Original Article

MiR-203 inhibits tumor invasion and metastasis in gastric cancer by ATM

Ping Zhou, Nan Jiang, Guo-Xia Zhang, and Qing Sun*

Department of Pathology, Qian-fo-shan Hospital Affiliated to Shandong University, Jinan 250014, China

*Correspondence address. Tel: +86-531-89268155; Fax: +86-531-89268191; E-mail: sq167660@163.com

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Abstract

Gastric cancer is one of the most common malignancies in the world. A number of miRNAs are aberrantly expressed during the progression of gastric cancer. In this study, we aimed to investigate the role of miR-203 in the invasion and metastasis of gastric cancer and the potential mechanism of the effect of miR-203 on the tumor progression of gastric cancer. Our results showed that miR-203 was significantly downregulated in gastric cancer tissues and cells, while ataxia telangiectasia mutated kinase (ATM) was upregulated in gastric cancer tissues and cells and was directly regulated by miR-203. Ectopic overexpression of miR-203 inhibited the colony formation, migration, and invasion of gastric cancer cells. In addition, miR-203 overexpression significantly suppressed the protein level of Snail and obviously promoted the protein level of E-cadherin in gastric cancer cells. ATM knockdown phenocopied the effect of miR-203 overexpression. These results suggested that miR-203 suppressed the migration and invasion of gastric cancer through regulating the level of ATM-mediated-Snail and E-cadherin. MiR-203 might be a novel therapeutic strategy for the treatment of gastric cancer.

Key words: ATM, gastric cancer, invasion, migration, miR-203

Introduction

Gastric cancer is one of the most common malignancies in the world [1]. In spite of the great advances in gastric cancer diagnosis and therapy, gastric cancer is still the second most frequent malignancy globally [2]. Due to the recurrence, invasion and metastasis, the prognosis of patients with advanced gastric cancer remains poor [3]. A better understanding of the mechanisms underlying tumor progression is urgently required to develop novel strategies for the diagnosis and treatment of gastric cancer.

DNA damage responses are critical for maintaining the genetic stability [4]. It plays vital roles in tumorigenesis and drug resistance [5,6]. Ataxia telangiectasia mutated kinase (ATM), as a central regulator of DNA damage responses, is reported to be hyperactive in late-stage breast cancer tissues with lymph-node metastasis and correlated with elevated expression of the epithelial-mesenchymal transition (EMT) marker, Snail [7]. EMT is the initial step for cancer invasion and metastasis. As the upstream molecule of E-cadherin, Snail is considered to be necessary for triggering EMT during tumor progression through suppressing E-cadherin transcription [8]. In addition, it was found that TGF-β1, Snail, E-cadherin, and N-cadherin were abnormally expressed in gastric cancer tissues and involved in the process of invasion and metastasis of gastric cancer [9].

MicroRNAs (miRNAs), as the important post-transcriptional regulatory factors, participate in many important biological processes [10–12]. Increasing evidence suggests that a number of miRNAs are aberrantly expressed during the progression of gastric cancer, including miR-200b, miR-104-3p, miR-27b, miR-663, miR222, miR-31, miR-574-3p, miR-455-3p, and miR-34a [13], and mediate various target genes to regulate proliferation, invasion, metastasis, and so on [14–22]. The serum miRNA levels of miR-21, miR-146a, and miR-148a have been used as biomarker candidates to predict the presence of lymph node metastasis in gastric cancer [23]. MiR-203 was found to be significantly low expressed in colorectal cancer and inhibit the cell proliferation in SGC-7901 cells [24]. Furthermore, Zhou et al. [25] confirmed that miR-203
regulated ATM protein expression by binding to the ATM 3' untranslated region (UTR) in colorectal cancer cells, and then induced oxaliplatin resistance. These results suggest that there may be an interaction between miR-203 and ATM in gastric cancer. Therefore, we propose that in gastric cancer, miR-203 may target ATM to activate Snail expression and repress E-cadherin transcription, finally regulate the invasion and metastasis of gastric cancer.

In this study, we aimed to investigate the role of miR-203 in the invasion and metastasis of gastric cancer and the potential mechanism of the effect of miR-203 on the tumor progression of gastric cancer. We explored the expression pattern of miR-203 in gastric cancer tissues and cell lines and confirmed its target gene ATM. Through the biological function analysis, it was proved that miR-203 suppressed the migration and invasion of gastric cancer through regulating the ATM mediated-Snail and E-cadherin expression.

Materials and Methods
Patient tissue samples and cell lines
Twenty-three gastric cancer samples and adjacent non-cancerous tissues were obtained from Qian-fo-shan Hospital Affiliated to Shandong University (Jinan, China). Pathological diagnosis was carried out according to the World Health Organization criteria. Informed consent for the collection of samples was obtained from all patients. Human gastric cancer cell line (SGC-7901 and HGC-27) and the normal gastric epithelial cell lines (GES-1) were obtained from American Type Culture Collection (ATCC, Manassas, USA). All cells were cultured in RPMI 1640 medium (Invitrogen, Carlsbad, USA) with 10% fetal bovine serum (Invitrogen) at 37°C in 5% CO2.

Immunohistochemistry assay
Briefly, sections about 3 μm were deparaffinized in xylene, rehydrated through graded ethanol, followed by blocking of endogenous peroxidase activity in 3% hydrogen peroxide for 20 min at room temperature. Sections were then incubated with rabbit polyclonal anti-ATM antibody (1:200; Santa Cruz Co, Santa Cruz, USA) overnight at 4°C in a humidified chamber. After being washed, slides were incubated with biotinylated secondary antibody (Santa Cruz) for 30 min, followed by incubation with streptavidin-biotin-peroxidase complex (LSAB; Dako, Carpinteria, USA) with 10% fetal bovine serum (Invitrogen) at 37°C in 5% CO2. The study protocol was approved by the Medical Ethics Committee of Qian-fo-shan Hospital Affiliated to Shandong University.

Cell transfection
Oligonucleotides has-miR-203 mimics (miR-203) and negative control (miR-NC) were designed as described previously [24]. All the RNA oligoribonucleotides were synthesized by GenePharma (Shanghai, China). ATM siRNA (siRNA-ATM) and negative control siRNA (siRNA-NC) were purchased from Cell Signaling Technology (Beverly, USA). SGC-7901 cells were, respectively, transfected with oligonucleotides using Lipofectamine 2000 (Invitrogen). Twenty-four hours after transfection, cells were harvested for qRT-PCR to determine the transfection efficiency.

Cell migration and invasion analyses
Cell migration and invasion were investigated using wound healing assay and Transwell assay as described previously [28]. Briefly, cells were plated into the 6-well plates and serum starved for 12 h to

Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR)
Total RNA was isolated from tissues and cells using total RNA Extraction kit (Life Technologies, Carlsbad, USA) and reverse transcribed using First-Strand RT-PCR kit (Invitrogen) according to the manufacturer’s protocol. The expression levels of ATM and miR-203 were analyzed by qRT-PCR using the SYBR Premix Ex TaqTM II kit (Takara, Dalian, China) on the ABI-7500 platform (Applied Biosystems, Foster City, USA). The reaction condition was as follows: 45 amplification cycles of 95°C for 5 s, 58°C for 20 s, and 72°C for 30 s. U6 RNA was used as an internal control. Primers were designed according to the previous report [25].

Western blot analysis
Protein concentrations were determined using a BCA kit (Tiangen, Beijing, China). Western blot analyses were performed using the Bio-Rad Protein Assay Kit (Bio-Rad, Hercules, USA) as described previously [7]. Proteins were separated by 10% SDS-PAGE gel and transferred to PVDF membranes (Millipore, Darmstadt, Germany). The membrane was blocked and incubated with specific primary antibodies, then incubated with secondary antibodies labeled with horseradish peroxidase (HRP) and detected by using the chemiluminescence kit (Thermo, Sunnyvale, USA). GAPDH was used as protein loading control. The antibodies against ATM, Snail, E-cadherin, and GAPDH were purchased from Sigma-Aldrich (St Louis, USA).

Luciferase reporter assay
Luciferase reporter assay was performed as described previously [26]. Briefly, human ATM 3' UTR containing the putative binding sites of miR-203 was inserted into the firefly luciferase reporter vector pmiR-RB-REPORTTM (RiboBio, Guangzhou, China). The wild-type 3'UTR of ATM was amplified from a commercial vector (GeneCopoeia, Rockville, USA). Based on the pmiR-RB-ATM-3'UTR construct, mutant ATM 3'UTR was generated by mutating three nucleotides that are recognized by miR-203. Cells were plated at the density of 1 × 10^5 cells/well on 24-well plates, and transfected with pmiR-RB-ATM 3'UTR or its mutant construct. After 48 h, these cells were transfected with has-miR-203 mimics or negative control oligonucleotides. Relative luciferase activity was measured 24 h post-transfection by using the Dual Luciferase Reporter Assay kit (Promega, Madison, USA).

Colony formation assay
Colony formation assay was performed as described previously [27]. Briefly, Cells were inoculated in 6-well plates at the density of 400 cells/well. Culture medium was changed every 3 days. After the cells were cultured at 37°C with 5% CO2 for 14 days, the cells were washed with phosphate buffer solution (PBS) and fixed with 5% paraformaldehyde for 20 min. Colonies were stained with GIMSA (Solarbio, Beijing, China) and counted under an inverted microscope (Nikon, Tokyo, Japan). Visible colonies were counted in four different fields and the mean value was calculated.

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made a ‘wound’ (clear space) using a sterile pipette tip. Then, serum-free medium containing 0 or 8 μg/ml harmine was added to the plates and the cells were incubated for 36 h at 37°C. Wound margins were observed using a phase contrast microscope and photographed at 0 h and 36 h.

Cell invasion assay was performed in Transwell chamber with a diameter of 6.5 mm containing polycarbonate filters with 8 μm pores coated with Matrigel (BD Biosciences, San Diego, USA) according to the manufacturers’ instructions. Cells were incubated at 37°C for 24 h, non-invading cells were mechanically removed with cotton swabs, and the invading cells on the bottom surface of the membrane were stained with 0.1% crystal violet for 30 min and then washed extensively with PBS. The invading cells were counted and photographed under a microscope (Nikon).

**Statistical analysis**

Statistical analysis was performed using SPSS software version 16.0. Experiments were repeated at least three times. Data were presented as mean ± standard deviation [29]. Intergroup difference was analyzed using t-test. \( P < 0.05 \) was considered statistically significant.

**Results**

**MiR-203 is downregulated in gastric cancer tissues and cell lines**

To explore the role of miR-203 in the development of gastric cancer, the expression pattern of miR-203 was first measured in gastric cancer tissues and cell lines. As shown in Fig. 1A, the expression level of miR-203 in gastric cancer tissues was significantly downregulated compared with the corresponding non-cancerous tissues (\( P < 0.001 \)). In addition, miR-203 was significantly low expressed in gastric cancer cell line SGC-7901 and HGC-27 compared with the normal gastric epithelial cell line GES-1 (Fig. 1B, \( P < 0.01 \)). These results confirmed that miR-203 was downregulated in gastric cancer tissues and cell lines.

In addition, the association of miR-203 expression with the clinicopathological features of patients with gastric cancer is summarized in Table 1. There was no correlation between miR-203 expression and patient age and sex (\( P > 0.05 \)). But significant association was found between miR-203 expression and tumor size, lymphatic invasion and borrhmann classification (\( P < 0.05 \)).

**ATM is upregulated in gastric cancer tissues and cell lines**

Given the previous finding that miR-203 could negatively regulate ATM expression in colorectal cancer by binding to the ATM 3'UTR [23], there may be an interaction between miR-203 and ATM in gastric cancer. In order to investigate the correlation between ATM and miR-203 in gastric cancer, the expression levels of ATM in gastric cancer tissues and cell lines were measured. The protein expression of ATM was significantly upregulated in gastric cancer tissues as revealed by immunoblotting and immunohistochemical assays (Fig. 1C,D). The results also showed that the mRNA level and protein level of ATM were obviously upregulated in gastric cancer cells (Fig. 1E,F, \( P < 0.01 \)).

**MiR-203 directly regulates ATM expression in gastric cancer**

To confirm whether miR-203 could regulate ATM expression by directly binding to the ATM 3'UTR, a luciferase reporter construct containing a specific deletion mutant at putative miR-203 binding site was generated (Fig. 2A). Luciferase activity assay revealed that miR-203 significantly inhibited the wt-ATM 3'UTR but not the mut-ATM 3'UTR luciferase activity in SGC-7901 cells (Fig. 2B, \( P < 0.001 \)). Furthermore, miR-203 was overexpressed in SGC-7901 cells by transfection with miR-203 mimics (Fig. 2C, \( P < 0.001 \)). Overexpression of miR-203 significantly reduced ATM mRNA and protein levels in SGC-7901 cells (Fig. 2D,E, \( P < 0.001 \)), while knockdown of miR-203 dramatically increased the protein level of ATM in SGC-7901 cells (Supplementary Fig. S1, \( P < 0.001 \)). Overall, these findings suggested that miR-203 directly regulated ATM expression in gastric cancer by binding to the ATM 3'UTR.

**MiR-203 inhibits colony formation, migration, and invasion of gastric cancer cells**

Since a previous study has demonstrated the important role of miR-203 in cell proliferation and migration of prostate cancer [30], we further investigated the function of miR-203 in gastric cancer. The effects of miR-203 overexpression on colony formation, migration, and invasion in SGC-7901 cells were examined. As shown in Fig. 3A, miR-203 overexpression significantly inhibited the proliferation of gastric cancer cells as revealed by colony formation assay (\( P < 0.001 \)). Wound healing assay showed that SGC-7901 cells transfected with miR-203 mimics presented an impaired migration capacity compared with those in control or negative control (Fig. 3B, \( P < 0.001 \)). In addition, transwell assay demonstrated that overexpression of miR-203 remarkably attenuated the invasiveness of gastric cancer cells (Fig. 3C, \( P < 0.001 \)). Taken together, these results indicated that miR-203 significantly suppressed proliferation, migration, and invasion of gastric cancer cells in vitro.

**MiR-203 regulates ATM-mediated-Snail and E-cadherin expression in gastric cancer**

The above evidence showed that miR-203 regulated ATM expression in gastric cancer, and that abnormal expression of some molecules (such as Snail and E-cadherin) was involved in the process of invasion and metastasis of gastric cancer. Thus, the potential mechanism of the role of miR-203 was explored in gastric cancer. The protein levels of Snail and E-cadherin in the gastric cancer tissues and adjacent non-cancerous tissues were shown in Supplementary Fig. S2. The protein level of Snail was significantly upregulated in gastric cancer tissues, while the protein level of E-cadherin was significantly downregulated in gastric cancer tissues (\( P < 0.001 \)). Western blot analysis showed that miR-203 overexpression significantly suppressed the protein levels of ATM and Snail, while the protein level of E-cadherin was obviously promoted in gastric cancer cells (Fig. 4, \( P < 0.001 \)). Furthermore, silencing ATM phenocopied the effects of miR-203 overexpression. Similar to that mediated by miR-203 overexpression, ATM knockdown significantly affected the gene expressions of Snail and E-cadherin (Fig. 4, \( P < 0.001 \)). Overall, these data indicated that miR-203 inhibited the migration and invasion of gastric cancer through regulating the expressions of ATM-mediated Snail and E-cadherin.
Discussion

Gastric cancer is one of the most common malignancies in the world and the second most common cause of death in cancer-related deaths. The major cause of the tumor recurrence and patient mortality of gastric cancer is metastasis. A better understanding of the molecular pathways regulating gastric cancer metastasis is conducive to the development of novel strategies for the diagnosis and therapy of the disease.
Recently, more and more evidence showed that aberrant expression of miRNAs is involved in the genesis and development of cancers [31]. miRNAs functioned as oncogenes or tumor suppressors, participated in regulating diverse biological processes, including proliferation, differentiation, apoptosis, migration, and invasion [10–12, 14, 15, 32]. Numerous studies have reported the aberrant expression of miR-203 in a wide range of human cancers, such as oral cancer [33], pancreatic adenocarcinoma [34], squamous cell carcinoma of esophagus [35], hepatocellular carcinoma [36], prostate cancer [30], and colorectal cancer [24]. In this study, we found that miR-203 was significantly downregulated in 23 gastric cancer tissues compared with the corresponding non-cancerous tissues. In addition, the expression level of miR-203 in gastric cancer cell line SGC-7901 was significantly decreased, which is consistent with the report of Chiang et al. [24]. The functional analysis showed that ectopic overexpression of miR-203 obviously suppressed the colony

### Table 1. Clinical characteristics of the patients with gastric cancer

<table>
<thead>
<tr>
<th>Variable</th>
<th>No. of patients (n = 23)</th>
<th>miR-203 low expression (n = 10)</th>
<th>miR-203 high expression (n = 13)</th>
<th>P</th>
</tr>
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<tbody>
<tr>
<td>Age</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;50</td>
<td>9</td>
<td>5</td>
<td>4</td>
<td>&gt;0.05</td>
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<tr>
<td>≥50</td>
<td>14</td>
<td>6</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>Sex</td>
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<td>13</td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>10</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Tumor size</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;1.5 cm</td>
<td>8</td>
<td>5</td>
<td>3</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>≥1.5 cm</td>
<td>15</td>
<td>10</td>
<td>5</td>
<td></td>
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<tr>
<td>Lymphatic invasion</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
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<td>10</td>
<td>6</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Negative</td>
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<td>4</td>
<td>3</td>
<td></td>
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<td>Borrmann classification</td>
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<td>10</td>
<td>7</td>
<td>3</td>
</tr>
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</table>

![Figure 2. ATM was a direct target of miR-203](image-url)

(A) The potential miR-203 binding sequence of ATM 3′UTR and the mutant. (B) SGC-7901 cells were co-transfected with miR-203 or miR-NC with wild-type or mutant ATM 3′UTR, and then luciferase activity was measured. (C) Expression of miR-203 in SGC-7901 cells transfected with miR-203 or control mimic was detected by qRT-PCR. (D) Expression of ATM in SGC-7901 cells transfected with miR-203 or control mimic was detected by qRT-PCR. (E) Protein level of ATM in SGC-7901 cells transfected with miR-203 or control mimic was detected by western blot analysis. GAPDH was used as protein loading control. Data were drawn from three independent experiments. Control means the SGC-7901 cells without any treatment. ***P < 0.001 compared with the control.
formation, migration, and invasion of gastric cancer cells. In colorectal cancer cells, miR-203 was proved to induce oxaliplatin resistance by negatively regulating ATM kinase [25]. MiR-203-mediated LASP1 overexpression increased cell proliferation and migration in high-risk prostate cancer [30]. These data suggested that miR-203 played an important role in the carcinogenic process.

Several studies have suggested that the aberrant expression of miR-203 contributes to carcinogenesis by inhibiting the expression of their target genes [25,30,33]. In this study, our results confirmed that miR-203 directly regulates ATM expression in gastric cancer by binding to the ATM 3’UTR. ATM is a target of miR-203 in gastric cancer. Similarly, Zhou et al. [23] showed that miR-203 negatively regulates ATM expression in colorectal cancer by binding to the ATM 3’UTR. ATM is a central regulator of DNA damage responses. Sun et al. [7] demonstrated that ATM is hyperactive in late-stage breast cancer tissues and is correlated with elevated expression of the EMT marker, Snail. ATM regulates Snail stabilization by phosphorylation on Serine 100, and activation of ATM-mediated Snail phosphorylation promotes tumor invasion and metastasis of breast cancer in vitro and in vivo [7]. These results suggest that miR-203 may regulate tumor invasion and metastasis through ATM-mediated DNA damage responses pathway. Our further study confirmed this hypothesis. MiR-203 overexpression significantly suppressed the protein level of Snail and obviously promoted the protein level of E-cadherin in gastric cancer cells. Similar to that mediated by miR-203 overexpression, ATM knockdown significantly affected the protein levels of Snail and E-cadherin in gastric cancer cells. Thiery et al. [8] revealed that as the downstream molecule of Snail, E-cadherin was suppressed by snail for triggering EMT during tumor progression. Based on these, we conclude that miR-203 inhibits the migration and invasion of gastric cancer through regulating the expression of ATM mediated-Snail and E-cadherin.

In summary, we showed that miR-203 was downregulated in gastric cancer tissues and cell lines, and abnormal high expression of miR-203 significantly suppressed colony formation, migration, and invasion of gastric cancer cells. ATM was identified as a target of miR-203. Enhanced miR-203 inhibited ATM activity, suppressed the ATM-Snail pathway, resulted in the increase of E-cadherin, and thereby prevented the migration and invasion of gastric cancer cells. These results suggest that miR-203 may be a novel therapeutic strategy for the treatment of gastric cancer.
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References

Figure 4. MiR-203 regulated ATM-mediated Snail and E-cadherin expression in gastric cancer cells. Protein levels of ATM, Snail, and E-cadherin in SGC-7901 cells transfected with miR-203, miR-NC, ATM siRNA or negative control siRNA were detected by western blot analysis. GAPDH was used as protein loading control. Data were drawn from three independent experiments. Control means the SGC-7901 cells without any treatment. **P < 0.01, ***P < 0.001 compared with the miR-NC. ###P < 0.001 compared with siRNA-NC.


