rad, Philadelphia, PA). Firstly, total protein was separated in 10% SDS-PAGE and then transferred to PVDF membranes (Millipore, Bedford, MA) at 80 V for 2 h at 4°C. The membranes were blocked in 5% non-fat dry milk in PBST and then incubated with primary antibodies in PBS, washed with PBST and then incubated with secondary antibodies conjugated with horseradish peroxidase in PBST for 1 hour at room temperature. Membranes were washed again in PBST for three times and protein bands were visualized using an ECL detection system on X-ray films.

Migration and invasion assay

Cell migration and invasion was carried out using the BD BioCoat Matrigel Invasion Chamber (BD Bioscience). Briefly, gastric cancer cells were transfected with miR-148a or the control
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and $2 \times 10^4$ cells in serum-free medium were placed into the upper chamber of the membrane of the insert for migration assay or the membrane coated with Matrigel for invasion assay after 24 h of transfection. In the low chamber, the medium contained 10% FBS. The cells migrated or invaded through the membrane were stained with 0.1% crystal violet and counted under microscope ($40\times$, three random fields per well) 24 h later.

Clinical specimens

Specimens of primary gastric cancer and their compared normal tissues were got from the patients diagnosed with gastric cancer in Affiliated Hospital of Luzhou Medical College (Sichuan, China). The patients did not receive any therapies before surgery. Both tumor and normal tissues were histologically confirmed by H&E (hematoxylin and eosin) staining. Informed consents from every patient were obtained, and the research protocols for sample collection were approved by the Ethics Committee of the hospital.

Statistical analysis

Data were expressed as the mean ± standard deviation of at least three independent experi-
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Results

miR-485-5p inhibited cell growth of gastric cancer

To investigate the roles of miR-485-5p in gastric cancer, firstly, expression of miR-485-5p in gastric cancer cell lines was examined by real time RT-PCR. The data showed that miR-485-5p expression was much lower in human gastric cancer cells including MCG803, MKN-28, MKN-45, SGC7901, BGC-803, BSG823 and BGC-823 compared with GES-1 normal cells, however in the latter four gastric cancer cell lines, miR-485-5p decreased significantly (Figure 1A). The result suggested that miR-485-5p might play inhibitory role in gastric cancer. BGC-823 and SGC7901 cells transiently transfected with miR-485-5p mimics for MTT and colony formation assays. Results of transfection effect indicated that miR-485-5p was increased significantly in BGC-823 and SGC7901 cells using real time RT-PCR (Figure 1B). MTT assay displayed that miR-485-5p inhibited cell proliferation in BGC-823 and SGC7901 cells (Figure 1C and 1D). It was also shown that colony formation rate decreased in the two cell lines compared with their controls (Figure 1E and 1F). To further observe the role of miR-485-5p in tumor growth in vivo, gastric cancer nude mice models were set up using BGC-823 cells with miR-485-5p overexpression or its control, tumor growth curve confirmed that miR-485-5p could inhibit the growth of gastric cancer greatly (Figure 1G). The above data indicated that miR-485-5p inhibited gastric cancer growth.

miR-485-5p suppressed gastric cancer metastasis, EMT and sphere formation rates

One of the reason for gastric cancer therapy failure is its metastasis. To investigate the roles of miR-485-5p in gastric cancer metastasis, BGC823 cells were infected with LV-miR-485-5p, and the results showed that up-regulation of miR-485-5p could significantly decreased
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migration (Figure 2A) and invasion (Figure 2B) of BGC-823 and SGC7901 cells. The sphere formation of BGC823 cells with LV-miR-485-5p was much lower than the control (Figure 2C). This indicated that miR-485-5p prevented BGC823 cell show stem-like cells, so did in SGC7901 cells (Figure 2D). In the progression of metastasis or sphere formation, there was companied with changes of associated protein and stem cell markers, which were detected by western blot and the results showed that MMP9, Twist1, CD44 and OCT4 decreased in the BGC-823 and SGC7901 cells with LV-miR-485-5p (Figure 2E). These results suggested that miR-485-5p was negative regulator in gastric cancer stem cell properties.

During the therapy of gastric cancer, it often occurs drug resistance. Above data indicated miR-485-5p might be a tumor suppressor in gastric cancer. To learn whether miR-485-5p involves in drug resistance, DDP resistant cell line SGC7901/DDP and their parent cell line SGC7901 were choosen to perform the experiment. When SGC7901 and SGC7901/DDP cells with miR-485-5p overexpression combined with DDP treatment, and cell vabiliy was further inhibited using DDP by miR-485-5p (Figure 3A). Increasing cell apoptosis after DDP treatment was observed in miR-485-5p-overexpressed gastric cancer cells (Figure 3B). These data indicated that miR-485-5p could reverse DDP resistance of SGC7901/DDP cells.

Flot1 was a target gene of miR-485-5p in gastric cancer cells

We then investigated the mechanisms by which miR-485-5p inhibit gastric cancer progression. Bioinformatic analysis showed that Flot1 was directly suppressed by miR-485-5p (Figure 4A).

Figure 4. Flot1 was a target gene of miR-485-5p in gastric cancer cells. A. Schematic representation of Flot1 3’UTR showing putative miRNA target sites. B. Relative luciferase activity of the indicated Flot1 reporter construct in gastric cancer cells, co-transfected with miR-485-5p or scramble mimics, was shown. In cells co-transfected with Flot1 3’-UTR vector and miR-485-5p mimic, the luciferase activity was suppressed relative to mutant construct groups. C and D. Quantitative RT-PCR and Western blot assays were performed to detect the expression of Flot1 upon transfection with miR-485-5p mimics or scramble mimics. *P < 0.05, **P < 0.01.
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As shown in Figure 4B, the luciferase activity of wide typed Flot1 in BGC-823 cells was much lower than in control cells. The luciferase activity of mutated Flot1 was rescued in BGC-823 cells. We next examined whether miR-485-5p could regulate endogenous Flot1 expression in BGC-823 cells. Compared with the control, endogenous Flot1 mRNA levels (Figure 4C) were down-regulated when cells were transfected with miR-485-5p. Flot1 protein increased in the cells with anti-miR-485-5p (Figure 4D).

miR-485-5p inhibited gastric cancer cell proliferation and metastasis by targeting Flot1

Next, given the fact that Flot1 was the target genes of miR-485-5p, we want to know the role of Flot1 in gastric cancer cell behavior. Flot1 mRNA was usually over-expressed (Figure 5A) and Flot1 protein was also increased in gastric cancer cell lines (Figure 5B). And then BGC-823 and SGC7901 cells were transfected with Flot1 siRNA and Flot1 mRNA was knocked down (Figure 5C). Next, we want to know whether...
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Figure 6. Low miR-485-5p expression was negatively related to Flot1 expression in gastric cancer. A. The expression of miR-485-5p in 60 pairs of gastric cancer tissues and their adjacent tissues was examined by quantitative RT-PCR. B. The average level of miR-485-5p expression was significantly reduced in tumor tissues compared with their adjacent tissues. C. Low miR-485-5p expression was related to metastasis of gastric cancer. D. Low miR-485-5p expression was related to TNM staging of gastric cancer. E. The expression of Flot1 mRNA in 60 pairs of gastric cancer tissues and their adjacent tissues was examined by quantitative RT-PCR. F. Low miR-485-5p expression was negatively related to Flot1 mRNA levels. The data represent the mean values of three independent experiments. *P < 0.05, **P < 0.01.

miR-485-5p regulates cell proliferation and metastasis in gastric cancer cells via targeting Flot1. The result showed that miR-485-5p combining with Flot1 siRNA inhibited cell proliferation much more significantly than Flot1 siRNA by MTT assay in BGC-823 and SGC7901 cells with Flot1 siRNA (Figure 5D and 5E). We also found that miR-485-5p combining with down-regulation of Flot1 inhibited cell migration much more significantly than only using Flot1 siRNA in BGC-823 and SGC7901 cells (Figure 5F).

Low miR-485-5p expression was negatively related to Flot1 expression in gastric cancer

Above data showed that miR-485-5p expression in gastric cancer cells was reduced, which suppressed the aggressive phenotypes of gastric cancer. Here, we examined the expression of miR-485-5p in gastric cancer samples from the patients. Data from qRT-PCR indicated that the expression levels of miR-485-5p in gastric cancer samples were lower compared with their compared normal samples (Figure 6A). The average level of miR-485-5p expression was significantly reduced in tumor tissues compared with their adjacent tissues (Figure 6B). There was negative relationship between miR-485-5p expression and metastasis (Figure 6C) or TNM stage (Figure 6D). These results confirmed that evidence that miR-485-5p was significantly downregulated in gastric cancer. Flot1 mRNA was also examined in gastric cancer tissues and the result was consistent with gastric cancer cell lines. In most of gastric cancer samples, Flot1 mRNA levels was increased (Figure 6E). Further analysis of relationship between miR-485-5p and Flot1 in gastric cancer showed that low miR-485-5p expression was negatively related to Flot1 protein levels in most gastric cancer tissues (Figure 6F).

Discussion

The expression of miR-485-5p is down-regulated in breast cancer and ovarian epithelial cancer, which suppressed cell proliferation, metastasis and promoted cell apoptosis [9-11]. miR-485-5p is also induced in soft tissue sarcoma cells under the hypoxic environment [11], which suggests that miR-485-5p may be controled by the tumor microenvironment. In our work, we found that miR-485-5p expression in gastric cell lines was much lower than it in the normal cells. Over-expression of miR-485-5p led to inhibition of cell growth, migration, invasion and stem-cell like properties in gastric cancer cells. Further research demonstrated that miR-485-5p expression was reduced in most of the tested gastric cancer tissues and the results was consistent with the cell lines.

miRNAs function in the gastric cancer cells by targeting many genes and it is very important to identify them. According the results from the prediction, miR-485-5p has many potential target genes. In our research, Flot1 was selected due to its high score which means there was high possibility to be regulated by miR-485-5p. Using dual luciferase system, Flot1 was validated as the target gene of miR-485-5p. Flot1 mRNA and protein were suppressed by miR-485-5p in gastric cancer cells. Flot1 expression was negatively correlated to miR-485-5p in gastric cancer samples. These results clearly indicated that Flot1 expression is regulated by miR-485-5p in gastric cancer.

Flot1 is a member of flotillin family, which is one of maker of lipid rafts. Flotillin family includes Folt1 and Flot2. They are ubiquitously expressed and play important roles in a wide variety of cellular processes such as membrane receptor signaling, membrane trafficking, actin cytoskeleton reorganizations, cell adhesion and cell motility [12, 13]. Flotillin involves in activating insulin and EGFR signal pathways by recruiting receptor kinases to lipid rafts [14]. Recent reports showed that flotillin play important roles in development and progression of cancer [12, 13]. Flot1 was often over-expressed in breast cancer, colorectal...
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cancer, liver cancer and esophageal squamous cell carcinoma [15-17]. Over-expression of Flot1 in esophageal squamous cell carcinoma cells could enhance cell proliferation. Down-regulation of Flot1 suppressed the proliferation and tumorigenicity of breast cancer cells both in vitro and in vivo [12]. Our study also showed that inhibition of Flot1 attenuates gastric cancer cell proliferation, migration, invasion and tumorigenesis and its cellular function is partially through miR-485-5p regulation.

In a conclusion, our study found that miR-485-5p was identified a tumor suppressor miRNA in gastric cancer cells and can negatively regulate Flot1 expression in vitro and in vivo. Taken together with clinical observations, our finding suggests that loss of miR-485-5p expression in gastric cancer is a significant biomarkers for metastasis and could be targets for the development of antimetastasis strategy in the treatment of gastric cancer. But the molecular mechanism of low expression of miR-485-5p in gastric cancer needs to further research.

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Disclosure of conflict of interest

None.

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