Original Article

microRNA-188 over-expression inhibited cellular proliferation and invasion of gastric cancer by targeting SOX4

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Abstract: Previous studies reported that microRNA-188 (miR-188) was down-regulated and acted as a tumor suppressor in oral squamous cell carcinoma, hepatocellular carcinoma and prostate cancer. However, the expression, biological roles and molecular mechanism of miR-188 in gastric cancer remain unclear. In this study, we measured the expression levels of miR-188 in gastric cancer tissues and cell lines. The results showed that miR-188 was significantly down-regulated in gastric cancer tissues and cell lines. Then following functional studies revealed that miR-188 over-expression inhibited proliferation and invasion of gastric cancer cells. Using bioinformatic analysis, luciferase report assay, qRT-PCR and western blot, SOX4 was identified as a novel direct target gene of miR-188 in gastric cancer. Moreover, SOX4 under-expression had the similar functions with miR-188 over-expression in gastric cancer, further suggesting that miR-188 inhibited proliferation and invasion of GC cells though directly targeting SOX4. These findings indicated that miR-188 was down-regulated and acted as a tumor suppressor in gastric cancer through directly targeting SOX4. miR-188/SOX4 based targeted therapy could be a novel therapeutic application to treat patients with gastric cancer.

Keywords: microRNA-188, gastric cancer, proliferation, invasion, SOX4

Introduction

Gastric cancer (GC) is the fourth most common cancer and the second leading cause of cancer mortality around the world [1]. Based on the GLOBOCAN 2012 estimates, approximately 951,600 new GC cases and 723,100 deaths due to GC occurred in 2012 [2]. The highest mortalities are in Eastern Asia, Eastern Europe, and South America, especially in China whose patients with GC account for 42% of all cases in the world [3]. Environmental and behavioral habits have been demonstrated to involve in the tumorigenesis of GC, including Helicobacter pylori infection, high-salted diet and smoking [4]. Currently, surgery is the primary therapeutic strategy for the early stage of GC. However, most patients are diagnosed in the advanced stage with lymphatic, peritoneal or distal organ metastases [5]. In the last few decades, with great progress in surgery, chemotherapy, radiotherapy and molecular targeted therapy, the prognosis of GC was improved [6]. However, the long-term prognosis of GC patients remains poor, especially for advanced stage GC with a 5-year overall survival rate of 25% or less [7]. Therefore, it is urgent to investigate the molecular mechanism underlying GC carcinogenesis and progression, and develop novel therapeutic targets to improve the survival rates of GC patients.

MicroRNAs (miRNAs) are a group of well-conserved, endogenous noncoding small RNA molecules that are approximately 20-25 nucleotides in length [8]. Up to now, hundreds of miRNAs have been found in plants, animals and viral RNA genomes [9]. They bind the complementary sites in the 3'-untranslated regions (3'-UTRs) of their target genes to post transcriptionally induce target genes degradation, translational suppression or protein degradation [10]. These miRNAs are known as important factors in multiple biological processes, includ-
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ing cell proliferation, apoptosis, differentiation, carcinogenesis, metastasis, and drug resistance [11-13]. Increasing evidences have demonstrated miRNAs are abnormally expressed in various kinds of human cancers, also including GC [14-16]. The abnormal expression of miRNAs and their profiles in human cancers could be applied not only in cancer prediction and prognosis, but also in tumor classification and progression [17]. In the context of cancers, miRNAs could act as oncogene or tumor suppressor in the carcinogenesis and progression of cancers by directly targeting tumor suppressors or oncogenes, respectively [18, 19]. Therefore, miRNAs have considerable potential in early screening, diagnosis and treatments of human cancers.

In this study, we revealed that miR-188 expression was lower in GC tissue and cell lines compared with corresponding non-tumor gastric tissues and human immortalized gastric epithelial, respectively. Restoration miR-188 expression inhibited the proliferation and invasion of GC cells. SOX4 was identified as a novel direct target gene of miR-188 in GC.

Material and methods

Tissue samples

Fresh frozen human GC tissue samples and corresponding non-tumor gastric tissues were obtained from 46 patients with GC who undergo radical gastrectomy at Affiliated Hospitals of Jilin University. None of these patients had received chemotherapy, radiotherapy and molecular targeted therapy prior to radical gastrectomy. This study was approved by Research Ethics Committee of Jilin University.

Cell culture

The human GC cell lines AGS, BGC-823, MKN-28, MKN-1, SGC-7901, and MKN-45 were purchased from American Type Culture Collection (ATCC, Rockville, MD, USA). The human immortalized gastric epithelial (GES-1) and human embryonic kidney cells (HEK239T) were obtained from Beijing Institute for Cancer Research (Beijing, China). All these cells were grown in RPMI 1640 or DMEM culture medium (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; Gibco) in a humidified atmosphere consisting with 5% CO₂ and 95% air at 37°C.

Cell transfection

miR-188 mimics and miRNA mimics negative control (NC) were ordered from GenePharma (Shanghai, China). The small interfering RNA (siRNA) targeting SOX4 (siRNA-SOX4) and its negative control siRNA (siRNA-NC) were purchased from SunBio (SunBio Medical Biotechnology, Guangzhou, China). For transfection, cells were seeded into 6-well plates at a density of 70%~80% confluence. After incubation overnight, cells were transfected with mimics or siRNA at a concentration of 50 nM using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA). Six hours after transfection, cells were continuously incubated with complete culture medium.

RNA extraction and quantitative real-time PCR (qRT-PCR)

Total RNA was extracted using TRIzol agent (Invitrogen), according to the manufacturer’s instructions. For miR-188 expression, cDNA was synthesized from RNA using Taqman microRNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA). Taqman microRNA assay kit (Applied Biosystems) was used to detect miR-188 and U6 expression. Quantitative (q)PCR was performed in triplicate on a 7900HT Real-Time PCR System (Applied Biosystems), and U6 was measured as an endogenous control for miR-188. For the detection of SOX4 mRNA expression, a SYBR Green Real-Time PCR Master Mix Kit (Applied Biosystems) was applied with GAPDH as an internal control. The relative expression of miR-188 and SOX4 mRNA were analyzed by use of the 2^ΔΔCt method.

Cell proliferation assay

Cell proliferation assay was performed to determine cell proliferation rate with Cell Counting Kit 8 (CCK8; Dojindo, Kumamoto, Japan) assay kit. Briefly, GC cells were seeded into 96-well plates at a density of 3000 cells per well. Following transfection, cells were incubated for 24 h, 48 h, 72 h, and 96 h in a humidified atmosphere consisting with 5% CO₂ and 95% air at 37°C. At each time points, 10 ul CCK8 assay solution was added into each well and incubated at 37°C for another 4 h. The absorbance was measured at 450 nm with a microplate reader (Bio-Rad, Hercules, CA, USA).
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miR-188 expression was down-regulated in GC

Luciferase reporter assay

PmirGLO-SOX4-3'UTR wild-type (WT) and mir-GLO-SOX4-3'UTR mutant (MUT) luciferase reporter vectors were obtained from GenePharma. For luciferase reporter assay, HEK293T cells were co-transfected with PmirGLO-SOX4-3'UTR WT or PmirGLO-SOX4-3'UTR MUT, and miR-188 mimics or NC. At 48 h after transfection, cells were collected and the relative luciferase activities were determined using Dual-Luciferase Reporter Assay System (Promega, Manheim, Germany) according to the manufacturer’s instructions. Experiments were performed in triplicate and replicated 3 times.

Statistical analysis

Data were presented as mean ± S.D. Statistical analyses were performed with student’s t-test by using SPSS 15.0 (SPSS Inc.; Chicago, IL, USA). Double-tailed P value less than 0.05 was considered to be statistically significant.

Results

miR-188 expression was down-regulated in GC

We first examined miR-188 expression in GC tissues and corresponding non-tumor gastric tissues. As shown in Figure 1A, miR-188 was
significantly down-regulated in GC tissues compared with that in corresponding non-tumor gastric tissues (P<0.05). We then determined miR-188 expression levels in GC cell lines (AGS, BGC-823, MKN-28, MKN-1, SGC-7901, MKN-45) and human immortalized gastric epithelial (GES-1). As shown in Figure 1B, all GC cell lines expressed lower miR-188 level than GES-1 (P<0.05). Therefore, we concluded that down-regulation of miR-188 might play important roles in GC.

miR-188 acted as a tumor suppressor in GC cells

As miR-188 was expressed at lower levels in GC tissues and cell lines, we hypothesized that miR-188 might act as a tumor suppressor in GC. Therefore, miR-188 mimics or NC was transfected into BGC-823 and MKN-1 cells which showed lower miR-188 expression. As shown in Figure 2A, miR-188 mimics could markedly increased miR-188 expression levels in both BGC-823 and MKN-1 cells (P<0.05).

To investigate the effect of miR-188 on proliferation of GC, cell proliferation assay was performed. As shown in Figure 2B, the proliferation of BGC-823 and MKN-1 cells transfected with miR-188 mimics was reduced compared with that of BGC-823 and MKN-1 cells transfected with NC (P<0.05).
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Significantly lower in the miR-188 groups compared with NC groups (P<0.05). All these findings suggested that miR-188 acted as a tumor suppressor in GC.

SOX4 was a direct target of miR-188 in GC.

Due to the important functions of miR-188 in GC, we explored the underlying mechanisms. Using the publicly available databases miRanda (www.microrna.org) and TargetScan (www.targetscan.org), we found that SOX4 was a putative target gene of miR-188 (shown in Figure 3A).

To investigate whether miR-188 directly recognizes the 3'UTR of SOX4, PmirGLO-SOX4-3'UTR WT or PmirGLO-SOX4-3'UTR MUT was transfected into HEK293T cells in combination with miR-188 mimics or NC. The results showed that miR-188 mimics decreased the luciferase activities of PmirGLO-SOX4-3'UTR WT compared with NC groups, while the luciferase activities of PmirGLO-SOX4-3'UTR MUT was restored (shown in Figure 3B). It indicated that miR-188 could specifically bind to the 3'UTR of SOX4 and decrease its luciferase activities, while MUT vector could not be combined with miR-188 to decrease its luciferase activities (P<0.05).

We then used qRT-PCR and western blot to detect SOX4 expression levels in BGC-823 and MKN-1 cells transfected with miR-188 mimics or NC. As shown in Figure 3C and 3D, miR-188 over-expression significantly decreased SOX4 expression at both the mRNA and protein levels in BGC-823 and MKN-1 cells (P<0.05). Taken together, SOX4 was a direct target of miR-188 in GC.

We also performed cell invasion assay to evaluate the function of miR-188 in regulating the invasion ability of GC cells. As shown in Figure 2C and 2D, the average number of invaded cells penetrating the transwell membrane was significantly lower in the miR-188 groups compared with NC groups (P<0.05). All these findings suggested that miR-188 acted as a tumor suppressor in GC.

Effects of SOX4 knockdown on the proliferation and invasion of GC cells

To investigate the roles of SOX4 in GC cells, siRNA-SOX4 or siRNA-NC was transfected into
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Figure 4. SOX4 under-expression inhibited proliferation and invasion of GC cells. A. Western blot analysis for SOX4 protein of BGC-823 and MKN-1 cells transfected with siRNA-SOX4 or siRNA-NC. B. Cell proliferation assay was used to determine the cellular proliferation in BGC-823 and MKN-1 cells following transfection with siRNA-SOX4 compared with that in those transfected with siRNA-NC (P<0.05). As shown in Figure 4B, cell proliferation was significantly reduced in BGC-823 and MKN-1 cells transfected with siRNA-SOX4 compared with that in those transfected with siRNA-NC (P<0.05). As shown in Figure 4C and 4D, the average number of invaded cells penetrating the transwell membrane was significantly lower in the siRNA-SOX4 groups compared with siRNA-NC groups (P<0.05). These results may partly elucidated the miR-188 inhibited proliferation and invasion of GC cells though down-regulation of SOX4.

Discussion

The tumorigeneis and tumor development of GC is controlled by multiple factors, and a great deal of diverse molecules act as important factors in GC [20]. Previous studies have indicated that miRNAs are important in numerous aspects of human cancers and play important roles in carcinogenesis and cancer development [21-23]. miRNAs have been reported to act as tumor suppressors or oncogenes in human cancer, providing a new perspective on the treatment of cancer [18, 19]. However, possible roles of miRNAs in carcinogenesis and progression of GC remain largely unexplored.

In this study, we found that miR-188 was significantly down-regulated in GC tissues and cell lines (AGS, BGC-823, MKN-28, MKN-1, SGC-7901, MKN-45) compared with corresponding non-tumor gastric tissues and GES-1 which is a human immortalized gastric epithelial, respective-ly. In addition, restoration of miR-188 expression inhibited proliferation and invasion of GC cells. Furthermore, SOX4 was identified as a direct target of miR-
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188 in GC. SOX4 under-expression also inhibited proliferation and invasion of GC cells. These results suggested that miR-188 might act as a tumor suppressor in GC through directly targeting SOX4.

miR-188 has been mainly studied in several human cancers, including oral squamous cell carcinoma [24], heaptocellular carcinoma [25], prostate cancer [26] and acute myeloid leukemia [27]. Recent study demonstrated that miR-188 was down-regulated in oral squamous cell carcinoma, and miR-188 overexpression inhibited cell proliferation, cell cycle progression and invasion [24]. miR-188 was also down-regulated in hepatocellular carcinoma, and its expression levels were highly correlated with multiple nodules, microvascular invasion, overall and disease-free survival. Functionally, miR-188 suppressed proliferation and metastasis of hepatocellular carcinoma cells in vitro and in vivo [25]. Zhang et al reported that miR-188 was significantly down-regulated in prostate cancer with metastasis. Low expression levels of miR-188 were an independent prognostic factor for poor overall and biochemical recurrence-free survival. Ectopic of miR-188 expression inhibited prostate cancer cells proliferation, migration and invasion in vitro and suppressed tumour growth and metastasis in vivo [26]. These studies suggested that miR-188 may be important in these types of cancer and may serve as a potential therapeutic target for their treatments.

Since miRNAs function through targeting different mRNAs, we deduced that miR-188 might directly target mRNAs to inhibit proliferation and invasion of GC cells. Studies revealed that it may regulate oncogenic transcripts in human cells, including SIX1 [24], FGF5 [25], and LAPT4B [26]. In this study, SOX4 was validated to be a novel direct target gene of miR-188 in GC. Firstly, two publicly available databases (miRanda and TargetScan) predicted that SOX4 was a putative target of miR-188. Secondly, luciferase report assays demonstrated that miR-188 could specifically bind to the 3′UTR of SOX4. Thirdly, qRT-PCR and western blot revealed that miR-188 decreased SOX4 expression at both mRNA and protein levels in GC cells. Finally, SOX4 under-expression has the similar effects with miR-188 over-expression in GC. Identification of miR-188 target genes is critical for understanding its role in tumorigenesis and progression of GC.

The sex-determining region Y (SRY) box family, also referred to as the SOX family, comprises 20 highly conserved transcription factors [28]. SOX4, a member of the SOX family, is a 47-kDa protein that is encoded by a single exon gene [29]. Increasing evidences suggested that SOX4 was significantly up-regulated in many kinds of human cancers, such as breast cancer [30], prostate cancer [31], lung cancer [32], hepatocellular carcinoma [33] and GC [28]. In GC, expression levels of SOX4 were correlated with depth of invasion, nodal status, distant metastasis, stage, and vascular invasion. Patients with high expression levels of SOX4 achieved a significantly poorer disease-free survival rate, compared with patients with low SOX4 expression levels. Univariate Cox regression analysis showed that SOX4 over-expression was a clear prognostic marker for GC [28]. These results indicated that SOX4 was a suitable target for GC. Combination with findings in the present study, miR-188/SOX4 based targeted therapy may be a novel therapeutic strategy for patients with GC.

In conclusion, we have four major findings in this study: 1) miR-188 was significantly down-regulated in GC tissues and cell lines. 2) Ectopic expression of miR-188 inhibited proliferation and invasion of GC cells. 3) miR-188 could directly target SOX4, which was a novel target gene of miR-188. 4) SOX4 under-expression had the similar functions with miR-188 over-expression in GC.

Disclosure of conflict of interest

None.

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