Original Article

MiR-486-5p inhibits metastasis by targeting neuropilin-2 in gastric cancer

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Abstract: Previous studies have revealed that aberrant expressions of miRNAs participate in almost every step of carcinogenesis. Despite of large numbers of miRNAs disclosed, the function of miR-486-5p in the metastasis of gastric cancer is not elucidated so far. In the current study, we collected 65 gastric cancer samples and adjacent tissues to detect the miR-486-5p expression using qPCR, and applied luciferase reporter assay to verify the effect of miR-486-5p on the transcriptional activity of the Neuropilin-2 (NRP2) mRNA 3'UTR. Xenograft model was performed to test the effect of miR-486-5p on gastric cancer cell proliferation and metastatic ability in nude mice. Immunohistochemistry were used to confirm the relationship between NRP2 and lymphatic vessel density in these gastric cancer samples. Our results found that expression of MiR-486-5p was obviously decreased in the gastric cancer samples compared with the adjacent samples (P<0.01). Ectopic overexpression of miR-486-5p significantly inhibited the transcriptional activity and expression level of NRP2, as detected by luciferase and western blot, respectively. Furthermore, the gastric cancer cell SGC-7901 transfected with miR-486-5p showed obvious low tumorigenesis ability compared with the non-target control in the nude mice xenograft model (p<0.01). In xenograft tissues the expression of NRP2 had a positive correlation with the LYVE-1 expression, suggesting NRP2 promote lymphangiogenesis. Thus, our findings demonstrated that miR-486-5p function as a tumor suppressor in gastric cancer cell proliferation and lymphangiogenesis via targeting NRP2, and suggesting miR-486-5p a new potential target for developing diagnostics and therapy strategy in gastric cancers.

Keywords: miR-486-5p, NRP2, proliferation, metastasis

Introduction

Gastric cancer (GC) is the fourth most common malignancies worldwide. The incidence is much higher in male and in developing countries, including East Asian and Eastern European nations [1]. Despite advances in surgical and chemotherapies for GC, the current treatments available to patients with advanced GC are very limited because of the distant metastasis [2, 3]. Therefore, better understanding of the molecular mechanisms that involved in gastric cancer metastasis is essential for the development of novel therapeutic strategies.

Metastasis is a major feature of malignant tumors and cause of cancer-related deaths [4]. The main metastatic routes of gastric cancer include direct invasion, vascular metastasis, lymphatic metastasis and enterocoeelia metastasis [5]. Lymphatic metastasis is an increasingly important prediction of optimizing surgical treatment and as a prognostic factor [6]. The two established ways of tumor cells entry into the lymphatic system are invasion of existing lymphatic ducts and induction of lymphangiogenesis, and the latter is considered as the major way [5]. Thus, studies of lymphangiogenesis have a promising impact on the treatment [7].

Neuropilin-2 (NRP2) plays an important role in lymphangiogenesis in cancer. Previous study demonstrated that NRP2 expression in breast cancer correlates with lymph node metastasis and poor prognosis [8]. Moreover, NRP2 activation can promote colorectal carcinoma lymphangiogenesis via activating integrin α9 β1/FAK/Erk [9]. Hironao Yasuoka also declared that anti-NRP2 blocked lymph node metastasis...
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in papillary thyroid carcinoma [10]. However, the relationship between NRP2 with lymphangiogenesis in gastric cancer is not clear so far.

Increasing evidence has demonstrated that miRNAs take part in the regulation of cancer pathological processes, especially tumor metastasis [11-13]. To date, several deregulated miRNAs have been demonstrated to be involved in GC cell migration, invasion and metastasis, such as miR-1, miR-181a, miR-449a, miR-223, miR-30 and miR-194 [1, 14-17]. A series of studies have reported that miR-486-5p is frequently decreased in many cancers, including lung cancer, breast cancer and myeloid leukemia of Down Syndrome, and functions as a tumor suppressor by inhibiting tumor cell growth, invasion, metastasis, and tumorigenesis [18-20]. Based on our recent microarray analysis, we discovered that miR-486-5p was significantly downregulated in GC tissues. However, the effect and mechanisms of miR-486-5p deregulation involved in GC development and progression remain unknown. Therefore, in this study, we aim to investigate the roles and functions of miR-486-5p in gastric carcinogenesis.

Materials and methods

Cell culture

The gastric cancer cell line SGC-7901 was purchased from ATCC (Manassas, VA, USA) and maintained in RPMI-1640 (Hyclone Waltham, MA, USA) supplemented with 10% fetal bovine serum (Hyclone Waltham, MA, USA) in a humidified atmosphere of 5% CO₂ at 37°C.

Clinical samples

Sixty-five freshly-frozen biopsy GC and adjacent normal epithelium tissue samples (2 cm from the cancer location) were collected from the pathology archives of the Affiliated Hospital of Binzhou Medical University. The protocol of this study was approved by the Institutional Ethical Review Committee of Binzhou Medical University and written informed consent was obtained from each patient for the use of their tissue samples.

Plasmid transfection

SGC-7901 cells were transfected using Lipofectamine 2000 (Invitrogen, CA, USA) the day after cell seeding in accordance with the manufacturer's instructions. The miR-486-5p overexpression vector (GenePharma, Shanghai, China) and its control mimic (GenePharma, Shanghai, China) were used at a final concentration of 500 ng/μl. After 72 h post-transfection, quantitative real-time polymerase chain reaction (qRT-PCR) were used to verify transfection efficiency.

RNA isolation and quantitative RT-PCR

Total RNA was isolated from GC cell lines and clinical samples using TRIzol reagent (Invitrogen, CA, USA). The isolated RNA was reversely transcribed and amplified using the TaqMan miRNA assays RT-PCR kit (TaKaRa, Tokyo, Japan) according to the manufacturer's protocol. Quantitative real-time PCR was performed by using an Applied RT-3000 real-time PCR System. the primers for miR-486-5p and U6 internal control were synthesized by RiboBio company (RiboBio, Guangzhou, China). The mRNA expression levels were determined using the 2^ΔΔCt method.

Luciferase reporter assay

SGC-7901 cells were seeded in 24-well tissue culture plates the day before transfection. The cells were then co-transfected with pMIR-GLO-Vector (GenePharma, Shanghai, China) and miR-486-5p mimics (GenePharma, Shanghai, China) or non-target control mimics. After 72 h of transfection, the lysates were harvested and the luciferase activities were measured using the Dual Luciferase Reporter Assay kit (BeyotimeBiotechnology, Beijing, China).

Tumorigenicity assay in nude mice

Female BALB/c nude mice aging 4 to 6 weeks were purchased from HFK BIOSCIENCE CO.LTD (Beijing, China). 1×10⁷ SGC-7901 cells were suspended in 200 μl RPMI-1640, and then injected subcutaneously into the dorsal sites of the nude mice. Tumor formation was monitored about seven days. Some mice were peritumoral injected of miR-486-5p overexpression mimics every 3 days for 2 weeks. PBS or mocks were injected into some animals as negative controls. The tumor volume (mm³) was measured every 3 days and was calculated using the following formula: volume = 0.5×L×W² (in millimeters), where L represented the length of
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Results

Expression of miR-486-5p is decreased in gastric cancer tissues

To investigate the role of miR-486-5p in gastric cancers, miR-486-5p expression in the GC patients’ samples and adjacent normal tissues were first evaluated. Compared to the normal adjacent tissues, we found that miR-486-5p expression was obviously reduced in GC tissues (P<0.01) (Figure 1).

NRP2 is a target of miR-486-5p

The function of miRNA primarily relies on its target genes. To investigate whether NRP2 is a target of miR-486-5p in gastric cancer cells, the SGC-7901 cells were transfected with miR-486-5p expressing plasmid (the vector only as negative control). The transfection efficiency was verified by RT-PCR (Figure 2A). At 72 h post-transfection, the expression of NRP2 was determined by western blotting. The results showed that NRP2 significantly decreased in the miR-486-5p overexpression group (Figure 2B). To assess whether NRP2 was a direct target of miR-486-5p, the cells were co-transfected with NRP2 mRNA 3’-UTR vector and miR-486-5p expressing plasmid (vector only as the negative control). After 72 h of transfection, the lysates were harvested and the luciferase activities were measured. We found that transfection of vector-miR-486-5p significantly suppressed the luciferase activity of the vector-NRP2 (Figure 2C, 2D). Taken together, these...
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findings demonstrated that miR-486-5p could negatively regulate the expression of NRP2 by directly targeting the NRP2 mRNA 3’UTR.

**MiR-486-5p suppresses gastric tumor growth in vivo**

To explore the function of miR-486-5p in gastric cancer cell proliferation, a SGC-7901 cell line stably overexpressing miR-486-5p was established using plasmid transfection, and then used to conduct an experimental xenograft through injecting it into the dorsal sites of nude mice. Three weeks later, the mice were sacrificed and the size and mass of xenograft was quantified. The total weight and size of tumors generated from the miR-486-5p expressing SGC-7901 cell were significantly lower than those generated from the SGC-7901 cells transfected with vector control. (Figure 3A, 3B).

To determine whether miR-486-5p affects gastric cancer cell proliferation by targeting NRP2 in vivo, we investigated the NRP2 expression level in the tumor cells. NRP2 expression was significantly decreased in miR-486-5p-transfected tumors compared with its levels in the control group (Figure 3C, 3D). These results suggested that overexpression of miR-486-5p inhibited tumor growth by targeting NRP2 in vivo.

**MiR-486-5p suppresses gastric tumor lymphangiogenesis via targeting NRP2 in vivo**

To determine the in vivo significance of our in vitro observations, NRP2 expression levels of xenograft derived from miR-486-5p overexpression SGC-7901 cells were significantly lower than those from the xenograft derived from blank and negative control SW620 cells (Figure 4A). Correspondingly, NRP2 expression levels were found to be positively correlated with the lymphatic vessel density of the xenograft (Figure 4B, 4C). These findings suggested miR-486-5p could attenuate the lymphangiogenesis of GC in vivo.
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Discussion

miRNAs are a class of small non-coding RNAs (approximately 22 nt) that bind to multiple target mRNAs to control gene expression post-transcriptionally by inhibiting translation [21]. Recently, miRNAs are increasingly becoming hotspots for their multiple biological functions. A single miRNA can influence the expression of hundreds of target genes, and miRNAs have been reported as key regulatory molecules in various diseases, including cancer [22]. Among them, reduced miR-486-5p expression is a frequent molecular event in human malignancies. Furthermore, Evidence showed that miR-486-5p overexpression inhibited progression and metastasis via targeting protumorigenic ARHGAP5 in lung cancer [18]. Moreover, miR-486-5p expression in gastric carcinoma is related to clinicopathological features and prognosis [23]. However, its relation to progression and metastasis in gastric cancer are unclear. In this study, we first demonstrated that miR-486-5p expression was lower in GC tissues compared with adjacent normal tissues by qRT-PCR, and Down-regulation of miR-486-5p promoted lymphatic metastasis.

Neuropilin-2 is a co-receptor for vascular endothelial growth factor-D (VEGF-D) that is expressed on the surface of endothelial cells [10]. Recently, Nrp2 was shown to play a role in breast cancer cells migration as well as in the induction of tumor growth and invasion of pancreatic adenocarcinoma cells [24, 25]. Moreover, Neuropilin-2 (NRP2) as a cell surface receptor involved in tumor-associated angiogenesis and lymphangiogenesis, has recently been shown to be expressed in melanoma [26]. Juan-juan Ou et al also indicated that NRP2 activation can promotes colorectal cancer lymphangiogenesis via activating integrin α9β1/FAK/Erk signaling independent of VEGF-C/VEGFR3 pathway [18]. However, the relationship between NRP2 and gastric cancer tumor growth and lymphangiogenesis remain unclari-
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In our study, we first suggested that NRP2 expression levels are directly targeted by the miR-486-5p in gastric cancer cells. Then, our study for the first time revealed miR-486-5p expression levels are significantly correlated with the expression level of NRP2 in vitro and vivo. Most importantly, the density of lymphatic microvessels and tumor mass of xenografts derived from miR-486-5p overexpression SGC-7901 cells were significantly lower than those from the xenografts derived from control SGC-7901 cells. Our findings for the first time revealed that miR-486-5p mediates GC angiogenesis and lymphangiogenesis via regulating NRP2 manner.

In summary, our present study showed that miR-486-5p expression was decrease in GC tissues. We have identified that miR-486-5p attenuated the proliferation and lymphangiogenesis of GC cells in vivo by directly targeting NRP2. So, miR-486-5p may provide a potential therapeutic target of metastatic GC in the future. We hope that our investigation can facilitate further exploration of the molecular mechanisms of miR-486-5p in GC.

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Disclosure of conflict of interest

None.

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