Epigenetic silencing of miRNA-9 is correlated with promoter-proximal CpG island hypermethylation in gastric cancer *in vitro* and *in vivo*

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Abstract. Silencing of protein-coding tumor suppressor genes (TSGs) by CpG island hypermethylation is a common occurrence in gastric cancer (GC). Here, we examine if tumor suppressor microRNAs (miRNAs) are silenced in a similar manner. Real-time quantitative PCR (RTQ-PCR) was employed to investigate the expression level of four candidate miRNAs in GC tissues (n=30) and cell lines. Basing on RTQ-PCR results and bioinformatics approach, miR-9 was chosen for further study on epigenetic regulation. Bisulfite genomic sequencing PCR (BSP) was performed to assess the methylation status of miR-9 in GC tissues. In both GC cell lines and animal models, demethylation was performed either by treatment with 5-aza-2'-deoxycytidine (5-AZA-CdR) or by siRNA targeting DNMT1. We also analyzed the relationship between miRNAs and several clinicopathological features. Candidate miRNAs (miR-9, miR-433, miR-19b, and miR-370) were found strongly downregulated in GC tissues and cell lines. Their expression was increased following 5-AZA-CdR treatment. CpG island methylation of miR-9 was significantly higher in GC tissues compared to normal controls. After two demethylation treatments, miR-9 methylation degree was significantly decreased and miR-9 expression was obviously restored in GC cells and animal models. Deregulation of miR-9 was positively correlated with tumor lesion size. Three other miRNAs, miR-19b, miR-433, and miR-370 were associated with lymph node metastasis, decreased curvature, and poorly differentiated carcinoma. miR-19b and miR-433 were positively correlated with male gender. Of four candidate miRNAs downregulated in GC, miR-9 is epigenetically regulated by DNA methylation both *in vitro* and *in vivo*.

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Introduction

microRNAs (miRNAs) are a class of small, endogenous, non-coding RNA molecules that are typically 20-25 nucleotides in length. miRNAs negatively regulate specific gene products by translational repression or mRNA degradation via binding to partially or perfectly complementary sequences in the 3' untranslated regions of target genes (1-3).

In human tumors, some miRNAs are upregulated and function as oncogenes, while others are downregulated functioning as tumor suppressor genes (TSGs). Recent studies have shown that 50% of miRNAs are located within fragile sites, thus supporting the fact that many of these miRNAs may be lost during tumorigenesis (1,4-14). Consistent with this, significant data indicate that many miRNAs exhibit decreased expression in tumors (1,5,6,8,10,13,15-25). Thus, although miRNAs have been shown to be both pro- and anti-tumorigenic, the majority seems to function as TSGs by negatively regulating protein-coding oncogenes and genes regulating cell proliferation and apoptosis (1,13,16,20-22,26-30).

The promoter regions of many genes, including a number of TSGs, sometimes are embedded in CpG islands regions within the DNA that are subject to methylation. In normal condition, these regions tend to be unmethylated. However, in a transformed setting, many of these CpG islands become hypermethylated, resulting in silencing of gene expression. Although hypermethylations of CpG islands has been mostly described for protein-coding genes, a similar mechanism

may be responsible for silencing expression of miRNAs that possess antitumorigenic properties; a mechanism such as this could potentially enhance tumorigenesis (13,16,21,22,30-37).

Gastric cancer (GC) has a poor prognosis, in large part, because patients often present with advanced disease. Limitations of early diagnosis and effective therapies unfortunately result in high lethality. Thus, additional research to improve both detection and treatment of GC is critical. Several studies have been performed examining the miRNA expression profile of multiple tumor types (6,11,14,20,27,28, 34,38-42). Evidence suggests that hypermethylation of CpG islands related with the promoters of miRNA genes is a common event in GC (20,28,34,37,38,40,42-48). Previously, we examined several miRNAs, including 19 downregulated and seven upregulated, in GC (6). Here, we specifically follow up on the downregulated miRNAs and investigate the mechanism of their decreased expression. Four downregulated miRNAs contain CpG islands within 5,000 bp upstream of the transcriptional start site, and these were selected as initial candidate genes. We measured miRNA expression levels in GC samples to validate the miRNA expression profile data. To assess the importance of methylation in expression of these genes, we treated GC cells with a demethylating agent. Based on these initial results, miR-9 was selected for additional epigenetic research. Thus, we studied the role of promoter methylation in regulating miR-9 expression both *in vitro* and *in vivo*.

Materials and methods

Cell lines and animals. Cell lines used in this study included human GC cell lines SGC-7901 and BGC-823 and normal gastric epithelium cell line GES-1. The cells were purchased from the Centre of Cell Cultures of Chinese Academy of Medical Sciences, Shanghai, China. All cell lines were cultured in RPMI-1640 medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal calf serum (FCS; Gibco), 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine, and 1 mM sodium pyruvate. Cells were housed in humidified incubators at 37˚C in an atmosphere with 5% CO_2. Cells were maintained as a monolayer by serial passaging after trypsinization with 0.1% trypsin (Beyotime, Jiangsu, China).

Five to six-week-old male Balb/c nu-nu mice, weighing 18-20 g, were purchased from the Shanghai Experimental Animal Center of the Chinese Academy of Medical Sciences, China. They were maintained in cages in a pathogen-free environment (temperature 25-27˚C, humidity 45-50%) and supplied with food and water *ad libitum*. All animals received humane care in accordance with institutional policies on Human Care and Use of Laboratory Animals and with the approval of the Ethics Committee of Chongqing Medical University.

GC samples. A total of 30 GC samples were obtained via surgery from patients that had provided informed consent to the General Surgery Department of the Second Affiliated Hospital, Chongqing Medical University, Chongqing, China. Matched controls (non-cancer gastric mucosa) were obtained from all patients. None of the patients had received any pre-operative treatment. Clinicopathologic information, such as age, gender, stage, grade, pathological diagnosis, and lymph node metastasis, was available. The study was approved by the Ethics Committee of Chongqing Medical University.

miRNA microarray and bioinformatics. We previously profiled GC samples for miRNA expression by microarray analysis (6). Data from that experiment prompted us to focus on miRNAs that were decreased in GC since these miRNAs may be functioning as tumor suppressors in the disease.

CpG Island Searcher (http://cpgislands.usc.edu/) and CpG plot (http://www.ebi.ac.uk/emboss/cpgplot) were used to determine which miRNAs were embedded in or located near (<500 bp 5'-upstream) a CpG island. Over 90% of human miRNA promoters are located 1,000 bp upstream of the mature miRNA (16,31). Promoter miRNA gene clusters were predicted using a combination of Promoter 2.0 (http://www.cbs.dtu.dk/services/Promoter/), Promoter Scan (http://www-bimas.cit.nih.gov/molbio/proscan/), and Neural Network Promoter Prediction (NNPP) (http://www.fruitfly.org/seq_tools/promoter.html).

RNA isolation and reverse transcription. Total RNA was extracted using TRIzol (Sigma-Aldrich Chemical Co., Milwaukee, WI, USA). Concentration and purity were assessed with an ultraviolet spectrophotometer at wavelengths of 260 and 280 nm. RNA was reverse transcribed into cDNA using the reverse transcription kit (Takara Bio, Inc., Dalian, China). RT primers are listed in Table I. A master mix (20 μl total) containing 5X PrimeScript Buffer (4 μl), PrimeScript RT Enzyme Mix I (1 μl), RT specific primer (1 μl), total RNA (1 μg), and nuclease-free water (13 μl) was prepared on ice. The reaction was performed at 42˚C for 15 min followed by 85˚C for 5 sec.

Real-time quantitative PCR (RTQ-PCR). RNA samples isolated from both GC tissues (n=30) and cell lines were converted into cDNA and analyzed by RTQ-PCR. GC cell lines and tumors from animal models following treatment with 5-aza-2'-deoxycytidine (5-AZA-CdR) or transfection with siRNA-DNMT1 were also examined by this method. RTQ-PCR was performed using the SYBR-Green real-time PCR master mix kit (Takara Bio, Inc.) and the IQ5 PCR instrument. In brief, a master mix (25 μl) was prepared on ice with 12.5 μl 1X SYBR-Green buffer, 1 μl each primer, 2 μl cDNA, and 8.5 μl nuclease-free water. The cDNA was initially denatured at 95˚C for 30 sec followed by 40 cycles of denaturation at 95˚C for 5 sec, annealing at 59˚C for 30 sec, and extension at 72˚C for 30 sec. Primer sequences were designed using software Primer 5.0, and sequences are listed in Table II. U6 snRNA served as an endogenous control. All experiments were performed in triplicate.

Each run was accompanied by a melting curve analysis to confirm the specificity of amplification and absence of primer dimers. Relative quantification of miRNA expression was calculated by the $2^{-\Delta\Delta ct}$ method.

Bisulfite genomic sequencing PCR (BSP). DNA was isolated from GC tissues (human samples, n=30; animal samples, n=10) and cell lines (including matched normal controls). GC cell lines and tumor tissues from animal models (n=10) following 5-AZA-CdR treatment or siRNA-DNMT1 transfection were

Table I. Reverse transcription primers.

Gene name	Primer sequence
U6	5'-CGCTTCACGAATTTGCGTGTCAT-3'
hsa-miR-9	5'-GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGACTCATACAG-3'
hsa-miR-433	5'-GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGACACACCG-3'
hsa-miR-19b	5'-GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGACTCAGTT-3'
hsa-miR-370	5'-GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGACACCAGG-3'

Table II. RTQ-PCR primers.

Gene name	Primer sequence	Annealing temperature (˚C)	Product length (bp)
U6	F: 5'-GCTTCGGCAGCACATATACTAAAAT-3' R: 5'-CGCTTCACGAATTTGCGTGTCAT-3'	59	89
hsa-miR-9	F: 5'-GGGTCTTTGGTTATCTAGC-3' R: 5'-TGCGTGTCGTGGAGTC-3'	59	63
hsa-miR-433	F: 5'-GGATCATGATGGGCTGGT-3' R: 5'-CAGTGCGTGTCGTGGAGT-3'	59	64
hsa-miR-19b	F: 5'-CGTGTGCAAATCCATGC-3' R: 5'-CAGTGCGTGTCGTGGAG-3'	59	65
hsa-miR-370	F: 5'-GCCTGCTGAGATGGAATCTGATGTC-3' R: 5'-CCAGTGCGTGTCGTAGAGTCATCAA-3'	59	63

RTQ-PCR, real-time quantitative PCR.

Table III. Specific BSP primers for miRNA-9.

Gene name	Primer sequence	Product length (bp)	Tm (˚C)
miR-9-1	F: 5'-GGTAGAGTTAATTAGAGGATGGTTTG-3' R: 5'-ACCAAAAATCACCCAAAATTATAAA-3'	498	57
miR-9-2	F: 5'-TGATTTTTGGTTTTTTTTGAAT-3' R: 5'-TCCACTACCCTTCTCTAAAAAA-3'	504	58

BSP, bisulfite genomic sequencing PCR.

also included. Genomic DNA was isolated using the Genomic DNA Extraction kit (Takara Bio, Inc.) according to the manufacturer's instructions. Bisulfite modification (EZ DNA Methylation-Gold™ kit, D5005/50; Zymo Research Corp., Irvine, CA, USA) was performed to convert unmethylated cytosine to uracil; methylated cytosine nucleotides are unaffected by the procedure. Bisulfite-modified miRNA promoters were amplified using specially designed primers listed in Table III. The reaction volume 50 μl included 10X buffer (5 μl), $MgCl_2$ (2 μl), dNTP (1 μl), each primer (2 μl), DNA (5 μl), Platinum Taq (0.3 μl), and ddH_2O (32.7 μl). Amplification was carried out as follows: 5 min 95˚C; 42 cycles of 30 sec at 95˚C, 30 sec at 57˚C, and 40 sec at 72˚C; and a 10 min final extension at 72˚C. Per sample, five independent colonies for each tested region were picked and sequenced. The extent of methylation was assessed by identifying the number and position of methylated cytosine residues.

5-AZA-CdR treatment in vitro and in vivo. Cell lines were treated with 5 μM 5-AZA-CdR (Sigma-Aldrich Chemical Co.) for 48 h.

Nude mice were subcutaneously injected with 10^7 GC cells on either side of the flank. Two weeks post-implantation, tumors reached a size of ~1.2 cm^3. Orthotopic GC models (n=10) were constructed according to our previously described protocol (49). Animals received intravenous injection of 5-AZA-CdR at 0.6 mg/kg once per day for 4 weeks after transplantation (50). Mice were euthanized and fresh

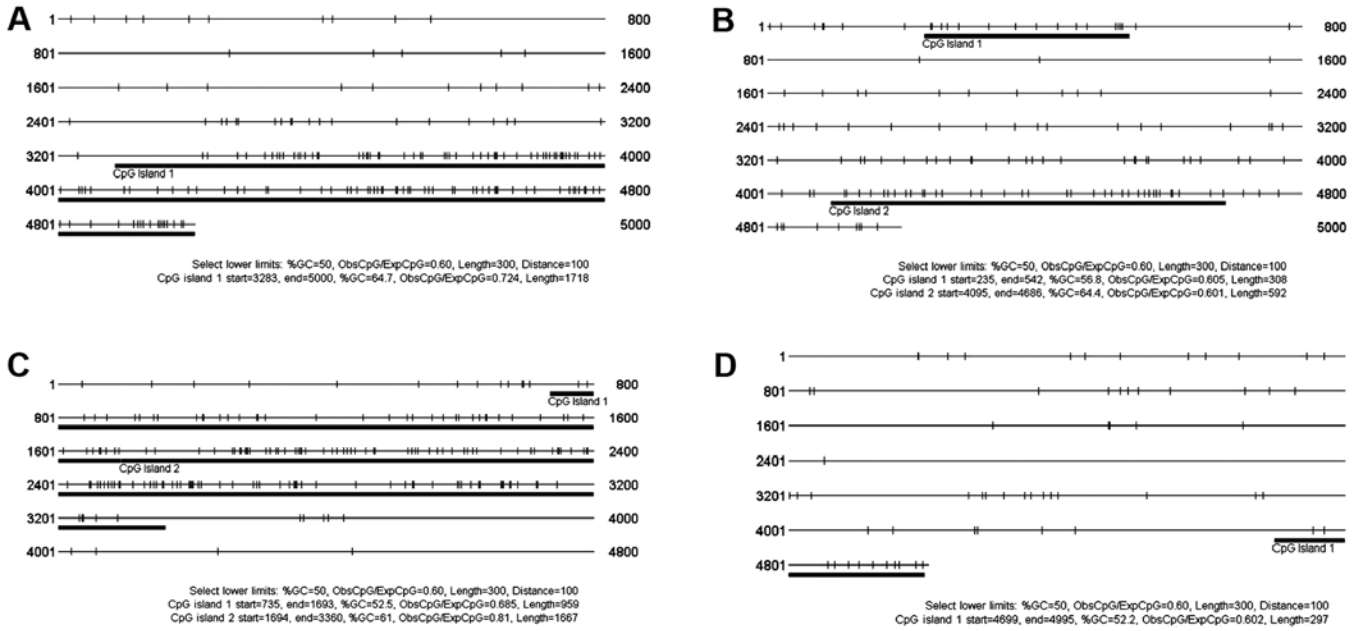

Figure 1. Schematic representation of CpG islands in the 5,000-bp promoters of select microRNAs (miRNAs). CpG Island Searcher (http://cpgislands.usc.edu/) and CpG plot (http://www.ebi.ac.uk/emboss/cpgplot) were used to predict the position of CpG islands. Strong lines depict CpG islands. (A) miR-9 CpG islands. (B) miR-433 CpG islands. (C) miR-370 CpG islands. (D) miR-19b CpG islands.

tumor fragments, free of any necrotic region, were harvested and preserved in liquid nitrogen until use.

siRNA-DNMT1 synthesis and transfection into cell lines and animal models in vivo. An siRNA-DNMT1 plasmid was synthesized by Jingsai Bio Co., Ltd., (Hubei, China). Gastric cell lines were seeded at a density of $2x10^5$ cells/well in 6-well plates (Costar, Cambridge, MA, USA) and cultured overnight. siRNA-DNMT1 plasmid (5 μl) was transfected into cells using Lipofectamine 2000 (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. Cells were incubated for 48 h, at which time, the transfection efficiency was >80%.

Animal models (n=10) using transfected GC cell lines were established as described above. Four weeks after implantation, fresh tumor tissues were harvested and preserved as described.

Statistical analysis. Data are presented as mean ± SD. RTQ-PCR data were analyzed by Student's t-test. The correlation between miRNA expression and clinicopathological factors was analyzed using Fisher's exact test. Differences in mean methylation levels were analyzed using the χ^2 test. Statistical significance was set at $p<0.05$.

Results

Selection of candidate miRNAs of interest. We previously profiled GC samples for miRNA expression by microarray analysis (6). A total of 26 differentially expressed miRNAs were identified, of which 19 were downregulated and seven were upregulated. We prioritized studying miRNAs that were repressed in GC and that had CpG islands within 5,000 bp upstream of the transcription start site. The final list of potential target genes was determined using a bioinformatics approach (51). Potential targets included miR-9, miR-433, miR-19b, and miR-370 (Fig. 1A-D).

Validation of expression of four miRNAs in GC tissues and cell lines. We measured expression of miR-9, miR-433, miR-19b, and miR-370 and found that they were all strongly repressed in GC samples compared to normal gastric mucosa. All miRNAs, except miR-19b, displayed significant differences in expression ($p<0.05$) (Fig. 2A). Compared to the normal gastric epithelial cell line GES-1, the four tested miRNAs all showed decreased expression in the GC cell lines SGC-7901 and BGC-823. However, miR-9 was the only miRNA of the four whose expression level was statistically significantly decreased ($p<0.05$) (Fig. 2B).

miRNA expression is increased following treatment with demethylating agent. To assess the importance of methylation in expression of the four candidate miRNAs, we examined their expression in two GC cell lines following treatment with 5-AZA-CdR. In both cell lines, miRNA expression was increased after demethylation. In SGC-7901, miR-9 and miR-19b were both significantly increased following 5-AZA-CdR treatment ($p<0.01$); in contrast, the increased expression of miR-370 and miR-433 was not statistically significant (Fig. 3A). In the BGC-823 cell line, statistical significance was achieved for all four miRNAs ($p<0.01$) (Fig. 3B).

Analysis of DNA methylation in the miR-9 CpG island. Approximately 90% of human miRNA promoters are located 1,000 bp upstream of the mature miRNA (16,31). To identify promoters harboring CpG islands, a manual search of the candidate miRNA promoters was performed via a bioinformatics approach. miR-9 promoter was predicted to embed in CpG islands based on this analysis (Fig. 4). Moreover, we

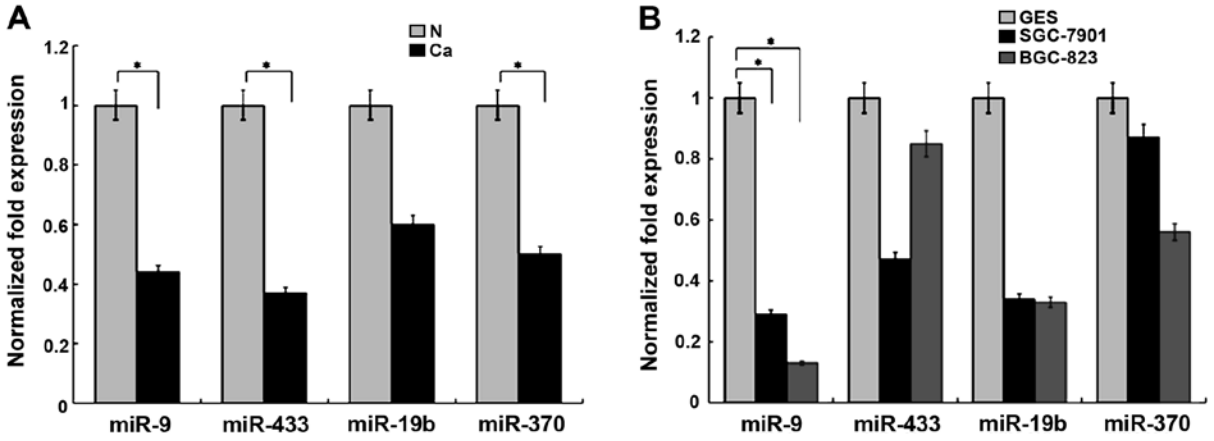

Figure 2. Expression of four microRNAs (miRNAs) in gastric cancer (GC) tissues and cell lines. (A) Expression levels of four miRNAs (miR-9, miR-433, miR-19b, and miR-370) was examined by real-time quantitative PCR (RTQ-PCR) in 30 GC patients. (B) Expression of these same four miRNAs was also examined by RTQ-PCR in GC cell lines (SCG-7901 and BGC-823) and normal controls. All experiments were performed in triplicate. Data were analyzed by Student's t-test and shown as mean ± SD. *P<0.05. Ca, GC tissue; N, normal gastric mucosa.

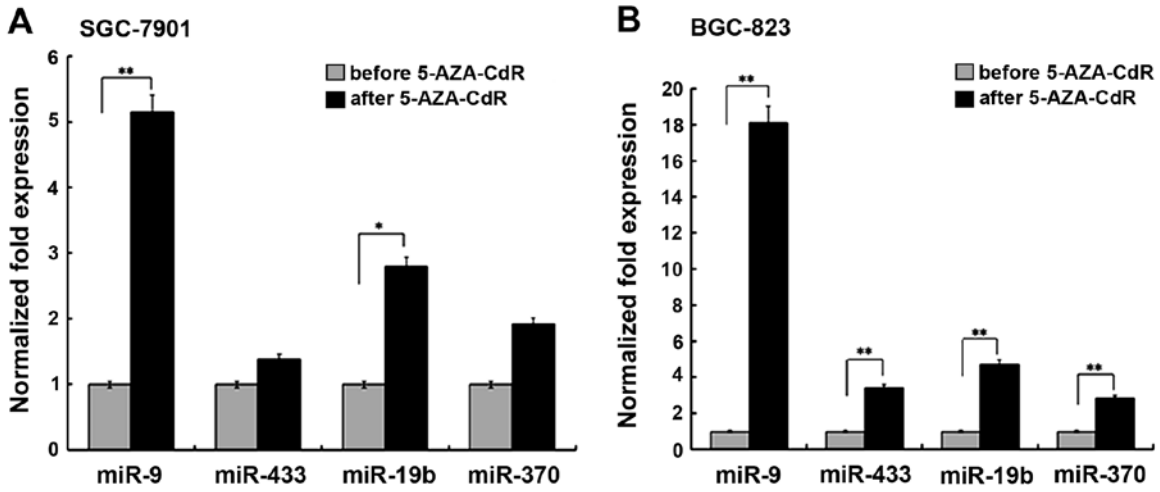

Figure 3. microRNAs (miRNAs) were re-expressed in gastric cancer (GC) cell lines after demethylation treatment. (A) Expression level of four miRNAs (miR-9, miR-433, miR-19b, and miR-370) was examined by real-time quantitative PCR (RTQ-PCR) after treating SGC-7901 cells with 5 μM concentrations of 5-aza-2'-deoxycytidine (5-AZA-CdR). (B) miRNAs expression was detected by RTQ-PCR following the same treatment in the BGC-823 cell line. All experiments were performed three times. Data were analyzed by ANOVA and shown as mean ± SD. *P<0.05, **p<0.01.

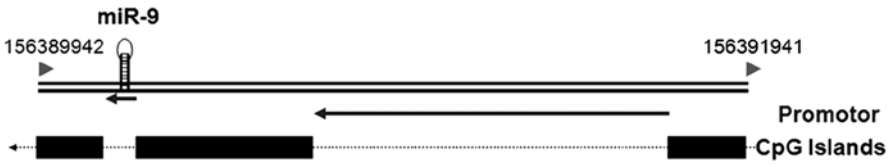

Figure 4. Schematic representation of the miR-9 promoter showing a CpG island ~1,000 bp upstream of the mature microRNA (miRNA).

found that miR-9 was consistently deregulated in both GC tissue and cell lines. Thus, miR-9 was chosen for further study.

We investigated the methylation status of the miR-9 promoter (500-1,500 bp upstream of the transcription start site) via amplification of two regions termed miR-9-1 and miR-9-2. CpG island methylation of miR-9 in GC tissues was significantly higher than the methylation level in normal gastric mucosa (p<0.05 and p<0.005) (Fig. 5A and B). Cell line data were consistent with this. That is, miR-9 methylation in the GC cell lines SGC-7901 and BGC-823 was significantly higher than methylation in normal controls (p<0.01 and p<0.005) (Fig. 5C and D).

Demethylation, induced by either 5-AZA-CdR or siRNA-DNMT1, increases miR-9 expression in GC cell lines. We assessed the importance of miR-9 methylation on its expression by two methods. First, SGC-7901 and BGC-823 cells

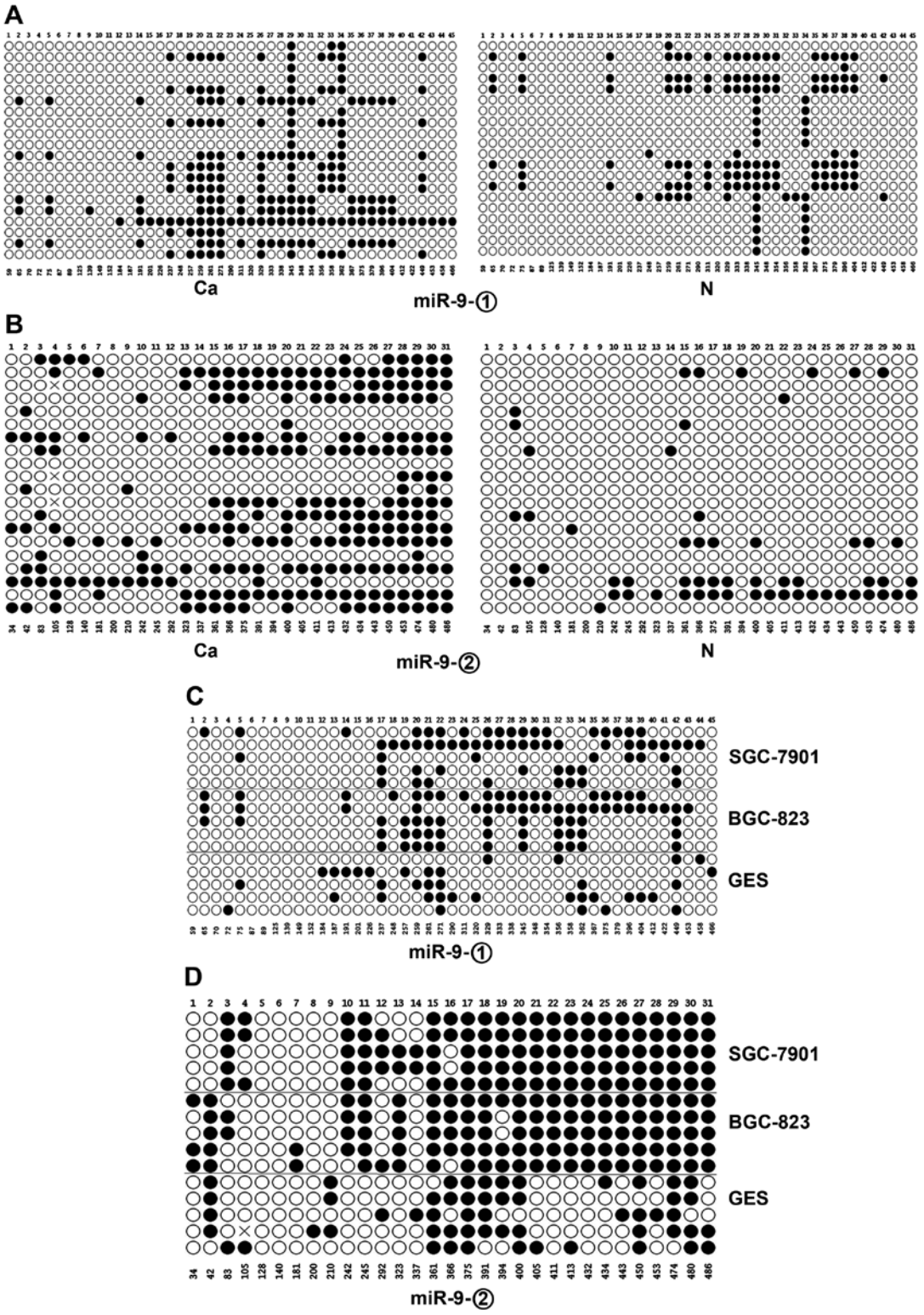

Figure 5. Methylation analysis of miR-9 CpG islands via amplification of two segments termed miR-9-1 and miR-9-2. (A and B) Bisulfite genomic sequencing PCR (BSP) was used to analyze CpG island methylation of miR-9 (miR-9-1 and miR-9-2) in gastric cancer (GC). miR-9 methylation in GC tissues was higher than in normal gastric mucosa. (C and D) BSP was performed and miR-9 methylation was examined. miR-9 methylation was higher in GC cell lines (SGC-7901, BGC-823) than in the normal control (GES). Ca, GC tissue; N, normal gastric mucosa. Each circle represents one clone. Black circles, methylated; white circles, unmethylated.

were treated with 5-AZA-CdR. In both cell lines, methylation of miR-9 CpG islands was decreased following treatment ($p<0.05$, $p<0.005$) (Fig. 6A and B). This was concomitant with increased miR-9 expression ($p<0.01$) (Fig. 4A and B). SGC-7901 and BGC-823 cells were also transfected with siRNA targeting DNMT1 as another means of relieving DNA methylation. Consistent with the 5-AZA-CdR results, transfection of siRNA-DNMT1 decreased CpG island methylation in both cell lines ($p<0.05$) (Fig. 6C and D) and increased expression of miR-9 ($p<0.01$) (Fig. 6E and F).

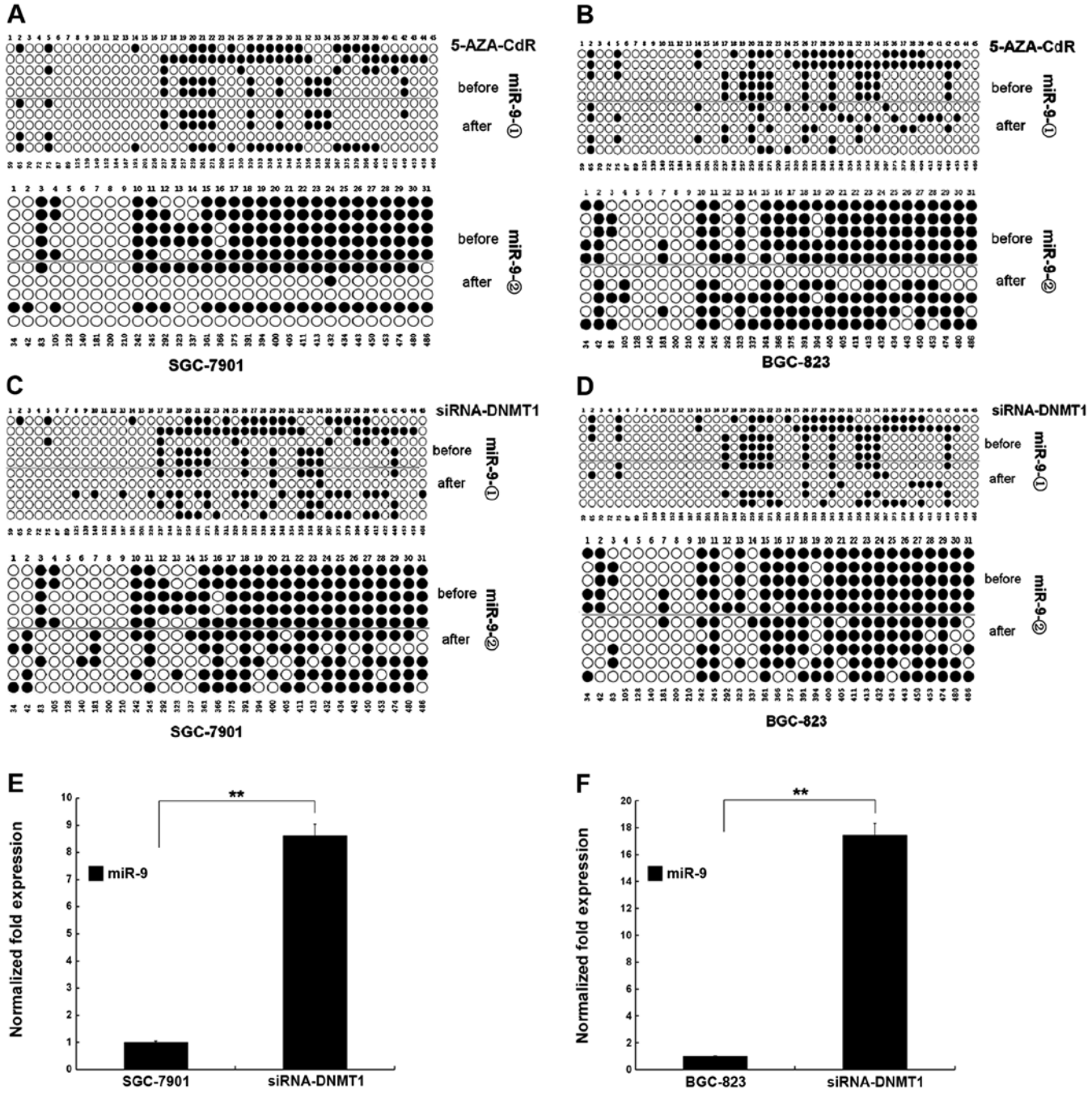

Figure 6. Changes in miR-9 CpG island methylation after different demethylation methods in SGC-7901 and BGC-823 cell lines. (A and B) miR-9 CpG islands (showed decreased DNA methylation after 5-aza-2'-deoxycytidine (5-AZA-CdR) treatment ($p<0.05$; $p<0.005$) in both cell lines. (C and D) Decreased miR-9 CpG island methylation was observed after transfecting siRNA-DNMT1 plasmid into SGC-7901 ($p<0.05$; $p<0.01$) and BGC-823 cells ($p<0.005$). (E and F) Expression of miR-9 was increased following transfection of siRNA-DNMT1 into SGC-7901 and BGC-823 cells ($^{**}p<0.01$, Student's t-test).

miR-9 methylation in the GC animal model. We next explored miR-9 methylation in our orthotopic GC animal model. In tumors, the degree of methylation of the miR-9-1 CpG island was decreased following 5-AZA-CdR treatment, but the change was not statistically significant. In contrast, methylation of miR-9-2 was significantly decreased by 5-AZA-CdR treatment ($p<0.01$) (Fig. 7A). Although expression of miR-9 showed an increasing trend following treatment, the change was not significant (Fig. 7B). Introduction of siRNA-DNMT1 into our GC animal models significantly decreased the levels of methylation of both miR-9-1 ($p<0.01$) and miR-9-2 ($p<0.05$) (Fig. 7C). This occurred concomitantly with increased miR-9 expression ($p<0.01$) (Fig. 7D).

Correlation between miRNAs and clinicopathological features. We performed correlation analysis between miRNA expression and several clinicopathological features, and the data are listed in Table IV. Of note, deregulation of miR-9 was positively correlated with tumor size ($p=0.026$) and lymph node metastasis ($p=0.041$). miR-433 correlated with gender ($p=0.031$), the position of local invasion ($p=0.006$), grade ($p=0.006$), and lymph node metastasis ($p=0.003$). miR-19b was

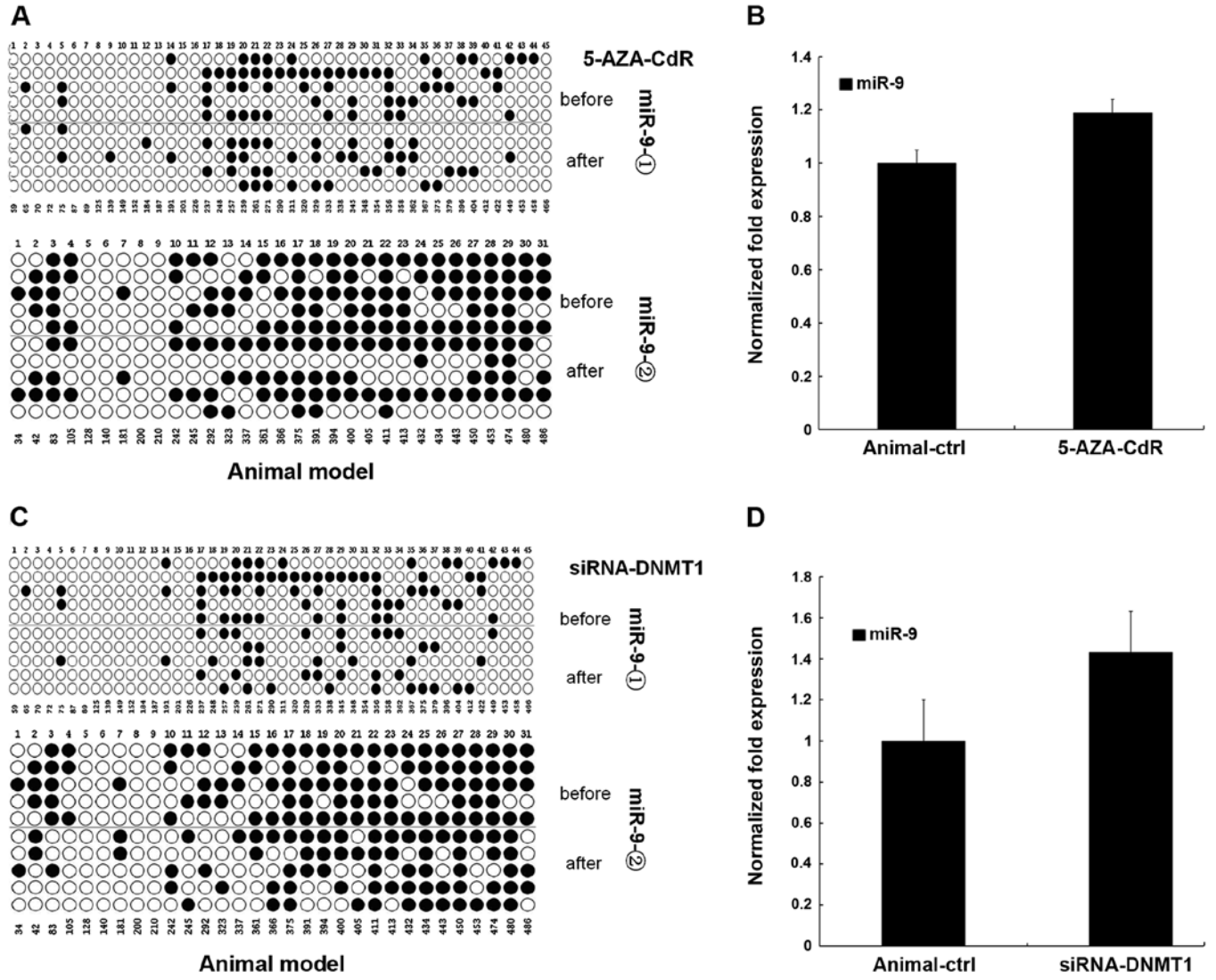

Figure 7. Changes in miR-9 CpG island methylation *in vivo* by two demethylation methods. (A) miR-9 CpG islands (miR-9-1 and miR-9-2) displayed decreased DNA methylation levels after 5-aza-2'-deoxycytidine (5-AZA-CdR) treatment ($p<0.05$) in the gastric cancer (GC) animal model. Changes in miR-9-1 were not statistically significant ($p>0.05$). (B) miR-9 was re-expressed after 5-AZA-CdR treatment in the GC model, but this was not statistically significant ($p>0.05$). (C) Methylation of miR-9 was significantly decreased in the GC model after transfection with siRNA-DNMT1 ($p<0.01$, $p<0.05$). (D) miR-9 expression was rescued in the GC model transfected with siRNA-DNMT1 ($^{**}p<0.01$, Student's t-test).

found to correlate with gender (p=0.031), the position of tumor involvement (p=0.001), and grade (p=0.031). Finally, miR-370 correlated with tumor position (p=0.001), grade (p=0.031), and lymph node invasion (p=0.012).

Discussion

miRNAs are heavily implicated in tumorigenesis in multiple cancer types (7-14). We identified four candidate miRNAs (miR-9, miR-433, miR-19b, and miR-370) whose expressions were all reduced in GC tissues and cell lines compared to normal healthy gastric epithelium. All of the miRNAs, except for miR-19b, displayed significant differences in expression, validating the previous miRNA profile data from GC patients. The only miRNA that showed consistent downregulation in GC cell lines SGC-7901 and BGC-823 (as in GC tissue) was miR-9. Recently, Du *et al* showed that miRNAs in GC cell lines might not be repressed to the same extent as they are in actual human tissue samples; in fact, they state that most cell lines likely exhibit normal miRNA expression (28). Their study may provide an explanation as to why miR-433, miR-19b, and miR-370 were not significantly repressed in the cell lines we examined. Many other groups have identified miRNAs that are specifically downregulated in GC samples (14,20,27,28,38,42,43,47).

The four candidate miRNAs included in this study had CpG islands within 5,000 bp upstream of the transcriptional start site. Thus, we hypothesized that expression of these miRNAs would be increased following treatment with the demethylation agent 5-AZA-CdR. This proved true, as expression of all four miRNAs (miR-9, miR-433, miR-19b, and miR-370) were increased after 5-AZA-CdR treatment; thus, methylation is an important epigenetic regulatory mechanism governing expression of these miRNAs. In both SGC-7901 and BGC-823 cells, the effect of 5-AZA-CdR on miR-9 and miR-19b was the greatest, suggesting that these two miRNAs are dominantly regulated by a methylation-dependent mechanism. Interestingly, we found that the ability of miR-370

Table IV. Correlation between miRNA and clinicopathological features in gastric cancer (n=30).

Clinicopathological features	Cases	miR-9		p-value	miR-433		p-value	miR-19b		p-value	miR-370		p-value
		LE	HE		LE	HE		LE	HE		LE	HE	
Gender													
Male	23	22	1	0.128	22	1	[a]0.031	22	1	[a]0.031	21	2	0.225
Female	7	5	2		4	3		4	3		5	2	
Age													
≤50 years	10	10	0	0.281	9	1	0.640	8	2	0.407	8	2	0.407
>50 years	20	17	3		16	4		18	2		18	2	
Tumor size													
≤3 cm	13	9	4	[a]0.026	10	3	0.367	12	1	0.409	12	1	0.409
>3 cm	17	17	0		15	2		14	3		14	3	
Tumor position													
Lesser curvature	23	22	1	0.128	22	1	[b]0.006	23	0	[b]0.001	23	0	[b]0.001
Greater curvature	7	5	2		3	4		3	4		3	4	
Pathological pattern													
Squamous carcinoma	0	0	0	None	0	0	None	0	0	None	0	0	None
Adenocarcinoma	30	27	3		25	5		26	4		26	4	
Pathological grade													
Well differientiated	7	5	2	0.128	3	4	[b]0.006	4	3	[a]0.031	4	3	[a]0.031
Poorly differientiated	23	22	1		22	1		22	1		22	1	
Lymph node metastasis													
Yes	19	19	0	[a]0.041	19	0	[b]0.003	18	1	0.126	19	0	[a]0.012
No	11	8	3		6	5		8	3		7	4	
Liver metastasis													
Yes	0	0	0	None	0	0	None	0	0	None	0	0	None
No	30	27	3		25	5		26	4		26	4	
Peritoneum dissemination													
Yes	0	0	0	None	0	0	None	0	0	None	0	0	None
No	30	27	3		25	5		26	4		26	4	
Clinical stages													
I, II	29	26	3	0.900	24	5	0.833	25	4	0.867	25	4	0.867
III, IV	1	1	0		1	0		1	0		1	0	

[a]P<0.05, [b]p<0.01. miRNA, microRNA; LE, low expression; HE, high expression.

and miR-433 to be demethylated was different in different GC cell lines. Consistent with this, Guo *et al* reported that, following 5-AZA-CdR treatment of other GC lines (HGC-27 and MGC-803), miR-433 was re-expressed to different degrees (38). Thus, aberrant expression of miRNAs in different tumor cell lines may result from tumor heterogeneity (28). One study showed that miRNA expression could be rescued by deacetylation even if miRNA hypermethylation was maintained (25). The exact role of DNA hypermethylation of miR-370 and miR-433 in SGC-7901 cells requires additional research.

In cancer, many protein-coding genes with tumor suppressor qualities are silenced by CpG island methylation. Here, we examined if tumor suppressor miRNAs may be silenced in a similar manner. Methylation of miR-9 at two promoter CpG islands was significantly higher in GC tissues and cell lines compared to normal controls. This finding supports our hypothesis that tumor suppressor miRNAs can be silenced by DNA methylation in tumors, similar to the silencing of protein-coding genes. We found that miR-9 is epigenetically regulated by hypermethylation of promoter-proximal CpG islands; this may be the dominant mechanism of miR-9 silencing in GC.

miR-9 is the best characterized miRNA regulated by methylation in cancer. Lehmann *et al* (35) showed that deregulation of hsa-miR-9-1, mediated by CpG island methylation, was an early event during breast tumorigenesis. Lujambio *et al* (31) determined that methylation-mediated silencing of the miR-148a, miR-34b/c, and miR-9 promoters was cancer-specific and closely correlated with lymph node

metastasis. Du *et al* (28) found that hypermethylation repressed expression of seven miRNAs in GC; interestingly the degree of miR-9 methylation was the most significant among them. Another study also showed that miR-9 methylation correlated with decreased expression in GC (20). Taken together, a number of studies have shown the importance of miRNA methylation in GC development (20,34,38,52,53). The data we present here contribute to this base of knowledge.

We validated, by two independent methods, that miR-9 expression is epigenetically regulated *in vitro*. The degree of CpG island methylation of miR-9 was significantly decreased after 5-AZA-CdR treatment or siRNA-DNMT1 in both GC cell lines and animal models; this was concomitant with an increase in miR-9 expression. Our data are supported by the study of others highlighting the importance of miRNA methylation (7,8,10,54-56).

We examined the importance of miR-9 methylation both in cell lines and in animal models. In all cases, miR-9 methylation was decreased and expression was increased following administration of a demethylating agent. However, the changes in miR-9 methylation and expression *in vivo* were not as dramatic as the *in vitro* alterations. The discrepancies could be due to the method of 5-AZA-CdR treatment *in vitro* versus *in vivo*, including dosage, duration, and pathway. While some have reported on 5-AZA-CdR treatment in mice, it is difficult to evaluate the effects in different laboratories (57). To the best of our knowledge, this is the first report describing the use of a demethylation drug in a GC animal model. Administration of 5-AZA-CdR in GC animal models likely requires further optimization. Although there were some differences in treatment with 5-AZA-CdR compared to siRNA-DNMT1, we effectively showed that miR-9 is epigenetically regulated by hypermethylation in GC.

We found that our four candidate miRNAs were significantly positively correlated with several clinicopathological features. Low levels of miR-9 were associated with tumor size, indicating that miR-9 may be an independent diagnostic factor in GC. Deregulated expression of miR-9, miR-433, and miR-370 was correlated with lymph node metastasis, which contributed to poor prognosis. In addition, decreased expression of these three miRNAs (miR-19b, miR-433, and miR-370) was shown to be associated with less curvature of the stomach; this shape is the most common position for both GC and poorly differentiated carcinoma, which is the most common pathological type of late stage GC. miR-19b and miR-433 positively correlated with male gender, inferring that the aberrant expression of these two miRNAs might be common events in male GC patients. Recently, Tsai *et al* showed that miR-9 expression was associated with tumor grade, metastasis, and survival rate in GC (20). Yanaihara *et al* (4) examined 104 lung cancer samples and analyzed potential correlation between six over-expressed miRNAs (miR-205, miR-99b, miR-203, miR-202, miR-102, and miR-204-prec) and clinicopathological features; however, no miRNA was found to be associated with gender. Similar analyses have been performed in multiple tumor types (4,12,13).

In conclusion, we showed that four miRNAs were downregulated in GC, and that their expression can be restored following treatment with 5-AZA-CdR. Additionally, these four miRNAs were positively correlated with several clinicopathological features. Of the four candidate miRNAs, miR-19b and miR-370 are the least studied in GC. To strengthen our claims, we performed experiments with two different demethylation methods; together, our data support that miR-9 is epigenetically silenced by CpG island methylation in GC. Due to technical limitations, the *in vivo* experiments should be revisited in more detail in the future. Future studies should also focus on how miRNAs regulate oncogenesis and which genes are the key miRNA targets.

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