Original Article

MicroRNA-122 inhibits proliferation and invasion in gastric cancer by targeting CREB1

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Abstract: MicroRNA-122 (miR-122) has been implicated in tumor development and progression in various types of cancers. However, the biological function and regulatory mechanisms of miR-122 in gastric cancer (GC) remain largely unknown. We aimed to determine the biological role and underlying mechanism of miR-122 in GC. Real time quantitative RT-PCR (qRT-PCR) was performed to detect the expression of miR-122 in GC tissues and cell lines. CCK8, wound healing, and transwell assays were conducted to determine the effect of miR-122 on cell proliferation, migration, and invasion, respectively. Target molecules were identified by luciferase activity, quantitative RT-PCR, and western blotting. We found that miR-122 expression was significantly decreased in both GC tissues and cell lines and that reduced expression was significantly associated with aggressive clinicopathological features in patients. We also found that overexpression of miR-122 markedly inhibited proliferation, migration, and invasion in GC cell lines. In addition, cAMP responsive element binding protein 1 (CREB1) was identified as a direct target of miR-122, and its expression was negatively correlated with miR-122 expression in GC tissues \((r = -0.711, P < 0.001)\). CREB1 overexpression rescued the suppressive effect of miR-122 on GC cell proliferation, migration, and invasion. Moreover, we demonstrated that miR-122 inhibited GC tumorigenesis in vivo by repressing CREB1 expression. These findings suggest that miR-122 might function as a tumor suppressor in GC and could serve as a promising candidate for therapeutic applications regarding GC treatment.

Keywords: miR-122, gastric cancer, CREB1, proliferation, invasion

Introduction

Gastric cancer (GC) is the fourth most common cancer and the second leading cause of cancer-related death worldwide [1, 2]. Despite considerable improvement in cancer diagnosis and treatment, patients with advanced GC have poor prognosis primarily due to frequent tumor metastasis and tumor recurrence after surgical resection [3]. Therefore, it is imperative to elucidate the molecular mechanisms involved in GC carcinogenesis and metastasis to identify novel biomarkers for diagnosis and to develop targeted therapies.

MicroRNAs (miRNAs) are a class of small, endogenous, non-coding, single stranded RNA species, approximately 19-25 nucleotides in length, that regulate target-gene expression at the post-transcriptional levels by binding to the 3'-untranslated region (UTR) of target mRNA [4]. miRNAs have been widely studied and have been showed to be involved in various biological processes such as proliferation, cell cycle regulation, differentiation, apoptosis, and metastasis [5, 6]. In recent years, miRNAs have emerged as an important class of regulators of gene expression involved in cancer development and progression, and these molecules function as either oncogenes or tumor suppressors in different types of cancer through the regulation of cellular proliferation, metastasis, and apoptosis [7-9]. In GC, numerous miRNAs are involved in its occurrence and progression by regulating the expression of specific target genes; this suggests that miRNAs could serve as a diagnostic marker or target of therapeutics for GC [10, 11].

Recently, miR-122 has been the focus of many studies, and has been shown to function as a
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Table 1. Correlation between the clinicopathologic characteristics and expression of miR-122 in GC

<table>
<thead>
<tr>
<th>Variables</th>
<th>No. of cases</th>
<th>miR-122 expression</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Low (n%)</td>
<td>High (n%)</td>
</tr>
<tr>
<td>Age (years)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt; 60</td>
<td>20</td>
<td>11 (55.0)</td>
<td>9 (45.0)</td>
</tr>
<tr>
<td>≥ 60</td>
<td>30</td>
<td>16 (53.3)</td>
<td>14 (46.7)</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Man</td>
<td>28</td>
<td>15 (53.6)</td>
<td>13 (46.4)</td>
</tr>
<tr>
<td>Woman</td>
<td>22</td>
<td>12 (54.5)</td>
<td>10 (45.5)</td>
</tr>
<tr>
<td>Tumor size</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt; 5 cm</td>
<td>31</td>
<td>15 (48.4)</td>
<td>16 (51.6)</td>
</tr>
<tr>
<td>≥ 5 cm</td>
<td>19</td>
<td>12 (63.2)</td>
<td>7 (36.8)</td>
</tr>
<tr>
<td>TNM stage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I-II</td>
<td>37</td>
<td>15 (40.5)</td>
<td>22 (59.5)</td>
</tr>
<tr>
<td>III-IV</td>
<td>13</td>
<td>12 (92.3)</td>
<td>1 (7.7)</td>
</tr>
<tr>
<td>Lymph node metastasis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>39</td>
<td>16 (41.0)</td>
<td>23 (59.0)</td>
</tr>
<tr>
<td>Yes</td>
<td>11</td>
<td>11 (100.0)</td>
<td>0 (0.0)</td>
</tr>
</tbody>
</table>

The miR-122 mimic (miR-122) and corresponding negative control (miR-NC) were purchased from GenePharma (Shanghai, China). The coding domain sequence of human cAMP responsive element binding protein 1 (CREB1) mRNA was amplified by PCR using human cDNA as a template, and inserted into the pcDNA 3.0 vector (Invitrogen, Grand Island, NY, USA). Transfection was performed using Oligofectamine™ Transfection Reagent (Invitrogen) according to the manufacturer’s instructions.

RNA extraction and real-time PCR (qRT-PCR) assays for miR-122 and CREB1 detection

Total RNA including miRNA was isolated from the cultured cells and tissues using TRIzol reagent (Invitrogen) following the manufacturer’s instructions. Total RNA was quantified using a NanoDrop ND-100 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) at 260 nm. Total RNA (2 μg) was reverse-transcribed into cDNA using SuperScript III (Invitrogen) according to the manufacturer’s instructions. For the detection of miR-122 levels, qRT-PCR was performed using an ABI PRISM7900 Sequence Detection System (Applied Biosystems) and TaqMan microRNA assay kits (Applied Biosystems, Foster City, CA, USA) with specific primers for miR-122 (Applied Biosystems) and endogenous U6 snRNA as the con-

Materials and methods

Human GC clinical specimens

A total of 50 paired GC and corresponding adjacent noncancerous tissues were collected after receiving written informed consent from patients who underwent gastric resection at the Department of the Gastrointestinal Surgery, the People's Hospital of Jilin Province (Changchun, China) from March 2014 to March 2016. Matched adjacent noncancerous gastric epithelial tissues were obtained at least 5 cm from the tumor site. All tissues specimens were snap frozen in liquid nitrogen. The basic clinical characteristics of these patients were documented (Table 1). The study protocol was approved by the ethics committee of the People’s Hospital of Jilin Province (Changchun, China).

Cell culture and transfection

Four human GC cell lines (BGC-823, SGC-7901, MKN-45, and AGS) and the immortalized gastric epithelial cell line GES-1 were all bought from the American Type Culture Collection (ATCC, Rockville, Md., USA). All cells were grown in Roswell Park Memorial Institute 1640 medium (RPM1640, HyClone, Logan, UT, USA) supplemented with 10% fetal bovine serum (HyClone), 50 U/ml penicillin or 50 μg/ml streptomycin in a humidified atmosphere containing 5% CO\textsubscript{2} at 37°C.
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trol. For the detection of CREB1 expression, qRT-PCR was performed using Fast SYBR Green Master Mix (Applied Biosystems) with an ABI PRISM7900 Sequence Detection System. The primers for CREB1 and GAPDH (as the control) used in this study were described previously [18]. The relative miR-122 or CREB1 mRNA expression levels were quantified by measuring cycle threshold (Ct) values and normalized using the2^{-ΔΔCt} method.

Cell proliferation, migration, and invasion assays

Cell proliferation was determined using Cell Counting Kit-8 assay kits (CCK-8, Dojindo, Kumamoto, Japan). Briefly, transfected cells were seeded into 24-well plates at a density of 5 x 10^3 cells/well. At indicated time points (24, 48, and 72 h), 10 μl of CCK8 solution was added to each well and incubated at 37°C for 4 h. Optical density (OD) values were measured at 450 nm using a microplate reader (Bio-Tek Instruments Inc., Winooski, VT, USA).

Vector construction and luciferase assay

To construct a CREB1-3'UTR reporter plasmid, a wild-type 3'-UTR fragment from human CREB1 mRNA, containing the putative miR-122 binding sequence, was amplified by PCR and cloned into the pGL3 vector (Promega, Madison, WI, USA) downstream of the luciferase reporter gene, and this was named Wt-CREB1 3'UTR. The mutant-type CREB1 plasmid was constructed by mutating the complementary site of the seed region of miR-122 (CACUCCA to AAGCCGA) using a site directed mutagenesis kit (Takara, Japan), based on the wild-type CREB1 reported plasmid, and was named Mut-CREB1 3'UTR. For luciferase assays, cells were co-transfected with miR-122 mimic or miR-NC, Renilla luciferase pRL-TK vector, and Wt/Mut CREB1 3'UTR reporter plasmid using Oligofectamine™ Transfection Reagent. Forty-eight hours after transfection, luciferase activity was measured using a dual-luciferase assay system (Promega, Madison, USA) according to the manufacturer's instructions. Renilla-luciferase activity was assayed for normalization.

Western blot analysis

Total cellular and tissue proteins were extracted using lysis buffer (50 mM Tris-HCl (pH 7.5), 20 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.1% SDS, 5% glycerol, and protease inhibitors), and centrifuged for 15 min at 20,000 × g and 4°C. The protein concentration was determined using a BCA protein assay kit (Thermo, Waltham, MA, USA) with bovine serum albumin as a standard. Equal amounts of protein (30 μg) were separated using 8% SDS-polyacrylamide gels (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA, USA) and blocked for 1 h with 5% non-fat milk in TBS buffer (10 mM Tris, 150 mM NaCl). The membrane then was incubated overnight at 4°C with primary antibodies as follows: mouse anti-human CREB1 (1:1000 dilution, Santa Cruz Biotechnology, Santa Cruz, CA, USA); mouse anti-human GAPDH (1:5000 dilution, Santa Cruz Biotechnology) was used as an endogenous control. After washing with TBS buffer three times, the membrane was incubated with horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (1:3000; Santa Cruz Biotechnology, Texas, USA) for 2 h at room temperature. The
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Protein bands were detected with Pierce® ECL chemiluminescence reagents (Thermo Fisher, USA) and exposed on X-ray film.

Xenograft model experiment

Male BALB/c nude mice aged 5-6 weeks were purchased from Laboratory Animal Center of Jilin University (Changchun, China) and maintained under specific pathogen free (SPF) conditions. All animal procedures were performed in accordance with protocols approved by the Committee on Animal Welfare of the People’s Hospital of Jilin Province. A total of $2 \times 10^6$ SGC-7901 cells, transfected with miR-122 mimic or miR-NC, were suspended in 0.2 ml Matrigel Matrix (BD Biosciences) and then subcutaneously injected into the flanks of each mouse. The length (L, cm) and width (W, cm) of tumors were measured every 3 days starting on the 15th day after inoculation, using calipers. Tumor volume was calculated using the formula $V = (W^2 \times L)/2$. Thirty days later, the mice were euthanized and tumor tissues were removed and weighed. A portion of the tumor tissue was snap frozen in liquid nitrogen and stored at -80°C for analysis of miR-122 and CREB1 expression.

Statistical analysis

All statistical analyses were performed with Statistical Product and Service Solutions (SPSS) 16.0 software (SPSS, Chicago, IL). GraphPad Prism 5.01 software (GraphPad Software, Inc., San Diego, CA, USA) was used to draw graphs. Differences were determined by performing a two-tailed student’s t-test or a one-way ANOVA. The relationship between miR-122 levels and clinical pathological variables was analyzed using a Pearson’s $\chi^2$ test. Correlations between miR-122 and CREB1 mRNA expression were analyzed using Pearson analysis. $P < 0.05$ was regarded as statistically significant.

Results

miR-122 is downregulated in both GC cell lines and clinical tissues

To examine miR-122 levels, we first examined its expression in four human GC cell lines (BGC-823, SGC-7901, MKN-45, and AGS) and compared it to that in the normal gastric mucosal epithelial cell line GES-1, used as a control. Results of qRT-PCR analysis showed that miR-122 was aberrantly downregulated in all GC cell lines, compared to its expression in normal GES-1 cells (Figure 1A). To validate whether aberrant downregulation of miR-122 also occurred in GC clinical specimens, we examined miR-122 expression in 50 GC tissues and corresponding adjacent noncancerous tissues. As shown in Figure 1B, the expression of miR-122 in GC tissues was significantly lower than that in adjacent non-cancerous tissues ($P = 0.0007$). To further investigate the clinicopathological significance of miR-122 levels in GC samples, 50 patients were divided into two subgroups based on mean value (0.425) in all GC samples; specifically, the low miR-122 group ($< 0.425$, 27 cases) and the high miR-122 group ($> 0.425$, 23 cases).
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Correlations between miR-122 expression and clinicopathologic parameters were then identified using a Pearson's χ² test. We found that reduced miR-122 expression was significantly associated with lymph node metastasis and TNM stage, but not with age, gender, or tumor size (Table 1).

miR-122 inhibits GC cell proliferation, migration, and invasion

To determine the biological function of miR-122 in GC, we introduced a miR-122 mimic into SGC-7901 cells to alter the levels of total miR-122; these cells were found to express the lowest levels of miR-122 among the four GC cell lines tested (Figure 1A). qRT-PCR analysis demonstrated that miR-122 expression was higher in SGC-7901 cells transfected with the miR-122 mimic compared to that in cells transfected with miR-NC (Figure 2A). Then, cell proliferation was assessed in SGC-7901 cells transfected with the miR-122 mimic or miR-NC using a CCK-8 assay. Our results revealed that proliferation was obviously suppressed in SGC-7901 cells. *P < 0.05, **P < 0.01.

Figure 2. miR-122 inhibits gastric cancer (GC) cell proliferation, migration, and invasion. (A) miR-122 expression was upregulated in SGC-7901 cells transfected with a miR-122 mimic compared to cells transfected with miR-NC. (B-D) Overexpression of miR-122 significantly decreased proliferation (B), migration (C), and invasion (D) in SGC-7901 cells. *P < 0.05, **P < 0.01.
miR-122 targets CREB1 to inhibit gastric cancer

Results also demonstrated that reduced miR-122 was associated with lymph node metastasis (Table 1), suggesting that it might regulate metastasis in GC. To determine if this was indeed the case, we performed wound-healing and transwell invasion assays using SGC-7901 cells transfected with the miR-122 mimic or miR-NC. Results showed that miR-122 overexpression suppressed cell migration (Figure 2C) and invasion (Figure 2D) in SGC-7901 cells. Taken together, results suggested that miR-122 functions to inhibit GC cell proliferation, migration, and invasion.

CREB1 is a direct target of miR-122 in GC

Potential targets of miR-122 were predicted using three bioinformatics databases (TargetScan, miRanda, and PicTar). Analysis results indicated that there are putative miR-122 binding sites located in the 3'-UTR of the CREB1 transcript (Figure 3A). To verify whether CREB1 is a direct target of miR-122 in GC cells, human wide-type or mutant-type CREB1 3'UTR plasmids containing the binding sites of miR-122 or mutant sites were constructed (Figure 3A). Then, together with the miR-122 mimic or miR-NC, these were co-transfected into SGC-7901 cells and cells were cultured for 48 h, before measuring luciferase activities. miR-122 overexpression obviously decreased luciferase activity from the wide-type CREB1 3'UTR; however, activity from the mutant-type CREB1 3'UTR was not changed (Figure 3B). In addition, qRT-PCR and western blotting analysis further revealed that miR-122 significantly decreased CREB1 expression on both the mRNA (Figure 3C) and protein level (Figure 3D) in SGC-7901 cells. These results indicated that miR-122 is able to bind directly to CREB1 and inhibit its expression in GC cells.
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CREB1 is upregulated and inversely correlated with miR-122 levels in GC tissues

We next examined CREB1 mRNA levels by qRT-PCR in 50 pairs of GC tissue specimens and adjacent non-cancerous tissues. As shown in Figure 4A, CREB1 mRNA expression was significantly upregulated in GC specimens compared to that in adjacent non-cancerous tissues. In addition, an inverse correlation between miR-122 and CREB1 mRNA levels was further confirmed by Pearson correlation analysis in 50 GC tissues (r = -0.711, P < 0.0001; Figure 4B).

Overexpression of CREB1 reverses the tumor suppressive effect of miR-122 in GC

To evaluate if CREB1 is responsible for the functional effects of miR-122 in GC cells, rescue experiments were performed. First, SGC-7901 cells were transfected with the miR-122 mimic or miR-NC and pcDNA3.1-CREB1. The transfection efficiency was confirmed by western blot analysis (Figure 5A). Subsequently, CCK8, wound healing, and transwell invasion assays were performed in the aforementioned cells. Results showed that CREB1 overexpression reversed the miR-122-mediated inhibitory effect on cell proliferation, migration, and invasion in SGC-7901 cells (Figure 5B-D). These results clearly demonstrated that miR-122 inhibits cell proliferation, migration, and invasion in GC cells, at least in part by targeting CREB1.

miR-122 suppresses xenograft tumor growth by targeting CREB1

To confirm whether miR-122 affects gastric tumorigenesis in vivo, SGC-7901 cells transfected with miR-122 or miR-NC were subcutaneously injected into mice. As shown in Figure 6A-C, overexpression of miR-122 significantly inhibited tumor growth in vivo. In addition, we also measured miR-122 and CREB1 expression in tumor tissues. We found that miR-122 expression was upregulated (Figure 6D), whereas CREB1 expression both on the mRNA and protein level was downregulated in the miR-122-overexpressing group compared to that in the miR-NC group (Figure 6E and 6F). Taken together, these results indicate that miR-122 inhibits tumor growth in vivo by targeting CREB1.

Discussion

Recently, numerous miRNAs have been determined to be involved in GC progression and metastasis, suggesting that miRNAs can serve as effective diagnostic and prognostic molecu-
miR-122 targets CREB1 to inhibit gastric cancer

**Figure 5.** Overexpression of CREB1 reversed the tumor suppressive effect of miR-122 in gastric cancer (GC). A. CREB1 protein expression was determined in SGC-7901 cells transfected with miR-122 with or without a CREB1 overexpression plasmid by western blotting. GAPDH was used as the internal control. B-D. Cell proliferation, migration, and invasion were assessed in SGC-7901 cells transfected with miR-122 with or without CREB1 overexpression by CCK8, wound healing, and transwell invasion assays. *P < 0.05, **P < 0.01.

**Figure 6.** miR-122 suppresses xenograft tumor growth in vivo by targeting CREB1. A. Tumor growth curve for each group. B. The mice were killed at 30 days after inoculation, and tumor tissues was excised and imaged. C. Mean tumor weight for each group. D. miR-122 expression was measured in tumor tissues by quantitative real-time PCR. E, F. CREB1 expression on the mRNA and protein level was detected in tumor tissues. GAPDH was used as an internal control. *P < 0.05, **P < 0.01.
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Liu et al., Department of [0x0] [20], has been identified as an oncogene in CREB1 tumor suppressor in GC. Detailed evidence that miR-122 functions as a knowledge, this is the first report providing the tumorigenicity of GC cells. We also showed that miR-122 suppresses proliferation, migration, and invasion in SGC-7901 cells. These results suggest that miR-122 plays a crucial role in inhibiting GC progression.

Recently, studies have demonstrated that miR-122 expression is frequently downregulated and that this molecule functions as a tumor suppressor in multiple tumor types [12-16]. For example, Wang et al. found that miR-122 decreases bladder cancer cell migration, invasion, and colony formation in vitro and inhibits bladder cancer growth and angiogenesis in vivo, in addition to sensitizing bladder cancer cells to cisplatin-induced apoptosis by targeting VEGF-C [15]. Couluoarn et al. reported that miR-122 expression is downregulated in HCC cells, and that reintroduction of miR-122 into HCC cells can reverse the tumorigenic properties of these cells [19]. Qin et al. demonstrated that miR-122 inhibits migration, invasion, and epithelial-mesenchymal transition in non-small-cell lung cancer cells through the suppression of PI3K/AKT activation by reducing endogenous expression of insulin-like growth factor 1 receptor [12]. Here, we found that miR-122 was downregulated in the GC tissue samples, when compared to that in adjacent normal tissues, and that low expression of miR-122 was associated with lymph node metastasis and advanced tumor stage, suggesting that it might be involved in the progression and metastasis of GC. Functional assays showed that miR-122 overexpression significantly inhibited cell proliferation, migration, and invasion in SGC-7901 cells. We also showed that miR-122 suppresses the tumorigenicity of GC cells in vitro. To our knowledge, this is the first report providing detailed evidence that miR-122 functions as a tumor suppressor in GC.

CREB1, located on human chromosome 2q34 [20], has been identified as an oncogene in many types of cancer such as bladder [21], lung [22], breast [23], and colorectal [24] cancer. Aberrant CREB1 expression has been linked to development, progression in multiple tumor types through the regulation of downstream gene expression, which includes cell apoptosis-related genes Bcl-2 and Bax; cell cycle-related genes cyclinA1, cyclinB1, and cyclinD2; the cell invasion-related gene MMP9; signal transduction proteins, activated transcription factor 3; NF-kB; and other growth-related genes [25-27]. For GC, CREB1 has been reported to be upregulated and to correlate with lymph node metastasis, distant metastasis, and tumor stage as well as poor outcome in patients with GC, suggesting that it is oncogenic in GC [28]. In the present study, CREB1 was confirmed to be a direct target of miR-122 based on luciferase activity assays, qRT-PCR, and western blotting. Furthermore, mRNA expression of CREB1 was found to be inversely correlated with miR-122 levels in GC tissues. Of note, exogenous overexpression of CREB1 partially reversed the effects of miR-122 on cell proliferation, migration, and invasion in GC cells. We also found that miR-122 suppresses tumorigenicity in GC cells in vivo by repressing CREB1. Taken together, these data suggested that miR-122 exerts its suppressive effect on GC cells partially by inactivation of CREB1.

In summary, the present study provided evidence that miR-122 expression is downregulated in GC tissues and cell lines, and that decreased miR-122 expression is associated with TNM stage and lymph node metastasis in patients with GC. In addition, we found that restoration of miR-122 suppressed proliferation, migration, and invasion of SGC-7901 cells, and inhibited tumor growth in vivo partially by inactivating CREB1. As this study was limited by the number of GC samples and cell types, more elaborate studies will be necessary to further investigate the potential biological role of miR-122 in GC progression. However, the findings herein are encouraging and suggest that miR-122 might be a potential target for the treatment of GC in the future.

Disclosure of conflict of interest

None.

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