miR-200b and miR-200c as prognostic factors and mediators of gastric cancer cell progression

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\textbf{Running head:} miR-200b/c are potential targets for gastric cancer therapy
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Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

H. Tang, M. Deng, X. Xie and Q. Su designed the experiments, interpreted the data, and wrote the manuscript. H. Tang, M. Deng, Y. Tang, X. Xie and J. Guo carried out experiments. H. Tang and F. Ye collected the human samples and clinical data.
**Translational Relevance**

Understanding the molecular mechanisms underlying gastric cancer progression contributes to the development of novel avenues for researching targeted therapies. In this study, we found that miR-200b/c were strongly down-regulated in gastric cancer, and their expression levels were associated with lymph node metastasis and clinical stage, as well as overall survival and relapse-free survival of gastric cancer patients. Functional studies revealed that miR-200b/c acted as new tumour suppressors in gastric cancer. Moreover, we found that miR-200b/c directly targeted DNMT3A, DNMT3B, and SP1, which resulted in marked reduction of the expression of DNA methyltransferases DNMT1, DNMT3A, and DNMT3B. This effect, in turn, led to a decrease in global DNA methylation and reexpression of p16, RASS1A1 and E-cadherin via promoter DNA hypomethylation. By understanding the function and molecular mechanism of miR-200b/c in gastric cancer, miR-200b/c may have therapeutic potential for the suppression of gastric cancer proliferation and invasion.
Abstract

Purpose: The purpose of this study was to investigate the clinicopathological significance and potential role of miR-200b and miR-200c in the development and progression of gastric cancer.

Experimental Design: We examined miR-200b and miR-200c expression in 36 paired normal and stomach tumour specimens, as well as gastric cancer cell lines, by qRT-PCR. Additionally, miR-200b and miR-200c were detected by in situ hybridisation using gastric cancer tissue microarrays (TMAs), and the association between miR-200b and miR-200c levels and clinicopathological factors and prognosis were analysed. A luciferase assay was conducted for target evaluation. The functional effects of miR-200b and miR-200c on gastric cancer cells were validated by a cell proliferation assay and cell invasion and migration assays.

Results: miR-200b and miR-200c were downregulated in the gastric cancer specimens and cell lines tested. miR-200b and miR-200c levels were significantly correlated with the clinical stage, T stage, lymph node metastasis and survival of patients. Ectopic expression of miR-200b and miR-200c impaired cell growth and invasion. In addition, when overexpressed, miR-200b and miR-200c commonly directly targeted \textit{DNMT3A}, \textit{DNMT3B} and \textit{SP1} (a transactivator of the \textit{DNMT1} gene), which resulted in marked reduction of the expression of DNA methyltransferases DNMT1, DNMT3A, and DNMT3B at the protein level. This effect, in turn, led to a decrease in global DNA methylation and reexpression of p16, RASS1A1 and E-cadherin via promoter DNA hypomethylation.
Conclusion: Our findings suggest that miR-200b and miR-200c, as valuable markers of gastric cancer prognosis, may be a promising approach to human gastric cancer treatment.
Introduction

An increasing number of studies have demonstrated that miRNAs can function as oncogenes or tumour suppressors and are often dysregulated in tumours(1, 2). In this regard, oncogenic miRNAs are frequently upregulated, whereas tumour suppressive miRNAs are frequently downregulated in tumours. The oncogenic miR-183/182/96 cluster of miRNAs is upregulated in a variety of tumours(3-5), and it regulates oxidative apoptosis and sensitises gliomal cells to chemotherapy (6). In contrast, we previously reported that miR-34a is greatly downregulated in breast cancer cells and tissues and inhibits breast cancer proliferation and invasion(7, 8). In addition, miR-216b is greatly downregulated in nasopharyngeal carcinoma and inhibits tumour growth by targeting KRAS(9). The miR-200 family consists of five members (miR-200a, miR-200b, miR-200c, miR-141 and miR-429) that are clustered and expressed as two separate polycistronic pri-miRNA transcripts with the miR-200b-200a-429 cluster at chromosomal location 1p36 and miR-200c-141 cluster at chromosomal location 12p13. miR-200 is a miRNA family with tumour suppressive functions in a wide range of cancers, including breast cancer(10), colorectal cancer (11), pancreatic cancer(12) and endometrial carcinoma(13), but by now, the role of miR-200 (miR-200a, miR-200b and miR-200c) in gastric cancer remained undefined.

DNA methylation consists of an enzymatic addition of a methyl group at the carbon 5 position of cytosine in the context of the sequence 5’-cytosine-guanosine (CpG) and is mediated by DNA methyltransferases (DNMT1, DNMT3A and DNMT3B)(14, 15). The promoter regions of approximately 50% of human genes contain regions of DNA
with a cytosine and guanine content greater than expected, and hypermethylation of these regions mediates gene transcriptional silencing(15, 16). Silencing of structurally normal tumour suppressor genes by aberrant DNA hypermethylation has been reported in haematological malignancies, including gastric cancer(17, 18). Accumulating evidence supports a role for miRNAs as both targets and effectors in aberrant mechanisms of DNA hypermethylation. We previously reported that in glioma, miR-185 is strongly downregulated and directly targets $DNMT1$, thereby leading to down-regulation of $PCDHA8$, $ANKDD1A$, $GADI$, $HIST1H3E$, $PHOX2B$, $SIX3$, and $SST$, reduction of global DNA methylation (GDM), and reexpression of the DNA-hypermethylated and silenced tumour suppressor genes $PCDHA8$, $ANKDD1A$, $GADI$, $HIST1H3E$, $PHOX2B$, $SIX3$, and $SST$(19).

In the present study, we showed that miR-200b and miR-200c were downregulated in gastric cancer cell lines and specimens. Moreover, the miR-200b and miR-200c levels were associated with clinical stage and lymph node metastasis. Decreased expression of miR-200b and miR-200c was significantly correlated with poor relapse-free survival (RFS) and overall survival (OS). Further study revealed that the ectopic expression of miR-200b and miR-200c in gastric cancer cells impaired proliferation and invasion not only through direct targeting of $DNMT3A$ and $3B$ but also by decreasing the $DNMT1$ expression indirectly via down-regulation of $SP1$, which is a known transactivating factor of the $DNMT1$ gene. These results support a previously unreported role of miRNAs in aberrant DNA methylation in gastric cancer and provide a pharmacological rationale for the use of synthetic miR-200b and
miR-200c for therapeutic DNA hypomethylation of gastric cancer.

Materials and Methods

Cell culture

The gastric epithelial cell line GES-1 was purchased from the Beijing Institute for Cancer Research (Beijing, China). The gastric cancer cell lines MGC-803, BGC-823, MKN-28, SGC-7901, HGC-27, AGS and MKN-45 were obtained from the American Type Culture Collection (ATCC, Rockville, MD). Cell lines involved in our experiments were reauthenticated in Beijing Microread Genetics Co., Ltd by STR profiles analysis every 6 months after resuscitated. These cells were maintained at 37 °C in an atmosphere of 5% CO2 in RPMI-1640 medium supplemented with 10% foetal bovine serum, penicillin and streptomycin (Gibco BRL, NY, USA). All transfections were performed using Lipofectamine 2000 (Invitrogen, Carlsbad, USA).

Clinical samples

All tissue samples used in the present study were collected from the Hunan Provincial Tumour Hospital (Changsha, Hunan, China). Written informed consent was obtained from all study participants. This study was approved by the Ethics Committee of Sun Yat-Sen University Cancer Center Health Authority and the University of South China Health Authority. The collection and use of tissues followed the procedures that are in accordance with the ethical standards as formulated in the Helsinki Declaration.
Tissue samples from 36 gastric cancer patients were used for quantitative real-time PCR (qRT-PCR) analysis. Resected cancerous tissues (Tumour) and paired matched normal gastric tissues (Normal) were immediately cut and stored in RNAlater (Ambion). The tissue microarrays (TMAs) consisted of 126 cases of gastric carcinoma and 41 cases of normal stomach mucosa used for in situ hybridisation analysis. All data, including age, sex, histological grade, tumour size, invasion depth (T stage), and lymph node metastasis were obtained from clinical and pathological records.

**Quantitative RT-PCR analysis (qRT-PCR)**

Total RNAs were extracted from cells with TRIzol reagent (Invitrogen, Carlsbad, USA). Reverse transcription and qRT-PCR reactions were performed by means of a qSYBR-green-containing PCR kit (Qiagen, Germantown, USA). Fold change was determined as $2^{-\Delta \Delta Ct}$. The Ct is the fractional cycle number at which the fluorescence of each sample passes the fixed threshold. The dCt was calculated by subtracting the Ct of snRNA U6 from the Ct of the miRNA of interest. The ddCt was calculated by subtracting the dCt of the reference sample (paired nontumourous tissue for surgical samples, and GES-1 cells for seven gastric cancer cell lines) from the dCt of each sample. The primers for qRT-PCR detection of *DNMT1*, *SP1*, *P16*, *E-cadherin* or *RASSF1A* mRNA were synthesised by Invitrogen. All real-time PCR was performed with the Bio-Rad IQTM5 Multicolor Real-Time PCR Detection System (USA).

**In situ hybridisation (ISH) analysis**

In situ hybridisation procedures were carried out as previously described(20).
miR-200b and miR-200c miRCURY™ LNA custom detection probes (Exiqon, Vedbaek, Denmark) were used for ISH. The 5′-3′ sequences (enhanced with LNA) were TCATCATTACCAGGCAGTATTA and TCCATCATTACCGGCAGTATTA with a DIG label at both the 5′ and 3′ ends. Hybridisation, washing, and scanning was carried out according to the manufacturer’s instructions. The intensities of miR-200b and miR-200b staining was scored by 0–4, according to the standards of 0-1 (no staining), 1-2 (weak staining), 2-3 (medium staining) and 3-4 (strong staining). The percentages of miR-200b and miR-200b cells in three representative high-power fields of individual samples were analyzed. Those expression scores equaled to scores of the intensities × the percentages of positive cells, and the maximum was 4 and the minimum was 0. Individual samples were evaluated by at least two pathologists in a blinded manner, and those expression scores of greater or equal to 2 was defined as high expression, less than 2 was low expression.

**Cell proliferation assay**

Cells transfected with scramble or miR-200b or 200c mimics (Ambion, Austin, USA) were plated in 12-well plates at the desired cell concentrations. Cell counts were estimated by trypsinising the cells and performing analysis using a Coulter Counter (Beckman Coulter, Fullerton, USA) at the indicated time points in triplicate.

**Cell invasion and migration assays**

Cell migration was examined by wound-healing assays. An artificial “wound” was created on a confluent cell monolayer, scratching assay was treated by 10 µg/ml mitomycin C for 2 hours, and photographs were taken using an inverted microscope.
(Olympus, Tokyo, Japan) after 24 hours.

The cell invasion assay was conducted as described previously(20). Briefly, cells were seeded onto the basement membrane matrix present in the insert of a 24-well culture plate (EC matrix, Chemicon, Temecula, CA). After an additional 48 hours, the non-invading cells and EC matrix were gently removed with a cotton swab. Invasive cells located on the lower side of the chamber were stained with crystal violet, counted and imaged.

**SP1 silencing**

The sense sequence of siRNA oligonucleotides targeting the SP1 transcripts was as follows: si-SP1: 5'-CACAAACACTGCCCACCG-3' (Invitrogen). Scrambled siRNA was used as a negative control. Cells were plated in culture dishes for 24 h and transfected with siRNA using Lipofectamine 2000. After 48 h, the cells were harvested for use in other assays or for RNA and protein extraction.

**Vector construct**

*SP1*-expressing vector was constructed. Full-length *SP1* cDNA was purchased from GeneCopeia TM (USA) and was subcloned into the eukaryotic expression vector pcDNA3.1(+). The vector pcDNA3.1(+) was used as a negative control.

**Luciferase assays**

The 3’ untranslated regions (UTRs) of the *SP1, DNMT1, DNMT3A* and *3B* genes were amplified by PCR from genomic DNA and inserted into the pGL3 control vector (Promega, Madison, WI) using the XBA1 site immediately downstream from the stop codon of luciferase. The primer sets used were: *SP1* FW
5’-CCTTCAGGGATTCTCAACTG-3’ and SP1 RV
5’-GTCCAAAAGGCATCAGGGTA-3’; DNMT1 FW:
5’-GGAGGAGGAAGCTGCTAAGG-3’ and DNMT1 RV:
5’-TTGGTTTATAGGAGAGATTTATTTG-3’; DNMT3A FW:
5’-GCTCTAGACGAAAAGGGTTGGACATCAT-3’ and DNMT3A RV:
5’-GCTCTAGAGCGAGGGAGTCTCCTTTTA-3’; and DNMT3B FW:
5’-GCTCTAGATAGGTAGCAACGTGGCTTTT-3’ and DNMT3B RV:
5’-GCTCTAGAGGCCCAACTTTGTGCAAC-3’. We also generated several inserts with deletions of 4 bp from the site of perfect complementarity of the DNMT3A, DNMT3B, and SP1 gene using the QIAGEN XL-site directed Mutagenesis Kit (QIAGEN, Valencia, CA). MGC-803 cells were cotransfected using nucleoporation (Amaxa Biosystems) according to the manufacturer’s protocol (solution V, program T-016) using 5 μg of the firefly luciferase report vector and 0.5 μg of the control vector containing Renilla luciferase, pRL-TK (Promega). For each nucleoporation, 50 nM of the miR-200b and miR-200c or a scrambled oligonucleotide was used. Firefly and Renilla luciferase activities were measured consecutively using the dual luciferase assay (Promega) 48 hours after transfection.

Western blot

Protein concentration in the lysates was measured with the Protein BCA Assay Kit (Bio-Rad, USA), and 20 μg of protein mixed with 2×SDS loading buffer was loaded per lane. The proteins in the lysates were separated by 12% SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Millipore,
USA). Next, the membranes were incubated for 12 h at 4°C with an antiserum containing antibodies against DNMT1, DNMT3A, DNMT3B, SP1, P16, E-cadherin, and RASSF1A purchased from Santa Cruz Biotechnology (Santa Cruz, CA). A peroxidase-conjugated secondary antibody and ECL western blotting detection reagents were used to visualise the target proteins (ECL New England Biolabs, USA), which were quantified with a Bio Image Intelligent Quantifier 1-D (Version 2.2.1, Nihon-BioImage Ltd., Japan). An anti-β-actin antibody (Boster, Wuhan, China) was used as a protein loading control.

**GDM analysis**

GDM analysis procedures were carried out as previously described(19). Genomic DNA was isolated from MGC-803 and AGS gastric cancer cells using a genomic DNA extraction kit, according to the manufacturer’s instructions (TaKaRa). The contents of global DNA methylation in individual samples were determined by high performance liquid chromatography/diode array detectors (HPLC-DAD).

**Statistical Analysis**

Comparisons between groups were analysed by the $t$ test and $\chi^2$ test. Overall survival curves and relapse-free curves were plotted according to the Kaplan-Meier method, with the log-rank test applied for comparison. Survival was measured from the day of the surgery. Variables with a value of $P<0.05$ by univariate analysis were used in subsequent multivariate analysis based on the Cox proportional hazards model. All differences were statistically significant at the level of $P<0.05$. Statistical analyses were performed using the SPSS13.0 software.
Results

miR-200b and miR-200c are downregulated in gastric cancer

Using a qRT-PCR method, miR-200a, miR-200b and miR-200c were detected in 36 pairs of gastric cancer tissues and their matched adjacent tissues, as well as in gastric cell lines. Among 36 patients with gastric cancer, approximately 89% (p=0.000, 32 of 36 patients) and 83% (p=0.000, 30 of 36 patients) of tumours revealed notable reduction in the miR-200b and miR-200c levels, respectively (Figure 1A). Similarly, both miRs were reduced in all gastric cancer cell lines compared to the gastric epithelial cell line GES-1 (Figure 1B). However, the miR-200a level was only slightly reduced in approximately 42% (p > 0.05, 15 of 36 patients) of tumours (Figure S1A). Similarly, miR-200a was slightly reduced in partial gastric cancer cell lines compared to the gastric epithelial cell line GES-1 (Figure S1B). To verify the biological role of miR-200b and miR-200c in human gastric carcinogenesis further, we performed in situ hybridisation to evaluate miR-200b and miR-200c levels in 126 gastric tumours and 41 normal stomach tissues with TMA and found that miR-200b and 200c were strongly downregulated in stomach tumours compared with normal tissues. Correlation analysis showed that the level of miR-200b is positively related to the expression of miR-200c in normal stomach mucosa and primary gastric cancer tissue (Figure 1C, D). These data indicate overt downregulation of miR-200b and miR-200c in gastric cancer.

Decreased miR-200b and 200c levels are correlated with advanced clinical stage, lymph node metastasis and poor clinical outcomes
Next, we determined the potential clinicopathological implications of altered miR-200b and miR-200c expression. Clinical samples were divided into low expression and high expression groups based on miR-200b and miR-200c expression scores greater or less than 2. Out of 41 total normal stomach samples, 32 (78%) and 34 (83%) had high expression of miR-200b and 200c, respectively (Table S1). In contrast, 63% (80 of 126) and 60% (75 of 126) of gastric carcinoma specimens had low to negative expression of miR-200b and 200c, respectively (Table S1). Thus, miR-200b and 200c are underexpressed in gastric cancers compared with normal stomach mucosa. This is consistent with the above data. In the 126 individuals with gastric carcinoma, the miR-200b level inversely correlated with invasion depth, clinical stage and lymph node metastasis (P=0.043, P=0.000 and P=0.032, respectively) (Table 1). A similar result was found for miR-200c (P=0.040, P=0.001 and P=0.022, respectively) (Table 1). However, neither miR-200b nor 200c levels in gastric cancer patients correlated with age, gender, tumour size or cell differentiation. Our results suggest that miR-200b and 200c could play critical roles in carcinogenesis and progression of gastric cancer.

To analyse the significance of miR-200b and 200c further in terms of clinical prognosis, a Kaplan-Meier survival analysis was performed using patient overall survival and disease free survival (Figure 2). The results demonstrated that patients with low miR-200b expression had shorter mean months of OS and DFS than did patients with high miR-200b expression (P=0.000 for OS, P =0.002 for DFS, Figure 2A). We also observed that miR-200c-low patients had shorter mean months of OS
and DFS than did miR-200c-high patients (P =0.000 for OS, P =0.001, Figure 2B). In addition, low expression of both miR-200b and miR-200c was significantly associated with a shorter OS and DFS (P =0.000 for OS, P =0.003 for OS) (Figure 2C). Our results indicated that expression levels of miR-200b and miR-200c were significantly associated with patient OS and DFS.

We used Cox proportional-hazards regression to evaluate the association between miR-200b and miR-200c expression and prognosis further (Table S2, S3). In univariate analysis, the levels of miR-200b and 200c were significantly associated with prognosis. The final multivariate model revealed that reduced miR-200b and 200c levels in tumours were independent predictors of shorter survival. Lymph-node metastasis (P<0.05) was an independent significant prognostic factor as well. A similar trend was found for TNM stage (P<0.05).

Overexpression of miR-200b or miR-200c inhibits cell proliferation and invasion

To assess the biological effects of overexpressing miR-200b and miR-200c in gastric cancer cells ectopic miR-200b and miR-200c mimics were transfected into gastric cancer cells. qRT-PCR analysis showed that the transfection and knockdown were successful (Figure 3A). We determined that overexpression of either miR-200b or 200c in MGC-803 and AGS cells markedly attenuated cell proliferation compared with scramble (Figure 3A). Expression of miR-200b or 200c significantly inhibited the MGC-803 cells’ capability for migration (Figure 3B). Moreover, ectopic expression of either miR-200b or 200c in MGC-803 and AGS cells markedly attenuated cell invasion compared with control cells (Figure 3C).

miR-200b and miR-200c directly target DNMT3A and 3B and indirectly target
To understand how miR-200b and miR-200c suppress gastric cancer growth and invasion, we used two algorithms (Targetscan and Miranda) to help identify miR-200b and miR-200c targets in human gastric cancers. Among these candidate target genes, *DNMT3A* and *DNMT3B* were predicted by both algorithms (Figure 4A). We confirmed this finding in gastric cancer cells by performing luciferase reporter assays. *DNMT3A* and *DNMT3B* complementary sites were cloned downstream of the firefly luciferase gene and cotransfected with miR-200b mimics, miR-200c mimics or scrambled oligonucleotide. Luciferase activity was measured after 48 hours of transfection. MGC-803 cells cotransfected with either *DNMT3A* or *DNMT3B* reporter constructs and miR-200b or miR-200c exhibited approximately 40% reduction of the luciferase activity with respect to those cotransfected with the scrambled oligonucleotide (Figure 4B). Additionally, mutation of the putative miR-200b and miR-200c sites in the 3’-UTR of *DNMT3A* and *DNMT3B* abrogated luciferase responsiveness to miR-200b and miR-200c (Figure 4B).

Transfection of the miR-200b or miR-200c mimics into MGC-803 and AGS cells resulted in a marked reduction of the protein levels of DNMT3A and DNMT3B (Figure 4C). In contrast to DNMT3A and DNMT3B, miR-200b and miR-200c are not predicted to hybridise with the DNMT1 3’UTR region. Nevertheless, transfection of the miR-200b and miR-200c mimics into MGC-803 and AGS cells resulted in a marked reduction of DNMT1 protein levels (Figure 4C). To further prove that DNMT1 is not a direct target of miR-200b or miR-200c, we cloned the DNMT1
3’UTR into a luciferase reporter vector. Next, as was performed for DNMT3A and DNMT3B, we cotransfected the DNMT1 3’UTR luciferase reporter vector with miR-200b mimics, miR-200c mimics or a scrambled oligonucleotide into MGC-803 cell lines. Consistent with the notion that DNMT1 is not a direct target of miR-200b or miR-200c, no difference in the luciferase activity was noted between cells treated with scrambled or miR-200b or miR-200c mimics (Figure 4B). Therefore, we reasoned that miR-200b and miR-200c down-regulated DNMT1 indirectly. SP1, a zinc finger transcription factor, binds directly to the promoter of DNMT1 and positively regulates the transcription of this gene in mice and humans(15, 21, 22). Here, we further confirmed the SP1-DNMT1 link by showing that SP1-siRNA treatment of MGC-803 and AGS cells and overexpression SP1 treatment of MGC-803 results in down-regulation of SP1 and, in turn, DNMT1 mRNA and protein levels as measured by quantitative RT-PCR and western blot (Figure 4D). Interestingly, the 3’UTR of SP1 contains 1 predicted binding site according to Targetscan 6.2 (Figure 4A). Therefore, we hypothesised that miR-200b and/or miR-200c down-regulate SP1 expression, and, in turn, inhibit DNMT1 transactivation resulting in a decrease of DNMT1 at the protein level. To validate this hypothesis, we cloned the 3’UTR region of SP1 into a luciferase reporter vector. The luciferase assay revealed that miR-200b and miR-200c directly bound to the SP1 3’UTR and remarkably reduced luciferase activity (Figure 4B). Additionally, mutation of the putative miR-200b or miR-200c sites in the 3’UTR abrogated the luciferase responsiveness to miR-200b and miR-200c. These results show that DNMT3A and DNMT3B are bona fide targets of
both miR-200b and miR-200c and support a mechanistic link between miR-200b/c-mediated down-regulation of SP1 and the subsequent decrease in DNMT1 expression.

**Overexpression of miR-200b and miR-200c reduces global DNA methylation and restores the expression of hypermethylated p16, E-cadherin and RASSF1A.**

At last, we investigated whether the enforced expression of miR-200b and miR-200c could functionally result in DNA hypomethylation. Global DNA methylation (GDM) was measured using an HPLC-DAD method as previously described(19) in MGC-803 and AGS cell lines after 48 hours of transfection. miR-200b or miR-200c mimics were used, and scrambled oligonucleotides served as negative controls. We observed a significant reduction in GDM for the MGC-803 and AGS cells treated with miR-200b and miR-200c mimics compared with the scramble controls (Figure 5A and 5B). The reduction in GDM in MGC-803 cells by miR-200b or miR-200c was comparable with that achieved with decitabine treatment at the same time point (Figure 5A). Several genes have been found to be methylated and silenced in gastric cancer and reexpressed after treatment with hypomethylating agents, including p16(23, 24), E-cadherin(25, 26) and RASSF1A(27, 28). To assess whether overexpression of miR-200b or miR-200c could also lead to reexpression of hypermethylated and silenced genes in gastric cancer, we measured the mRNA and protein levels of p16, E-cadherin and RASSF1A by qRT-PCR and western blot in the MGC-803 and AGS cell lines after transfection with miR-200b mimics, miR-200c mimics or a scrambled oligonucleotide. We showed that after transfection of
miR-200b and miR-200c mimics, p16, E-cadherin and RASSF1A mRNA and protein levels increased compared to transfection with scrambled oligonucleotides (Figure 5C, D).

Discussion:

It is known that the miR-200 family plays a significant role in growth, invasion and metastasis(29, 30). Several studies showed that miR-200b or/and miR-200c expression is dysregulated in the cells, tissues and plasma of certain human cancers(31-34). Moreover, miR-200b and miR-200c were expressed differently in epithelial ovarian cancer relapsers compared with non-relapsers (33). However, there are no studies on miR-200b or miR-200c in gastric cancer. In the current study, we showed that miR-200b and miR-200c levels in gastric cancer tissues were significantly lower than those in noncancerous tissues by qRT-PCR and ISH. Moreover, the miR-200b and miR-200c levels were associated with clinical stage and lymph node metastasis. Kaplan-Meier survival analyses revealed that gastric cancer patients whose primary tumours displayed low expression of miR-200b and miR-200c had shorter OS and RFS. In addition, multivariate analysis showed that reduced miR-200b or miR-200c levels in tumours were both strong independent predictors of shorter OS and RFS. Based on these data, miR-200b and miR-200c may be useful as prognostic markers to predict survival and relapse in gastric cancer patients.

In the current study, we characterised the role of miR-200b and miR-200c in the regulation of DNA methylation in gastric cancer. Our data showed that
miR-200b and miR-200c target DNMTs, thereby resulting in global DNA hypomethylation and reexpression of hypermethylated, silenced genes in gastric cancer. We also showed that miR-200b and miR-200c down-regulate DNMT1 by targeting SP1, which is a zinc finger transcription factor that regulates a large number of genes involved in the cell cycle, proliferation and invasion(35, 36). It has been shown that SP1 binds to the promoter of DNMT1 and transactivates the DNMT1 gene in mice(22). In this study, we demonstrated that miR-200b and miR-200c down-regulate SP1, thereby interfering with the SP1-dependent expression of DNMT1. The discovery that miR-200b and miR-200c down-regulate not only DNMT3A and 3B but also DNMT1 has important functional ramifications because selective genetic disruption of DNMT3B in colon cancer cell lines has been reported to reduce GDM by only 3%, while genetic disruption of both DNMT1 and DNMT3B completely abolished DNA methyltransferase activity and reduced GDM by 95%(15, 37). Consistent with these results, our study showed that miR-200b and miR-200c can efficiently modulate DNA hypomethylation by targeting both DNMT3A and DNMT3B. To the best of our knowledge, this report is the first indicating that overexpression of miR-200b or miR-200c both result in global DNA hypomethylation and gene reexpression of the hypermethylated and silenced p16, E-cadherin and RASSF1A genes in gastric cancer cell lines.

In this work, we also provide insights about the biological effects of overexpression of miR-200b or miR-200c in gastric cancer. Our in vitro data further demonstrated that miR-200b and miR-200c function as tumour suppressors in gastric cancer.
Several studies support our results. For example, miR-200b and miR-200c are downregulated in hepatocellular carcinoma and can attenuate cellular invasion\cite{38}. miR-200b and miR-200c regulate epithelial-to-mesenchymal transition in bladder cancer cells and reverse resistance to epidermal growth factor receptor therapy by targeting ERF-I\cite{31}. Therefore, restoring miR-200b or miR-200c expression in gastric cancer blasts induces partial proliferation and invasion. We also believe that these findings have relevant therapeutic implications. Synthetic miR-200b and miR-200c oligonucleotides combining with DNMT1 inhibitors decitabine may result in a synergistic hypomethylating effect, more genes re-expression, and improving the response for chemotherapy in gastric cancer patients.

In conclusion, miR-200b and miR-200c are valuable markers of gastric cancer prognosis and play an important role in the development and progression of human gastric cancer.

Acknowledgements

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Table 1. Analysis of the correlation between expression of miR-200b and miR-200c in primary gastric cancer and its clinicopathological parameters

<table>
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*aWell differentiated adenocarcinoma (Well), Moderately differentiated adenocarcinoma (Moderate), Poorly differentiated adenocarcinoma (Poor), Other histological type (Other).*
Figure legends

Figure 1. miR-200b and miR-200c expression levels are frequently downregulated in human gastric cancer.

(A) The expression levels of miR-200b (upper panel) and 200c (down panel) were detected in 36 gastric cancer patients by qRT-PCR. Data are shown as log2 of fold change of gastric cancer relative to adjacent normal tissues. (B) Relative expression of miR-200b (left panel) and 200c (right panel) in 7 cell lines derived from gastric cancer and one gastric epithelial cell line (GES-1) was determined by qRT-PCR. Data are presented as means ± SD from at least three separate experiments. (C) miR-200b and miR-200c expression was analysed in normal stomach mucosa (normal) and gastric cancer (tumour) samples with tissue microarray by in situ hybridisation. Expression scores are shown as box plots with the horizontal lines representing the median, the bottom and top of the boxes representing the 25th and 75th percentiles, respectively, and the vertical bars representing the range of the data (left panel). Expression scores indicate that miR-200b level is positively related to the expression of miR-200c in normal stomach mucosa (41 cases) and gastric cancer (126 cases) tissues (right panel). (D) Representative images of miR-200b and miR-200c expression by in situ hybridisation.

Figure 2. Low levels of miR-200b and miR-200c correlate with shorter survival.

OS and DFS curves for all studied patients with high or low miR-200b expression (A), high or low miR-200c expression (B), and the 4 possible combinations(C).
**Figure 3** Ectopic expression of miR-200b and miR-200c in gastric cancer cells inhibits growth, migration and invasion.

(A) MGC-803 and AGS cells transfected with miR-200b, miR-200c or scramble mimics (left panel). MGC-803 (middle panel) and AGS (right panel) cells transfected with miR-200b, miR-200c or scramble mimics were seeded in 12-well plates at the desired cell concentrations and maintained in medium containing 10% fetal bovine serum. The cells were counted at the indicated time points in triplicate and their growth rates were recorded. (B) Scratch wound assays were performed on MGC-803 cells transfected with miR-200b, miR-200c or scramble mimics. The results from three separate assays were averaged together and graphed. *P<0.05 (left panel). Representative images of the assays are shown. Original magnification:×200 (right panel). (C) The invasive properties of the MGC-803 and AGS cells were analysed by an invasion assay using a Matrigel-coated chamber. Migrated cells were plotted as the average number of cells per field of view from three different experiments, as described in the materials and methods. **P<0.01 (left panel). Representative images of the assays are shown. Original magnification:×200 (right panel).

**Figure 4.** miR-200b and miR-200c directly target DNMT3A and 3B and indirectly target DNMT1

(A) The 3’UTRs of *DNMT3A*, *DNMT3B*, and *SP1* have putative binding sites for miR-200b and miR-200c.

B: Luciferase assay on MGC-803 cells, which were cotransfected with miR-200b, miR-200c mimics, or scramble and a luciferase reporter containing the following:
DNMT1 3’-UTR (DNMT1-wt), DNMT3A 3’-UTR (DNMT3A-wt), DNMT3B 3’-UTR (DNMT3B-wt) or SPI 3’-UTR (SPI-wt) or mutant constructs in which the first four nucleotides of the miR-200b and miR-200c binding site were mutated: DNMT3A-mut, DNMT3B-mut or SPI-mut. An empty luciferase reporter construct was used as a negative control. *p <0.05 vs. scramble.

C: The effect of miR-200b or miR-200c on the protein expression of DNMT1, DNMT3A, DNMT3B and SPI by western blot. β-actin was used as a loading control (upper panel). The band intensities are quantified and normalized to β-actin intensites. Data represent the mean ± S.D from three independent experiments. *p<0.05, **p<0.01 (down panel).

D: The effect of the SPI siRNA and overexpression SPI on the mRNA expression of DNMT1 and SPI by qRT-PCR in MGC-803 or AGS cells. β-actin was used as a control. *p <0.05, **p <0.01 vs. scramble (upper panel). The effect of the SPI siRNA and overexpression SPI on the protein expression of DNMT1 and SPI by western blot in MGC-803 or AGS cells. β-actin was used as a loading control (middle panel). The band intensities are quantified and normalized to β-actin intensites. Data represent the mean ± S.D from three independent experiments. *p<0.05, **p<0.01 (down panel).

Figure 5. Overexpression of miR-200b and miR-200c reduced global DNA methylation and restored the expression of hypermethylated p16, E-cadherin and RASSF1A.

(A) MGC-803 and (B) AGS cell lines were transfected with miR-200b mimics,
miR-200c mimics or scrambled oligonucleotides. DNA was obtained from both cell lines after 48 hours, and GDM was measured by HPLC-DAD. The results from treatment with 2.5 μM decitabine, a hypomethylating agent, or phosphate-buffered saline (control) are also shown for the MGC-803 cell line as positive controls. Data represent the mean±S.D from three independent experiments. vs control **p<0.01, vs scramble #p<0.05.

(C) MGC-803 (left panel) and AGS (right panel) cell lines were transfected with miR-200b mimics, miR-200c mimics or a scrambled oligonucleotide. The mRNA expression of p16, E-cadherin and RASSF1A was detected by qRT-PCR. Data represent the mean±S.D from three independent experiments. *p<0.05, **p<0.01.

(D) The effect of the miR-200b or miR-200c mimics on the protein expression of p16, E-cadherin and RASSF1A by western blot in MGC-803 and AGS cells. β-actin was used as a loading control.
Figure 2

A

Overall survival probability

\[ P = 0.000 \]

200b high

200b low

months

B

Overall survival probability

\[ P = 0.000 \]

200c high

200c low

months

Disease free survival probability

\[ P = 0.002 \]

200b high

200b low

months

B

Overall survival probability

\[ P = 0.001 \]

200c high

200c low

months

Disease free survival probability

\[ P = 0.000 \]

200b high/200c low

200b high/200c high

months

\[ P = 0.003 \]

200b low/200c low

200b low/200c high

200b high/200c low

200b high/200c high
Figure 3

A

mRNA/U6 expression

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细胞数量（×10^4）

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B

创口愈合率（%）

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0 h

48 h

C

侵袭细胞数

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MGC-803

AGS
**Figure 5**

A

![Bar graph showing GDM ratios for different treatments.](image1)

B

![Bar graph showing GDM ratios for different miRNAs.](image2)

C

![Bar graph showing mRNA relative expression for p16, E-cadherin, and RASSF1A.](image3)

D

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<td>scramble, 200b, 200c</td>
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- **MGC-803**
  - scramble
  - 200b
  - 200c

- **AGS**
  - scramble
  - 200b
  - 200c

- **Images** of Western blot for p16, E-cadherin, RASSF1A, and β-actin for MGC-803 and AGS cell lines.
miR-200b and miR-200c as prognostic factors and mediators of gastric cancer cell progression

Hailin Tang, Min Deng, Yunyun Tang, et al.

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