miR-200b and miR-200c as Prognostic Factors and Mediators of Gastric Cancer Cell Progression S

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Abstract

Purpose: The purpose of this study was to investigate the clinicopathologic 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 tumor specimens, as well as gastric cancer cell lines, by quantitative real-time PCR. In addition, miR-200b and miR-200c were detected by ISH using gastric cancer tissue microarrays, and the association between miR-200b and miR-200c levels and clinicopathologic factors and prognosis were analyzed. 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 *DNMT3A*, *DNMT3B*, and *SP1* (a transactivator of the *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. *Clin Cancer Res;* 19(20); 5602–12. ©2013 AACR.

Introduction

An increasing number of studies have showed that micro-RNAs (miRNA) can function as oncogenes or tumor suppressors and are often dysregulated in tumors (1, 2). In this regard, oncogenic miRNAs are frequently upregulated, whereas tumor suppressive miRNAs are frequently downregulated in tumors. The oncogenic miR-183/182/96 cluster of miRNAs is upregulated in a variety of tumors (3–5),

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and it regulates oxidative apoptosis and sensitizes 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 tumor growth by targeting KRAS (9). The miR-200 family consists of 5 members (miR-200a, miR-200b, miR-200c, miR-141, and miR-429) that are clustered and expressed as 2 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 tumor 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*; refs. 14 and 15). The promoter regions of approximately 50% of human genes contain regions of

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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 downregulated 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 tumor 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.

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 tumor suppressor genes by aberrant DNA hypermethylation has been reported in hematologic 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 downregulation of PCDHA8, ANKDD1A, GAD1, HIST1H3E, PHOX2B, SIX3, and SST, reduction of global DNA methylation (GDM), and reexpression of the DNA-hypermethylated and silenced tumor suppressor genes PCDHA8, ANKDD1A, GAD1, HIST1H3E, PHOX2B, SIX3, and SST (19).

In this 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 downregulation 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 pharmacologic 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 (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% FBS, penicillin, and streptomycin (Gibco BRL). All transfections were conducted using Lipofectamine 2000 (Invitrogen).

Clinical samples

All tissue samples used in this 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 (tumor) and paired matched normal gastric tissues (normal) were immediately cut and stored in RNAlater (Ambion). The tissue microarrays (TMA) consisted of 126 cases of gastric carcinoma and 41 cases of normal stomach mucosa used for ISH analysis. All data, including age, sex, histologic grade, tumor size, invasion depth (T stage), and lymph node metastasis were obtained from clinical and pathologic records.

Quantitative real-time PCR analysis

Total RNAs were extracted from cells with TRIzol reagent (Invitrogen). Reverse transcription and qRT-PCR reactions were conducted by means of a qSYBR-greencontaining PCR kit (Qiagen). Fold change was determined as 2^{-ddCt} . 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 nontumorous tissue for surgical samples, and GES-1 cells for 7 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 synthesized by Invitrogen. All real-time PCR was conducted with the Bio-Rad IQTM5 Multicolor Real-Time PCR Detection System.

ISH analysis

ISH procedures were carried out as previously described (20). miR-200b and miR-200c miRCURY LNA custom detection probes (Exiqon) were used for ISH. The 5'-3'

sequences (enhanced with LNA) were TCATCATTAC-CAGGCAGTATTA and TCCATCATTACCCGGCAGTATTA with a DIG label at both the 5' and 3' ends. Hybridization, 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 3 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 2 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) were plated in 12-well plates at the desired cell concentrations. Cell counts were estimated by trypsinizing the cells and conducting analysis using a Coulter Counter (Beckman Coulter) 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 $10 \ \mu g/mL$ mitomycin C for 2 hours, and photographs were taken using an inverted microscope (Olympus) 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). After an additional 48 hours, the noninvading 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'-CACAAA-CACTGCCCACCG-3' (Invitrogen). Scrambled siRNA was used as a negative control. Cells were plated in culture dishes for 24 hours and transfected with siRNA using Lipofectamine 2000. After 48 hours, 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 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 (UTR) of the SP1, DNMT1, DNMT3A, and 3B genes were amplified by PCR from geno-

mic DNA and inserted into the pGL3 control vector (Promega) using the XBA1 site immediately downstream from the stop codon of luciferase. The primer sets used were: SP1 FW 5'-CCTTCAGGGATTTCCAACTG-3' and SP1 RV 5'-GTCCAAAAGGCATCAGGGTA-3'; DNMT1 FW: 5'-GGAG-GAGGAAGCTGCTAAGG-3' and DNMT1 RV: 5'-TTGGTTTA-TAGGAGAGATTTATTTG-3'; DNMT3A FW: 5'-GCTCTAGA-CGAAAAGGGTTGGACATCAT-3' and DNMT3A RV: 5'-GC-TCTAGAGCCGAGGGAGTCTCCTTTTA-3'; and DNMT3B FW: 5'-GCTCTAGATAGGTAGCAACGTGGCTTTT-3' and DNMT3B RV: 5'-GCTCTAGAGCCCCACAAAACTTGTC-AAC-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). 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 nmol/L 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), and 20 μ g of protein mixed with 2× SDS loading buffer was loaded per lane. The proteins in the lysates were separated by 12% SDS-PAGE and transferred to polyvinylidene difluoride membranes (Millipore). Next, the membranes were incubated for 12 hours at 4°C with an antiserum containing antibodies against DNMT1, DNMT3A, DNMT3B, SP1, P16, E-cadherin, and RASSF1A purchased from Santa Cruz Biotechnology. A peroxidase-conjugated secondary antibody and ECL western blotting detection reagents were used to visualise the target proteins (ECL New England Biolabs), which were quantified with a Bio Image Intelligent Quantifier 1-D (Version 2.2.1, Nihon-BioImage Ltd.). An anti- β -actin antibody (Boster) 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 GDM in individual samples were determined by high performance liquid chromatography/ diode array detectors (HPLC-DAD).

Statistical analysis

Comparisons between groups were analyzed by the *t* test and χ^2 test. OS 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 conducted 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 tumors revealed notable reduction in the miR-200b and miR-200c levels, respectively (Fig. 1A). Similarly, both miRs were reduced in all gastric cancer cell lines compared to the gastric epithelial cell line GES-1 (Fig. 1B). However, the miR-200a level was only slightly reduced in approximately 42% (P > 0.05, 15 of 36 patients) of tumors (Supplementary Fig. S1A). Similarly, miR-200a was slightly reduced in partial gastric cancer cell lines compared to the gastric epithelial cell line GES-1 (Supplementary Fig. S1B). To verify the biological role of miR-200b and miR-200c in human gastric carcinogenesis further, we conducted ISH to evaluate miR-200b and miR-200c levels in 126 gastric tumors and 41 normal stomach tissues with TMA and found that miR-200b and miR-200c were strongly downregulated in stomach tumors 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 (Fig. 1C and D). These data indicate overt downregulation of miR-200b and miR-200c in gastric cancer.

Decreased miR-200b and miR-200c levels are correlated with advanced clinical stage, lymph node metastasis, and poor clinical outcomes

Next, we determined the potential clinicopathologic 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. Of 41 total normal stomach samples, 32 (78%) and 34 (83%) had high expression of miR-200b and miR-200c, respectively (Supplementary 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 miR-200c, respectively (Supplementary Table S1). Thus, miR-200b and miR-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, 0.000 and 0.032, respectively; Table 1). A similar result was found for miR-200c (*P* = 0.040, 0.001, and 0.022, respectively; Table 1). However, neither miR-200b nor miR-200c levels in gastric cancer patients correlated with age, gender, tumor



Figure 1, miR-200b and miR-200c expression levels are frequently downregulated in human gastric cancer. A, the expression levels of miR-200b (top) and 200c (bottom) were detected in 36 gastric cancer patients by gRT-PCR. Data are shown as log2 of fold change of gastric cancer relative to adjacent normal tissues B relative expression of miB-200b (left) and 200c (right) in 7 cell lines derived from gastric cancer and 1 gastric epithelial cell line (GES-1) was determined by qRT-PCR. Data are presented as means \pm SD from at least 3 separate experiments. C, miR-200b and miR-200c expression was analyzed in normal stomach mucosa (normal) and gastric cancer (tumor) samples with TMA by ISH. 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). 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). D, representative images of miR-200b and miR-200c expression by ISH.

| Viable | Cases | miR-200b | | | miR-200c | | |
|---------------------------------|-------|----------|------|---------|----------|------|---------|
| | | Low | High | P value | Low | High | P value |
| Age (y) | | | | | | | |
| <60 | 73 | 45 | 28 | 0.376 | 42 | 31 | 0.364 |
| ≥60 | 53 | 35 | 18 | | 33 | 20 | |
| Gender | | | | | | | |
| Male | 70 | 46 | 24 | 0.347 | 45 | 25 | 0.150 |
| Female | 56 | 34 | 22 | | 30 | 26 | |
| Histological grade ^a | | | | | | | |
| Well and moderate | 32 | 24 | 8 | 0.086 | 22 | 10 | 0.153 |
| Poor and other | 94 | 56 | 38 | | 53 | 41 | |
| T stage | | | | | | | |
| T1-T2 | 71 | 40 | 31 | 0.043 | 37 | 34 | 0.040 |
| T3–T4 | 55 | 40 | 15 | | 38 | 17 | |
| TNM stage | | | | | | | |
| I–II | 51 | 22 | 29 | 0.000 | 21 | 30 | 0.001 |
| III–IV | 75 | 58 | 17 | | 54 | 21 | |
| Lymph node metastasis | | | | | | | |
| Present | 88 | 61 | 27 | 0.032 | 58 | 30 | 0.022 |
| Absent | 38 | 19 | 19 | | 17 | 21 | |

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

^aWell-differentiated adenocarcinoma (well), moderately differentiated adenocarcinoma (moderate), poorly differentiated adenocarcinoma (poor), other histologic type (other).

size, or cell differentiation. Our results suggest that miR-200b and miR-200c could play critical roles in carcinogenesis and progression of gastric cancer.

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

To analyze the significance of miR-200b and miR-200c further in terms of clinical prognosis, a Kaplan-Meier survival analysis was conducted using patient OS and disease-free survival (DFS; Fig. 2). The results showed 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; Fig. 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; Fig. 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; Fig. 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 (Supplementary Tables S2 and S3). In univariate analysis, the levels of miR-200b and miR-200c were significantly associated with prognosis. The final multivariate model revealed that reduced miR-200b and miR-200c levels in tumors 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). 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 (Fig. 3A). We determined that overexpression of either miR-200b or miR-200c in MGC-803 and AGS cells markedly attenuated cell proliferation compared with scramble (Fig. 3A). Expression of miR-200b or miR-200c significantly inhibited the MGC-803 cells' capability for migration (Fig. 3B). Moreover, ectopic expression of either miR-200b or miR-200c in MGC-803 and AGS cells markedly attenuated cell invasion compared with control cells (Fig. 3C).

miR-200b and miR-200c directly target DNMT3A and 3B and indirectly target DNMT1

To understand how miR-200b and miR-200c suppress gastric cancer growth and invasion, we used 2 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 (Fig. 4A). We confirmed this finding in gastric cancer cells by conducting 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



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). MGC-803 (middle), and AGS (right) 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% FBS. The cells were counted at the indicated time points in triplicate and their growth rates were recorded. B, scratch wound assays were conducted on MGC-803 cells transfected with miR-200b, miR-200c, or scramble mimics. The results from 3 separate assays were averaged together and graphed. **, P < 0.05 (left). Representative images of the assays are shown. Original magnification: ×200 (right). C, the invasive properties of the MGC-803 and AGS cells were analyzed by an invasion assay using a Matrigel-coated chamber. Migrated cells were plotted as the average number of cells per field of view from 3 different experiments, as described in the Materials and Methods. **P < 0.01 (left). Representative images of the assays are shown. Original magnification: ×200 (right).

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 (Fig. 4B). In addition, 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 (Fig. 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 (Fig. 4C). In contrast to DNMT3A and DNMT3B, miR-200b and miR-200c are not predicted to hybridize with the DNMT1 3'-UTR



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 *SP1* 3'-UTR (SP1-wt) or mutant constructs in which the first 4 nucleotides of the miR-200b and miR-200c binding site were mutated: DNMT3A-mut, DNMT3B-mut, or SP1-mut. An empty luciferase reporter construct was used as a negative control. "P < 0.05 versus scramble. C, the effect of miR-200b or miR-200c on the protein expression of DNMT1, DNMT3B, and SP1 by Western blot. β -Actin was used as a loading control (top). The band intensities are quantified and normalized to β -actin intensities. Data represent the mRNA expression of *DNMT1* and *SP1* siRNA and overexpression *SP1* on the mRNA expression of *DNMT1* and *SP1* siRNA and overexpression *SP1* on the protein expression of DNMT1 and SP1 by Western blot in MGC-803 and/or AGS cells. β -Actin was used as a loading control (middle). The band intensities are quantified and normalized to β -actin intensities. DAT is a loading control (middle). The band intensities are quantified and normalized to β -Actin was used as a control. "P < 0.05, "P < 0.01 versus scramble (top). The effect of the *SP1* siRNA and overexpression of DNMT1 and SP1 by Western blot in MGC-803 and/or AGS cells. β -Actin was used as a loading control (middle). The band intensities are quantified and normalized to β -actin intensities. Data represent the means \pm SD from 3 independent experiments. "P < 0.05, "P < 0.01 (bottom).

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 (Fig. 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 conducted 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 (Fig. 4B). Therefore, we reasoned that miR-200b and miR-200c downregulated 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 downregulation of SP1 and, in turn, DNMT1 mRNA and protein levels as measured by quantitative RT-PCR and Western blot (Fig. 4D). Interestingly, the 3'-UTR of SP1 contains 1 predicted binding site according to Targetscan 6.2 (Fig. 4A). Therefore, we hypothesized that miR-200b and/or miR-200c downregulate 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 (Fig. 4B). In addition, 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 downregulation of SP1 and the subsequent decrease in DNMT1 expression.

Overexpression of miR-200b and miR-200c reduces GDM and restores the expression of hypermethylated p16, E-cadherin, and RASSF1A

Finally, we investigated whether the enforced expression of miR-200b and miR-200c could functionally result in DNA hypomethylation. GDM was measured using an HPLC-DAD method as previously described (19) in MGC-803 and AGS cell lines after 48 hours of transfection.



Figure 5. Overexpression of miR-200b and miR-200c reduced GDM and restored the expression of hypermethylated p16, Ecadherin, 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 µmol/L decitabine, a hypomethylating agent, or phosphate-buffered saline (control) are also shown for the MGC-803 cell line as positive controls. Data represent the means \pm SD from 3 independent experiments versus control **P < 0.01, versus scramble [#]P < 0.05. C. MGC-803 (left) and AGS (right) 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 means \pm SD from 3 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, Ecadherin, and RASSF1A by Western blot in MGC-803 and AGS cells. B-Actin was used as a loading control.

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 (Fig. 5A and B). 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 (Fig. 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.

cer, we measured the mRNA and protein levels of p16, Ecadherin, 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 (Fig. 5C and 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 and/or 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 nonrelapsers (33). However, there are no studies on miR-200b or miR-200c in gastric cancer. In this study, we showed that miR-200b and miR-200c levels in gastric cancer tissues were significantly lower than those in noncancerous tissues by gRT-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 tumors 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 tumors 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 this study, we characterized 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 downregulate 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 showed that miR-200b and miR-200c downregulate SP1, thereby interfering with the SP1-dependent expression of DNMT1. The discovery that miR-200b and miR-200c downregulate 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%, whereas 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 to indicate that overexpression of miR-200b or miR-200c both result in global DNA hypomethylation and gene reexpression of the hyper-

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methylated 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 showed that miR-200b and miR-200c function as tumor 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 (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 ERRFI-1 (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 reexpression, 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.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: H. Tang, Q. Su, X. Xie

Development of methodology: H. Tang, M. Deng, Q. Su

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): X. Xie

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): H. Tang, Y. Tang, X. Xie, J. Guo, Y. Kong Writing, review, and/or revision of the manuscript: H. Tang, Y. Tang, X. Xie, J. Guo, Y. Kong, X. Xie

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): M. Deng, F. Ye

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