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miR-148a suppresses cell invasion and migration in gastric cancer by targeting DNA methyltransferase 1

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Abstract. Gastric cancer (GC) is the fourth most common malignant tumor globally. The highest incidence of GC is found in Eastern Asia, particularly in China. It is therefore imperative to further elucidate the molecular pathogenesis of GC in order to identify new biomarkers and targets for effective therapy. In the present study, we determined whether miR-148a was aberrantly downregulated in gastric cancer tissues and significantly correlated with aggressive clinicopathological characteristics in the MGC-803, HGC-27 and GES-1 cell lines using reverse transcription-quantitative PCR and western blot analysis. The cell lines were obtained from 60 patients who presented at our hospital between September 2010 and July 2015. The results showed that, miR-148a was aberrantly downregulated in GC tissues and its expression was relatively lower in the MGC-803 and HGC-27 GC cell lines than in the normal gastric epithelial cell line, GES-1. The clinicopathological analysis revealed that a decrease of miR-148a was significantly correlated with lymph-node metastasis (P<0.01) and tumor node metastasis (TNM) stage (P<0.05). The transwell assay showed that the re-expression of miR-148a significantly reduced cell migratory and invasive abilities *in vitro* (P<0.01). The luciferase assay confirmed that, DNA methyltransferase 1 (DNMT1) was a direct and functional target of miR-148a. The miR-148a inhibitor increased the expression of DNMT1 in HGC-27 cells and the re-expression of miR-148a reduced the expression of DNMT1 in MGC-803 cells as confirmed by western blot analysis. Furthermore, we found that the re-expression of DNMT1 reversed the inhibition of cell migration and invasion induced by miR-148a. Taken together, we demonstrated that miR-148a suppresses cell invasion and migration in gastric cancer by regulating DNMT1 expression. The miR-148a/DNMT1 axis may therefore be a new potential target for GC therapy.

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Introduction

Gastric cancer (GC) is the fourth most common malignant tumor globally (1) and the third leading cause of cancer-associated mortality worldwide (2). The highest incidence rate of GC is found in Eastern Asia, and particularly in China (3). GC is a consequence of multi-factors, including environmental and genetic factors. Infection with *Helicobacter pylori (H. pylori)* is a major risk factor, accounting for approximately 75% risk (4). It is accepted that *H. pylori* causes general inflammatory stress leading to malignancy (5), and activates multiple critical pathways in gastric epithelial cells (6). Although *H. pylori* infection is extremely prevalent, only a small minority of infected individuals are likely to develop gastric cancer. Thus, only *H. pylori* infection is not sufficient to cause cancer. Previous findings showed that genetic factors synergize in the development of gastric cancer (4). Therefore, it is of vital importance to elucidate the molecular pathogenesis of GC in order to identify new biomarkers and targets for effective therapy.

MicroRNAs (miRNA) are a class of endogenous, non-coding RNAs that are approximately 20-23 nucleotides in length. At present, over 1500 miRNAs have been identified in humans. miRNAs negatively regulate gene expression at the post-transcriptional level mainly by binding to the 3'-untranslated region (3'-UTR) of a target mRNA (7). miRNAs are involved in many cancer-related cell processes, including apoptosis, as well as migration/invasion and differentiation. miRNAs are aberrantly expressed in various types of cancer, including gastric, lung, liver and esophageal cancer (8-11). In addition, miRNAs function as either oncogenes or tumor suppressors (12). Increasing evidence has shown that miRNAs are involved in the oncogenesis and progression of GC. The microRNA-7/NF-κB signaling regulatory feedback circuit regulates gastric carcinogenesis (8). Chemotherapy-induced miRNA-29c/catenin-δ signaling suppresses metastasis in gastric cancer (13). MicroRNA-29c mediates the initiation of gastric carcinogenesis by directly targeting ITGB1 (14). miR-148a is reported to suppress tumor cell invasion and metastasis by downregulating ROCK1 (15). However, the mechanism of elucidating mRNA in gastric cancer remains to be elucidated.

DNA methylation is an epigenetic modification, mainly regulated by DNA methyltransferase family, which includes the subtypes DNMT1, DNMT2 and DNMT3. DNMT1 is considered the most abundant DNMT and is primarily involved in the maintenance of methylation during DNA replication (16). DNMT1 is overexpressed in various types of cancer, including gastric cancer (17). It was previously reported that DNMT1 is crucial in the silencing of several tumor suppressor genes (18).

In the present study, we aimed to determine and showed that miR-148a was aberrantly downregulated in gastric cancer tissues and was significantly correlated with aggressive clinicopathological characteristics. The re-expression of miR-148a significantly suppressed migration and invasion *in vitro*. We identified *DNMT1* as a functional and direct target gene of miR-148a and DNMT1 attenuated the suppression of miR-148a-medicated inhibition on cell migration and invasion. Therefore, miR-148a was able to suppress the migration and invasion of GC by directly targeting DNMT1, thus, providing a new potential therapeutic target for GC treatment.

Materials and methods

Tissue samples and cell lines. Sixty patients were involved in the present study. Gastric cancer tissues and their corresponding non-tumorous gastric tissues were collected from patients between September 2010 and July 2015 at the Department of Gastrointestinal Surgery, Weihai Central Hospital, Weihai, Shandong, China. Human tissues were immediately frozen in liquid nitrogen and stored at -80˚C refrigerator.

Written informed consent for samples was obtained from the patients. The study was approved by the Clinical Research Ethics Committee of Weihai Central Hospital.

Two gastric cancer cell lines (MGC-803, HGC-27), one normal gastric epithelial cell line (GES-1, as control), and HEK293T cells were used in the present study. The cells were cultured in RPMI-1640 medium (MGC-803 and HGC-27) or Dulbecco's modified Eagle's media (HEK293T and GES-1) supplemented with 10% FBS (HyClone, South Logan, UT, USA) at 37˚C in a humidified air atmosphere containing 5% CO_2.

Cell transfection. MGC-803 cells were plated in 6-well plates and transfection was performed after 24 h. DNMT1 (plasmid no. 36939) was purchased from Addgene (Cambridge, MA, USA), and was transfected into cells using X-tremeGENE HP Transfection Reagent (Roche Applied Science, Indianapolis, IN, USA). miR-148a mimics or inhibitor and the control were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Cells were collected at 48 h after transfection for reverse transcription-quantitative PCR (RT-qPCR) and western blot analysis. miR-148a mimics or inhibitor and the control were purchased from RiboBio (Guangzhou, China).

RT-qPCR. miRNA was extracted from tissue samples and cultured cells using mirVana™ miRNA isolation kit (Ambion, Austin, TX, USA). The expression level of miR-148a was quantified with specific primers and probes using TaqMan miRNA assays (Applied Biosystems, Carlsbad, CA, USA) and U6 small nuclear RNA was used as an internal control as per the manufacturer's protocol. The experiments were repeated three times. Total RNA from cultured cells and tissues was extracted using TRIzol Reagent (Invitrogen) as per the manufacturer's protocol. RT-qPCR assays were carried out to detect DNMT1 relative expression by using the PrimeScript RT Reagent kit (Takara, Dalian, China) and SYBR Premix Ex Taq (Takara) with the Roche LightCycler 480 system (Roche, Basel, Switzerland) according to the manufacturer's protocol. GAPDH was used as an internal control and was analyzed using the $2^{-\Delta\Delta ct}$ method. The experiments were repeated three times.

Western blot analysis. Cell proteins were extracted and separated using 10% SDS-PAGE and then transferred to nitrocellulose filter membranes (Millipore, Darmstadt, Germany). After blocking in 5% non-fat milk, the membranes were incubated overnight at 4˚C with rabbit polyclonal anti-DNMT1 antibody (cat. no. ab87654; dilution, 1:1,000; Abcam, Cambridge, MA, USA), and rabbit monoclonal anti-GAPDH antibody (cat. no. EPR16891; dilution, 1:1,000; Epitomics, Burlingame, CA, USA). The membranes were subsequently washed and incubated with goat polyclonal anti-rabbit IgG H&L secondary antibody (cat. no. ab150077; dilution, 1:2,000; Abcam). The proteins were visualized using a LI-COR Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE, USA).

Luciferase reporter assay. HEK-293T cells were used for the luciferase reporter assay. Cells were cultured in a 24-well plate and co-transfected with the pGL3-3'-UTR (500 ng) of DNMT1 or mutated 3'-UTR and miR-148a expressing vector or negative control vector. The 3'-UTR was amplified using the primers: Forward, CCGCTCGAGAAATAAAGGAGGAGGA AGCTGC and reverse, GGGTTTAAACGGTGGTTTATAG GAGAGAT (19). The 3'-UTR mutation was generated using the QuikChange II XL Site-Directed Mutagenesis kit (Stratagene, Santa Clara, CA, USA). At 48 h after transfection, the cells were collected and analyzed using the Dual Luciferase Assay System (Promega, Fitchburg, WI, USA) according to the manufacturer's instructions. The relative firefly luciferase activities were normalized to *Renilla* luciferase activities. The experiments were repeated at least three times.

In vitro migration and invasion assays. For the migration and invasion assay, $5x10^4$ cells were cultured in 24-well plates with 8-μm pore size chamber inserts (Corning Inc., Corning, NY, USA). For the migration assay, cells resuspended in 200 μl of serum-free medium were placed into the upper chamber with the non-coated membrane. For the invasion assay, the cells were placed into the upper chamber with the coated membrane. The lower chamber was filled with 10% FBS as a chemoattractant and incubated for 48 h for the migration assay and 72 h for the invasion assay. The cells on the upper surface of the membrane were removed and cells on the bottom surface of the chamber were fixed with 100% methanol for 20 min and stained with 0.1% crystal violet for 30 min. The assays were conducted in independent triplicates.

Statistical analysis. Statistical analysis was performed with GraphPad Prism version 6 for Windows (GraphPad Software Inc., La Jolla, CA, USA). Data were presented as means ± SD.

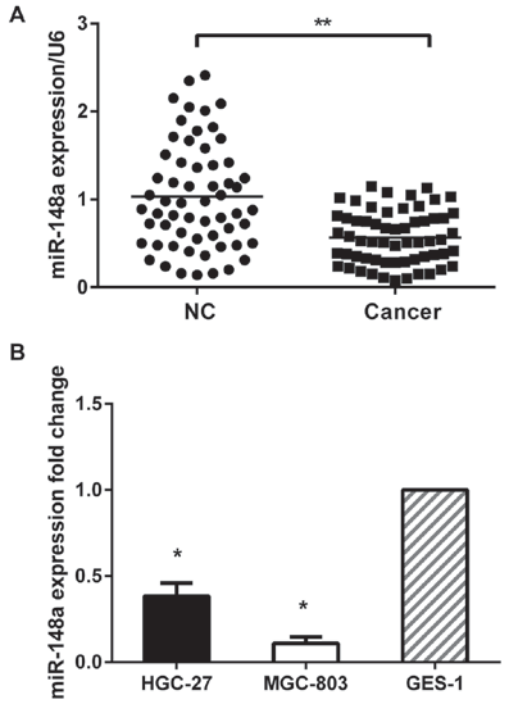

Figure 1. miR-148a is downregulated in gastric cancer. (A) The expression of miR-148a was significantly downregulated in cancer tissues compared with normal control. U6 RNA served as an internal control. (B) Relative expression of miR-148a in MGC-803 and HGC-27 cells compared with the normal gastric epithelial cell (GES-1). *P<0.05, **P<0.01.

The difference between two groups was analyzed using ANOVA and Chi-squared test. Differences were considered significant for P<0.05.

Results

miR-148a is downregulated in gastric cancer tissues. To investigate the role of miR-148a in the progression of gastric cancer, we first analyzed the expression level of miR-148a in 60 pairs of gastric cancer tissues and their corresponding normal tissues. The RT-qPCR analysis revealed that the expression of miR-148a was significantly downregulated in cancer tissues compared with the normal control (P<0.01) (Fig. 1A). We further analyzed the relative expression of miR-148a in HGC-27 and MGC-803 cells. The two cell lines had a low expression of miR-148a. Moreover, MGC-803 had a relatively lower expression of miR-148a than HGC-27 (Fig. 1B).

The clinicopathological analysis revealed that no significant correlations were observed between the miR-148a expression level and age, sex, location or tumor size. The low expression of miR-148a was significantly correlated with lymph-node metastasis (P<0.01) and gastric cancer with advanced stages (III+IV) had a lower level of miR-148a than early stages (I+II) (Table I). These findings suggested that the downregulation of miR-148a may be involved in gastric cancer development.

Re-expression of miR-148a suppresses gastric cancer migration and invasion in vitro. To understand better the biological function of miR-148a on the development of gastric cancer, we re-expressed miR-148a in MGC-803 cells. Transwell assays were performed to detect migratory and invasive ability induced by miR-148a. The successful re-expression of mature miR-148a in MGC-803 cells was confirmed by RT-qPCR (Fig. 2A). For the migration assay, the re-expression of miR-148a significantly reduced the migratory ability and cell numbers in MGC-803 cells (P<0.01) (Fig. 2B). For the invasion assay, the re-expression of miR-148a significantly decreased the invasive ability and cell numbers in MGC-803 cells (P<0.01) (Fig. 2B). Furthermore, deletion of miR-148a by a specific miR-148a inhibitor increased cell migratory and invasive ability in GC cell lines (P<0.01) (Fig. 2C).

Table I. Clinicopathological characteristics and miR-148a expression in 60 patients with gastric cancer.

Characteristics	Cases (n=60)	miR-148 expression High (n=13)	miR-148 expression Low (n=47)	P-value[a]
Age (years)				0.3468
≥55	28	8	20	
<55	32	5	27	
Sex				0.2134
Male	29	4	25	
Female	31	9	22	
Tumor size (cm)				0.5354
≥5	35	7	30	
<5	25	6	17	
Differentiation				0.9301
Well	21	4	17	
Moderately	18	4	14	
Poor	21	5	16	
TNM stage				**0.0242**
I + II	24	9	15	
III + IV	36	4	32	
Lymph-node metastasis				**0.0022**
Yes	38	3	35	
No	22	10	12	

[a]χ^2 test; TNM, tumor node metastasis. Bold, statistically significant.

miR-148a downregulates DNMT1 expression by directly targeting its 3'-UTR. To explore downstream targets of miR-148a, two online algorithms, TargetScan and miRanda, were used to predict its targets. DNMT1 was identified as one candidate target (Fig. 3A). To verify whether miR-148a could bind to 3'-UTR of DNMT1, luciferase report assays were performed in HEK293T cells. Our results showed that the luciferase activity of the wild-type (WT) 3'-UTR of DNMT1 was significantly decreased in cells co-transfected with miR-148a compared with that in miRNA control. While the luciferase activity of the mutated type (MT) 3'-UTR of DNMT1 was not obviously altered in cells co-transfected with miR-148a compared with that in miRNA control (Fig. 3B). Additionally, we detected whether the expression of DNMT1

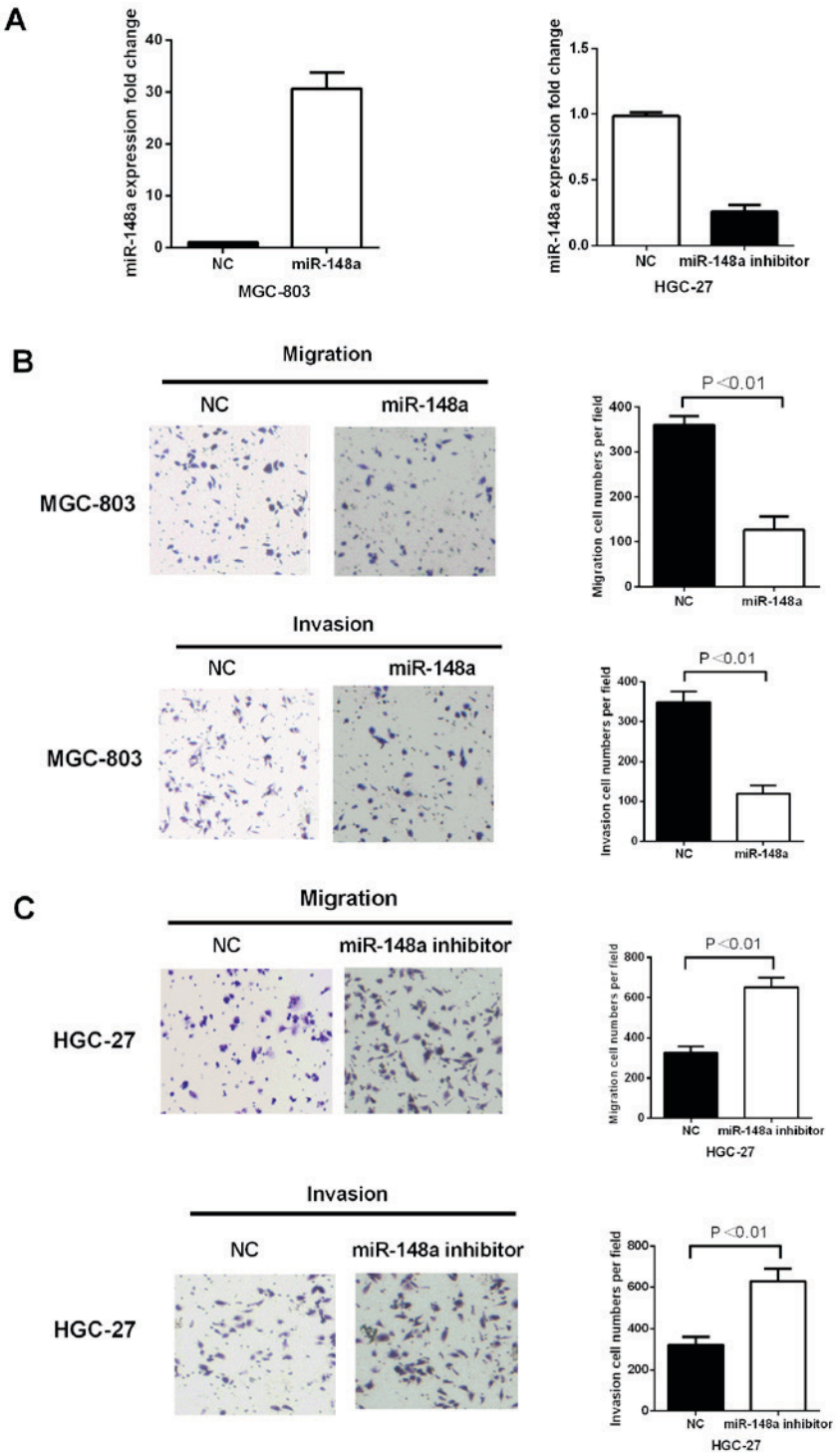

Figure 2. Re-expression of miR-148a suppresses gastric cancer migration and invasion *in vitro*. (A) The re-expression of miR-148a in MGC-803 cells was confirmed by RT-qPCR. (B) The re-expression of miR-148 significantly decreased the migratory and invasive abilities in MGC-803 cells. (C) The re-expression of miR-148 signifcantly decreased the migratory and invasive abilities in HGC-27 cells.

is regulated by miR-148a. In MGC-803 cells, the re-expression of miR-148a reduced the expression of DNMT1 at the protein and mRNA level, and in HGC-27 cells, the miR-148a inhibitor increased the level of DNMT1 at protein and mRNA levels (Fig. 3C and D).

DNMT1 is involved in cell migration and invasion. As miR-148a is downregulated in gastric cancer and targets DNMT1 by binding to its 3'-UTR, we then examined whether DNMT1 is involved in cell migration and invasion. MGC-803 cells were co-transfected with DNMT1 overexpression plasmid and miR-148a and then detected the mRNA and protein levels using RT-qPCR and western blot analysis. As shown in Fig. 4A and B, the downregulation of DNMT1 mediated by miR-148a was partially reversed by the ectopic expression of DNMT1. The transwell assay showed that the ectopic expression of DNMT1 partially attenuated miR-148-mediated inhibition on cell migratory and invasive abilities (Fig. 4C). Moreover,

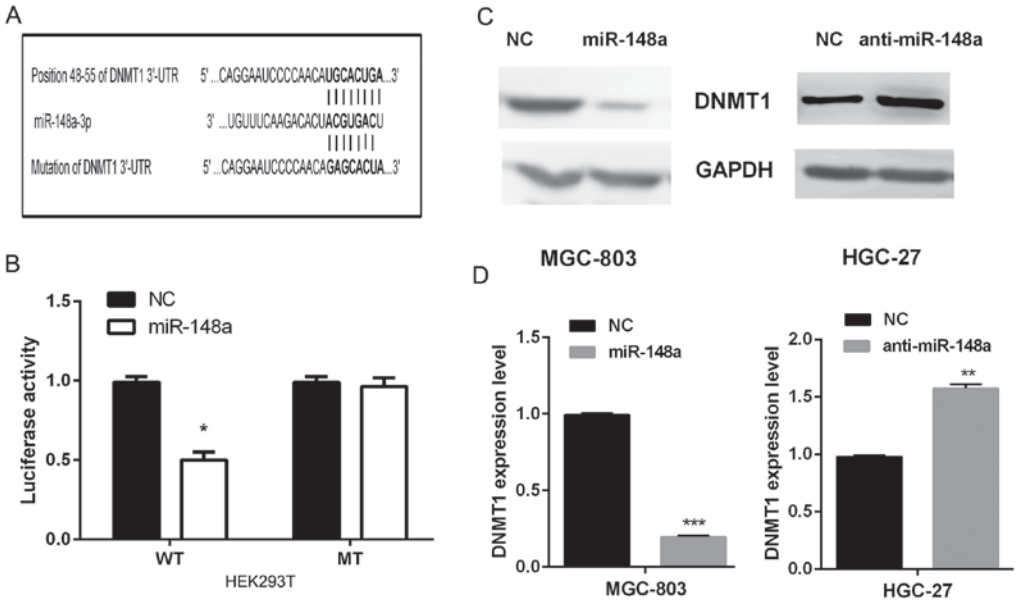

Figure 3. miR-148a downregulates DNMT1 expression by directly targeting its 3'-UTR. (A) The putative and corresponding mutant binding site of miR-148a in the DNMT1 3'-UTR. (B) Analysis of luciferase activity. HEK293T cells were co-transfected with *Renilla* plasmid and luciferase report plasmid containing either the wild-type or mutant DNMT1 3'-UTR, and either the miR-148a mimic or negative control. The firefly luciferase activity was normalized to *Renilla* luciferase activity. (C) The protein expression level and (D) the mRNA expression level of DNMT1. In MGC-803 cells transfected with miR-148a mimic, DNMT1 protein and mRNA expression levels were decreased. In HGC-27 cells containing miR-148a inhibitor, DNMT1 protein and mRNA expression was increased. GAPDH was used as internal control. *P<0.05, **P<0.01, ***P<0.001.

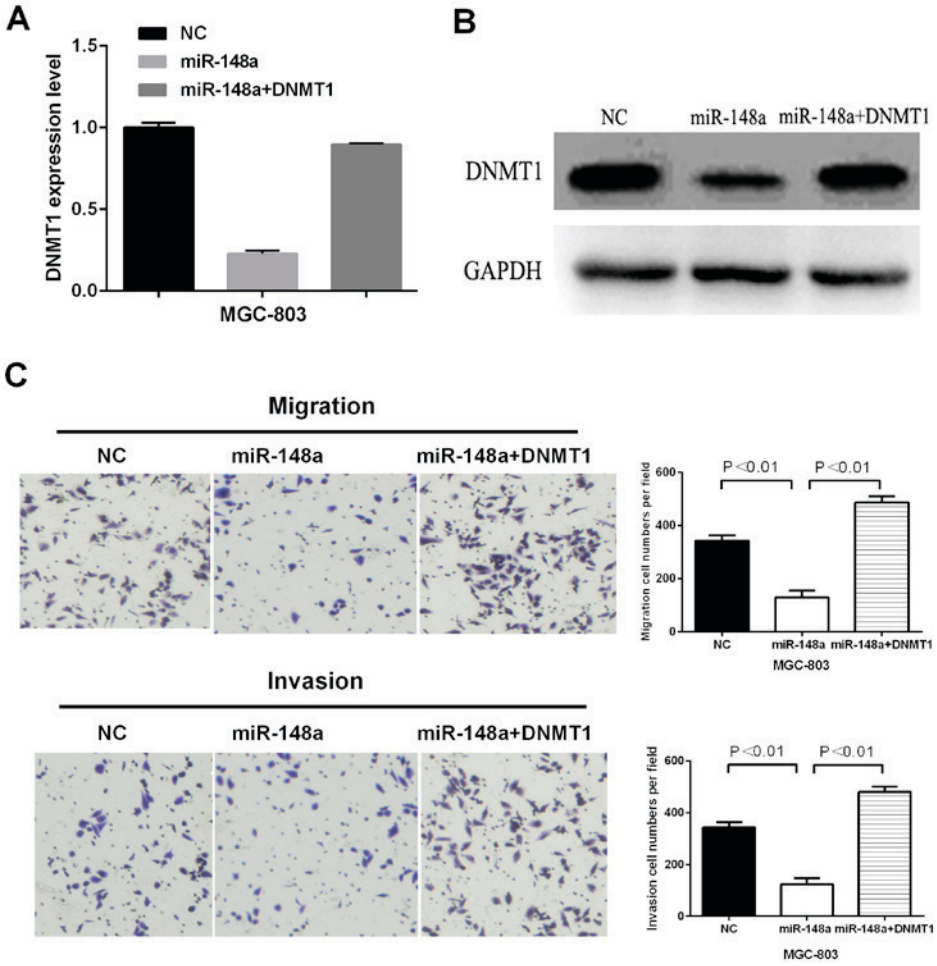

Figure 4. DNMT1 is involved in migration and invasion. (A) DNMT1 overexpression was confirmed by RT-qPCR. (B) DNMT1 overexpression was confirmed by western blot analysis. (C) Transwell assay shows the migratory and invasive abilities MGC-803 cells co-transfected with miR-148 and DNMT1 overexpression plasmid. The results showed that the cells were co-transfected with DNMT1 overexpression plasmid and miR-148 mimics.

the relative cell numbers were presented beside the images. Thus, DNMT1 may play an important role in gastric cancer cell migration and invasion.

Discussion

In the present study, we showed that miR-148a was aberrantly downregulated in GC tissues and was significantly correlated with lymph node metastasis and gastric cancer with advanced stages (III+IV) had a lower level of miR-148a than early stages (I+II). Re-expression of miR-148a was observed to suppress gastric cancer migration and invasion *in vitro*. In addition, DNMT1 was identified as a functional and direct target of miR-148a. Our results suggest that miR-148a plays important roles in suppressing tumor progression in GC by directly targeting DNMT1.

miRNAs perform as tumor suppressors or oncomiR. It is reported that miR-148a is downregulated in various types of cancer, including gastric cancer (15), and non-small cell lung cancer (20), suggesting that miR-148a may play a key role in tumorigenesis and tumor progression. In early pancreatic carcinogenesis, it is demonstrated that the silencing of miR-148a was induced by DNA hypermethylation, which was regulated by DNA methyltransferase-1 (21). In gastric cancer, it was revealed that the downregulation of miR-148a was significantly correlated with an advanced clinical stage, lymph node metastasis, and poor clinical outcome (22). In addition, the ectopic expression of miR-148a was validated by inhibiting tumor cell proliferation and migration *in vitro*, and inhibiting tumor formation *in vivo* (23).

miRNAs perform their function by downregulating the expression of their target mRNAs at the post-transcriptional level (24). Many target genes are involved in the promotion of cancer progression. Song *et al* reported that miR-148b can suppress cell growth by targeting cholecystokinin B receptor (CCK-BR) in colorectal cancer (25). Yu *et al* revealed that miR-148a functions as a tumor suppressor by targeting CCK-BR through the inactivation of STAT3 and Akt in human gastric cancer (23). Sakamoto *et al* demonstrated that microRNA-148a is downregulated in gastric cancer and targets MMP7 (22). By contrast, in this study, DNMT1 was identified as a functional target of miR-148a. The re-expression of miR-148a reduced DNMT1 expression in gastric cancer. In addition, the downregulation of miR-148a increased DNMT1 expression. The luciferase report assay showed that luciferase activity was recovered from mutated-type 3'-UTR construct. Furthermore, we revealed that DNMT1 upregulated cell migration and invasion, and the upregulation of DNMT1 attenuates miR-148a-mediated inhibition on cell migration and invasion. DNMT1 as a number of DNA methyltransferase, functions as an inhibitor of several suppressor genes in various types of cancer. Hino *et al* demonstrated that the activation of DNA methyltransferase 1 by EBV latent membrane protein 2A leads to promoter hypermethylation of *PTEN* gene in gastric carcinoma (26). Zhang *et al* suggested that the overexpression of DNMT1 induces tumor suppressor gene hypermethylation via the Akt-NFκB pathway in gastric cancer development (27). A high DNMT protein expression was identified in 83% of primary gastric cancer patients as detected by Mutze *et al* (28). DNMT1 expression was also significantly associated with tumor differentiation, and the overexpression of DNMT1 was associated with poor outcome after treatment (29). DNMT1 inhibitors have been demonstrated to be useful as sensitizers for various chemotherapeutic agents or radiation in different cell lines including gastric cancer cell lines (29). Collectively DNMT1 was identified as an important target for gastric cancer development and treatment.

In conclusion, the present findings show that miR-148a suppresses cell invasion and migration in gastric cancer by regulating DNMT1 expression. The miR-148a/DNMT1 axis can be considered a prospective novel target for gastric cancer therapy.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

HS conceived and designed the study and wrote the manuscript; XC contributed significantly to the analysis and manuscript preparation; HJ performed the data analyses; XW helped perform the analysis with constructive discussions; HY performed the histological examination of the stomach; PS sorted out experimental data; XS contributed to the conception of the study. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Written informed consent for samples was obtained from the patients. The study was approved by the Clinical Research Ethics Committee of Weihai Central Hospital.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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