miR-138 inhibits gastric cancer growth by suppressing SOX4

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Abstract. MicroRNA-138 (miR-138) has been reported to be downregulated and function as a tumor suppressor in several cancers. However, the role and molecular mechanisms of miR-138 in the progression of gastric cancer (GC) remain to be clarified. The aim of the present study was to determine the role of miR-138 in GC progression. In the present study we found that miR-138 expression was downregulated in GC tissues and cell lines. Statistical analysis demonstrated that low expression levels of miR-138 were associated with advanced tumor-node-metastasis (TNM) stage, and lymph node metastasis. Function assays demonstrated that overexpression of miR-138 impaired GC cell proliferation, colony formation, migration and invasion in vitro, as well as suppressed tumor growth in vivo. Through reporter gene, qRT-PCR and western blot assays, SRY-related high mobility group box 4 (SOX4), a master mediator in epithelial-mesenchymal transition (EMT), was confirmed to be a direct target of miR-138 in GC cells. Western blot assay revealed that miR-138 overexpression inhibited EMT procession in GC cells by upregulation of epithelial marker E-cadherin and downregulation of mesenchymal markers, N-cadherin and vimentin. Furthermore, the levels of miR-138 were inversely correlated with those of SOX4 expression in GC tissues. Overexpression of SOX4 rescued the inhibition effect in GC cells caused by miR-138. Collectively, these findings indicate that miR-138 may be a potential therapeutic target for GC.

Introduction

Gastric cancer (GC) is the fourth most common malignancy worldwide (1), and the second leading cause of cancer-related deaths worldwide (1). Despite recent advances in the diagnosis and management of GC, patients with advanced GC have a poor prognosis and the 5-year overall survival rate remains low at ~25% due to tumor recurrence, metastasis and poor response to chemotherapy and radiotherapy (2,3). In addition, the unclear pathophysiological mechanisms of GC have limited its clinical treatment options (4). Thus, it is essential to identify the molecular characterizations of GC in order to find new diagnostic strategies and potential drugs targets.

MicroRNAs (miRNAs), a class of small non-coding RNAs that regulate the gene expression by binding to the 3'-untranslated region (3'-UTR) of target mRNAs, have been reported to be involved in various biological processes, such as cell proliferation, the cell cycle, migration, invasion and apoptosis (5,6). Emerging evidence has indicated that miRNAs function as oncogenes or tumor-suppressor genes by regulating cancer cell proliferation, invasion, migration, differentiation and apoptosis (7-9). Recently, the aberrant expression of miRNAs has been revealed to play essential roles in tumorigenesis and progression of GC (10,11).

Growing evidence has revealed that microRNA-138 (miR-138) is frequently downregulated in hepatocellular carcinoma (12), colorectal cancer (13), osteosarcoma (14), glioma (15), non-small-cell lung (16) and oral squamous cell carcinoma (17), cervical (18) and breast cancer (19), suggesting a tumor-suppressive role of miR-138 in human tumorigenesis. However, the roles of miR-138 as well as its downstream targets in the regulation of GC progression remain unclear. Therefore, in the present study we aimed to investigate the expression levels of miR-138 and its clinical significance in GC. We also studied the molecular mechanism by which miR-138 mediated the growth and metastasis of GC.

Materials and methods

Tumor tissue sample. GC tissues and their matched adjacent normal tissues were collected from 48 patients during surgical resection at The First Hospital of Jilin University (Changchun, China) from July 2014 to July 2015. All samples were immediately snap-frozen in liquid nitrogen after surgical removal and stored at -80°C until use. The pathological diagnosis was made at the Department of Pathology of The First Hospital of Jilin University. Written informed consents were obtained from all patients. The present study was approved by the Ethics Committee of Jilin University and all aspects of the study complied with the Declaration of Helsinki.

Cell culture and transfection. Human GC HGC-27, MGC-803, SGC-7901 and BGC-823 cell lines and immortalized gastric epithelial cells GES-1 were purchased from the Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. HGC-27 cells were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum and antibiotics at 37°C in a 5% CO2 atmosphere. Other cell lines were grown in DMEM medium supplemented with 10% fetal bovine serum and antibiotics at 37°C in a 5% CO2 atmosphere. The cells were transfected with microRNA mimics with the pmaxGFP transfection vector (Life Technologies, Carlsbad, CA, USA) using Lipofectamine 2000 (Life Technologies) according to the manufacturer's instructions. Then, the cells were subjected to statistical analysis.

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Expression kit (Applied Biosystems). The miR-138-specific primer and the internal control U6 primer were purchased from Ambion. For the detection of SOX4 mRNA level, cDNAs were synthesized using a PrimeScript® RT reagent kit (Takara Biotechnology Co., Ltd., Dalin, China). qPCR was performed on an ABI7900 Real-Time PCR machine with miRNA-specific primers using TaqMan Gene Expression kit (Applied Biosystems). The miR-138-specific primer and the internal control U6 primer were purchased from Ambion. For the detection of SOX4 mRNA level, cDNAs were synthesized using a PrimeScript® RT reagent kit (Takara Biotechnology Co., Ltd., Dalin, China). qPCR was performed on ABI7900 sequence detector (Applied Biosystems) using the SYBR Premix Ex Taq kit (Takara Biotechnology Co., Ltd., Dalin, China). qPCR was performed as previously described. The primary antibodies used for the analysis were mouse anti-human SOX4 (dilution, 1:500), mouse anti-human E-Cadherin (dilution, 1:500), mouse anti-human N-cadherin (dilution, 1:500), mouse anti-human vimentin (dilution, 1:1,000), mouse anti-human SOX4 (dilution, 1:500), and mouse anti-c-Myc (dilution, 1:1,000). The secondary antibodies used were peroxidase-conjugated (HRP) antibodies against mouse (dilution, 1:5,000) and rabbit (dilution, 1:5,000) antibodies. The protein bands were detected with SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, Inc., Waltham, MA, USA) and visualized on an UVP BioImaging System (Upland, CA, USA).

Xenograft model experiment. Twenty male BALB/c nude mice aged 6 weeks were purchased from the Laboratory Animal Center of Jilin University, and maintained under specific pathogen-free (SPF) conditions in the animal care facility at Jilin University. All animal experiments in the present study were approved by the Committee on Animal Welfare of Jilin University. A total of 2x10^6 SGC-7901 cells transfected with
miR-138 mimic or miR-NC were suspended in 0.2 ml of Matrigel matrix (BD Biosciences), and then subcutaneously injected into the right side of the posterior flank of each mouse (n=10 each group), respectively. The length (L, cm) and width (W, cm) of the tumors were assessed every 5 days starting on the 10th day after inoculation using vernier callipers. The tumor volume was calculated using the formula: V = 0.5 x W^2 x L. The mice were sacrificed on the 35th day after injection and the tumor tissues were stripped, weighed and stored for further assays.

Statistical analysis. Data were analyzed using SPSS version 19.0 software (SPSS, Inc., Chicago, IL, USA) and GraphPad Prism 5 (GraphPad Software, Inc., San Diego, CA, USA). The difference was determined using two-tailed Student's t-test or one-way ANOVA. The relationship between miR-138 levels and SOX4 mRNA expression was tested with Pearson's correlation. Statistical significance was established at P<0.05.

Results

miR-138 is downregulated in GC tissues and cell lines. To explore the role of miR-138 in GC, we performed real-time quantitative RT-PCR in 48 pairs of GC and adjacent normal tissues. Compared with the adjacent normal tissues, miR-138 expression was significantly decreased in GC tissues (Fig. 1A). In clinical stages, miR-138 expression in cancer tissues of stages III and IV was significantly lower than in cancer tissues of stages I and II (Fig. 1B). Concomitantly, we compared the expression of miR-138 GC tissues with lymph node metastasis and GC tissues without lymph node metastasis. As the data revealed in Fig. 1C, the level of miR-138 in GC with lymph node metastasis was significantly lower than GC without lymph node metastasis. Moreover, we also investigated the expression of miR-138 in 4 human GC cell lines (HGC-27, MGC-803, SGC-7901 and BGC-823) and immortalized gastric epithelial cells GES-1 as control. The results demonstrated that miR-138 was significantly downregulated in GC cell lines compared with the human normal gastric cell line (Fig. 1D). These results revealed that miR-138 may be involved in gastric cancer tumorigenesis.

miR-138 suppresses the proliferation and colony formation of GC cells. To investigate the potential functions of miR-138 in GC cells, SGC-7901 cells, with the lowest expression of miR-138 of the 4 GC cell lines (Fig. 1D), were transfected with the miR-138 mimic or miR-NC to enhance miR-138 expression, and then cell proliferation and colony formation were determined. Firstly, we performed qRT-PCR to verify the efficacy of transfection. As indicated in Fig. 2A, transfection of cells with miR-138 mimic revealed a significant increase of miR-138 expression compared to cell transfection with miR-NC. The results from the CCK-8 assay revealed that overexpression of miR-138 significantly inhibited cell prolif-
miR-138 inhibits cell invasion and migration in GC cells. Since the expression of miR-138 was correlated with lymph node metastasis, we explored the effect of miR-138 on cell migration and invasion in GC cells. The results from the wound-healing assay revealed that overexpression of miR-138 markedly decreased the migration of SGC-7901 cells (Fig. 3A). Matrigel Transwell assays revealed that miR-138 overexpression significantly inhibited the invasion of SGC-7901 cells (Fig. 3B). These results implied that miR-138 is involved in metastasis.
SOX4 is a direct target of miR-138 in GC cells. To identify the potential target mRNA of miRNA-138, the bioinformatics algorithms PicTar, TargetScan and miRanda were used. All of these databases predicted SOX4 as a target of miR-138, and the 3'-UTR of SOX4 mRNA contained a highly conserved binding site from positions 1,233 to 1,239 with a miR-138 seed sequence (Fig. 4A). Luciferase activity analysis further revealed that miR-138 significantly inhibited the luciferase activity of the wild-type SOX4-3'-UTR reporter, but did not affect the mutant-type SOX4-3'-UTR reporter (Fig. 4B). Furthermore, qRT-PCR and western blot analysis revealed that the SOX4 expression at the mRNA and protein levels was downregulated in SGC7901 cells transfected with miR-138 compared to cells transfected with miR-NC (Fig. 4C and D). It has been demonstrated that SOX4 could act as a downstream regulator of epithelial-mesenchymal transition (EMT). Therefore, we wondered whether miR-138 regulates EMT. Thus, we also detected the expression of EMT epithelial marker E-cadherin and mesenchymal markers, N-cadherin and vimentin expression in SGC7901 cells after transfection with miR-138, suggesting that miR-138 regulates EMT progression. These data revealed that the SOX4 gene is one of the direct targets of miR-138 in GC cells.

Therefore we next detected the expression of SOX4 in GC tissues and the corresponding adjacent normal tissues by qRT-PCR. We found that SOX4 mRNA expression was upregulated in GC tissues compared to adjacent normal tissues (Fig. 5A). Through Pearson's correlation analysis, we found that SOX4 mRNA expression was inversely correlated with miR-138 in GC tissues (Fig. 5B; r=-0.455; P=0.002).

miR-138 modulates cell growth, migration and invasion by targeting SOX4. To verify whether the inhibitory effects of miR-138 are mediated through SOX4, we rescued the expression of SOX4 in miR-138 mimic-transfected cells. qRT-PCR and western blot assays revealed that restoration of SOX4 expression partially reversed the inhibitory effect on cell proliferation, colony formation, migration and invasion in SGC7901 cells induced by miR-138 overexpression (Fig. 6C-F). These results revealed that miR-138 modulates cell growth, migration and invasion by targeting SOX4.

miR-138 suppresses tumor growth in vivo. To determine the effects of miR-138 on tumorigenicity in vivo, transfected cells were injected into the flanks of nude mice to form ectopic tumors. We found that tumor growth was slower in the miR-138 transfected cell group than that of the miR-NC transfected cell group (Fig. 7A). In addition, the size and weight of the tumors from the miR-138 transfected cell group were significantly decreased.
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We also analyzed the expression of miR-138 and SOX4 in xenograft tumors. In the miR-138 transfected cell group, miR-138 expression was upregulated (Fig. 7D), whereas SOX4 expression was downregulated at the mRNA (Fig. 7E) and protein levels (Fig. 7F). These data indicated that miR-138 suppresses tumor growth in vivo by targeting SOX4.

Discussion

MicroRNAs (miRNAs) are important epigenetic regulators in the development and progression of human gastric cancer, and function as oncogenes or tumor suppressors in gastric cancer (GC) progression (10,11). Thus, identification of the exact biological role of miRNAs in GC progression and development may contribute to finding novel diagnosis markers and therapy targets for GC. In the present study we first confirmed that miR-138 was downregulated in GC tissues and cell lines, and its expression was closely associated with advanced tumor-node-metastasis (TNM) stage and lymph node metastasis. The in vitro and in vivo experiments revealed that restoration of miR-138 suppressed the cell proliferation, colony formation, migration and invasion of GC cells in vitro, as well as suppressed tumor growth by directly targeting
SOX4. These data implied that miR-138 may be a promising therapeutic target of GC.

MicroRNA-138 (miR-138), a family of microRNA precursors, has been reported to play crucial roles in various biological processes, such as embryological morphogenesis, cell proliferation, cell invasion and developmental events tied to stem cell differentiation (21). Several studies have demonstrated that miR-138 played a suppressive role in multiple human cancers (12-19). For instance, Liu et al revealed that miR-138 inhibited cell proliferation, colony formation, migration and invasion in hepatocellular carcinoma cells partially suppressing SOX9 expression (12). Xiao et al reported that miR-138 suppressed the growth and metastasis of non-small cell lung cancer cells partly via targeting YAP1 (22). Zhang et al demonstrated that miR-138 suppressed metastasis and EMT in breast cancer cells by targeting vimentin (19). In the present study, we found that the miR-138 expression level was significantly decreased in GC tissues compared to their matched adjacent normal tissues, and that decreased miR-138 was associated with advanced TNM stage and lymph node metastasis. However, a previous study reported that miR-138 was among 24 miRNAs that were significantly upregulated in GC tissues compared to normal gastric mucosae (23), yet, this previous study did not specify how many cases of GC tissues were used in the miRNA array analysis and did not verify their results by qRT-PCR (23). In addition, our results also revealed that miR-138 was downregulated in 4 GC cell lines compared to normal gastric cell lines, which further suggested that miR-138 may be downregulated in GC tissues and cell lines. We also found that enforced expression of miR-138 inhibited proliferation, colony formation, migration and invasion in GC cells, as well as suppressed tumor growth in nude mice. These results revealed that miR-138 functioned as a tumor suppressor by inhibiting GC progression.

It is well known that miRNAs exert their biological function primarily depending on the target gene (5,6). To explore the mechanisms underlying how miR-138 inhibited GC progression, we searched for potential targets of miR-138 in GC cells using several computational algorithms. Among the candidate target genes, we focused on SOX4 since it has been demonstrated to serve as an oncogene in various human cancers (24-26). SOX4, a member of the Sox (Sry-related high-mobility group box) family of transcription factors, has been reported to promote tumor cell growth and metastasis, through regulation of multiple signaling pathways, such as the Wnt, Notch1 and p53 pathway (27,28). In addition, SOX4 could serve as a master mediator in epithelial-mesenchymal transition (EMT) (29,30), which is a critical step in tumor progression and metastasis (31). For GC, a previous study revealed that SOX4 expression was upregulated in GC tissues, and that overexpression of SOX4 was significantly correlated with depth of invasion, nodal status, distant metastasis, stage and vascular invasion, suggesting that SOX4 could serve as an oncogene in GC. Furthermore, SOX4 has been reported to be a target of miR-138 in ovarian cancer (32), however, the interaction between miR-138 and SOX4 has not been experimentally validated in GC. In the present study, luciferase reporter assay, qRT-PCR and western blot assays further confirmed their targeting relationship, and found that the expression of SOX4 was negatively regulated by miR-138 in GC cells. We also found that miR-138 downregulated vimentin expression and upregulated E-cadherin expression, which contributed to EMT inhibition. In addition, an inverse correlation between miR-138 and SOX4 mRNA expression was observed in GC tissues. Notably, overexpression of SOX4 markedly reversed the inhibitory effect of miR-138 on GC cell proliferation, migration and invasion. Our in vivo experiments also revealed that miR-138 inhibited tumor growth in nude mice by regulating SOX4. These results demonstrated that miR-138 played a tumor-suppressive role in GC, at least in part, by suppressing SOX4.
In summary, we investigated the biological functions of miR-138 in GC for the first time, and found that miR-138 was downregulated in GC cell lines and tissues, and was closely associated with advanced TNM stage and lymph node metastasis, and that miR-138 could inhibit malignant progression of GC by targeting SOX4. These findings revealed that miR-138 may function as a diagnostic marker and a therapeutic target for GC.

References