Methylation-associated silencing of microRNA-34b/c in gastric cancer and its involvement in an epigenetic field defect

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Altered expression of microRNA (miRNA) is strongly implicated in cancer, and recent studies have shown that the silencing of some miRNAs is associated with CpG island hypermethylation. To identify epigenetically silenced miRNAs in gastric cancer (GC), we screened for miRNAs induced by treatment with 5-aza-2’-deoxycytidine and 4-phenylbutyrate. We found that miR-34b and miR-34c are epigenetically silenced in GC and that their downregulation is associated with hypermethylation of the neighboring CpG island. Methylation of the miR-34b/c CpG island was frequently observed in GC cell lines (13/13, 100%) but not in normal gastric mucosa from *Helicobacter pylori*-negative healthy individuals. Transfection of a precursor of miR-34b and miR-34c into GC cells induced growth suppression and dramatically changed the gene expression profile. Methylation of miR-34b/c was found in a majority of primary GC specimens (83/118, 70%). Notably, analysis of non-cancerous gastric mucosae from patients with multiple GC than in mucosae from patients with single GC (27.3 versus 20.8%; P < 0.001) or mucosae from *H. pylori*-positive healthy individuals (27.3 versus 20.7%; P < 0.001). These results suggest that miR-34b and miR-34c are novel tumor suppressor genes, DNA methylation is involved in an epigenetic field defect and that the methylation might be a predictive marker of GC risk.

Introduction

MicroRNAs (miRNAs) are small non-coding RNAs that regulate gene expression by inducing degradation or translational inhibition of partially complementary target messenger RNAs. The ~1000 miRNAs thought to be encoded in the human genome play pivotal roles in a wide array of biological processes, including cell proliferation and differentiation and apoptosis (1,2). In recent years, a number of studies have provided evidence that dysregulation of miRNA expression contributes to the initiation and progression of human cancer (1,2). Indeed, downregulation of a subset of miRNAs is a commonly observed feature of cancers, suggesting that these molecules may act as tumor suppressors (3). The first report of altered miRNA expression in cancer was the frequent chromosomal deletion and downregulated expression of miR-15 and miR-16, two miRNAs thought to target the antiapoptotic factor BCL2, in chronic lymphocytic leukemia (4). Another example is let-7, which negatively regulates expression of Ras oncogenes (5); its downregulation in tumors is thought to contribute to activation of the Ras signaling pathway.

Although the mechanism underlying miRNA dysregulation in cancer is not yet fully understood, recent studies have shown that the silencing of several miRNAs is tightly linked to epigenetic mechanisms, such as histone modification and DNA methylation. It was shown, for example, that pharmacological unmasking of silenced genes using histone deacetylase and DNA methyltransferase (DNMT) inhibitors restored miR-127 expression in a human bladder cancer cell line (6) and that genetic disruption of DNMTs restored expression of miR-124a in a colorectal cancer cell line (7). Notably, the DNA sequences encoding miR-127 and miR-124a are embedded within CpG islands that are hypermethylated in cancer cells. Similarly, we found that the frequent silencing of miR-34b and miR-34c in colorectal cancer is associated with CpG island hypermethylation (8). Taken together, these results suggest that, as with other tumor suppressor genes, DNA methylation is a major mechanism by which miRNA expression is silenced in cancer.

Gastric cancer (GC) is one of the most common causes of death from cancer among both men and women around the world (9). To date, we and many others have identified a wide variety of tumor suppressor genes and other tumor-related genes that are inactivated by aberrant DNA methylation in GC (10–12). Moreover, it was recently shown that epigenetic mechanisms are involved in the alteration of several miRNA genes in GC (13,14), though much remains to be learned about the dysregulation of miRNAs in this disease. In the present study, we aimed to identify miRNAs whose expression is epigenetically silenced in GC by screening for miRNAs whose expression is upregulated by DNA demethylation and histone deacetylase inhibition in GC cell lines. We found that miR-34b and miR-34c are frequent targets of epigenetic silencing in GC, and analysis in primary GC and non-cancerous gastric mucosa specimens revealed a significant involvement of miR-34b/c methylation in the development of an epigenetic field defect during stomach carcinogenesis.

Materials and methods

Cell lines and tissues

GC cell lines (MKN74, SNU1, SNU638, JRST, KatoIII, AZ521, MKN28, MKN45, NUGC3, NUGC4, AGS and NCI-N87) were obtained and cultured as described previously (13). SH101 cells were kindly provided by Dr K. Yamauchi at the National Cancer Research Institute and were described previously (14). To analyze restoration of silenced genes, cells were treated with 5-aza-2’-deoxycytidine (DAC; Sigma–Aldrich, St Louis, MO) or with a combination of DAC and 4-phenylbutyrate (PBA; Sigma–Aldrich). Cells were treated with 2 μM DAC for 72 h, replacing the drug and medium every 24 h. In the combined protocol, cells (MKN74 and AGS) were treated first with 2 μM DAC for 72 h and then with 3 mM PBA for an additional 72 h, replacing the drug and medium every 24 h. A total of 118 primary GC specimens were obtained from surgical resection (15,17) or endoscopic biopsy (82 male and 36 female; average age 66 years, ranging from 36 to 89). Non-cancerous gastric mucosae were obtained by endoscopic biopsy from 109 well-differentiated GC patients (82 male and 27 female; average age 69 years, ranging from 35 to 89).
Normal gastric mucosa were also obtained by endoscopic biopsy from 85 healthy individuals (61 male and 24 female; average age 58 years, ranging from 22 to 89). Biopsies of non-cancerous gastric mucosa or normal gastric mucosa were obtained from two independent sites (gastric body and antrum) in each individual. *Helicobacter pylori* infection was identified using a rapid urease test, a serum antibody test or a urea breath test. The updated Sydney System was used to estimate the degree of gastritis (18). Informed consent was obtained from all patients before collecting the specimens. Genomic DNA was extracted using the standard phenol–chloroform procedure. Total RNA was extracted using TRIZOL reagent (Invitrogen, Carlsbad, CA) and then treated with a DNA-free kit (Ambion, Austin, TX). Genomic DNA and total RNA from normal gastric mucosa from a healthy individual were purchased from BioChain (Hayward, CA).

miRNA microarray analysis

miRNA expression was analyzed using a color microarray according to the manufacturer’s instructions (Agilent Technologies, Santa Clara, CA). Briefly, 1 ng of small RNA or total RNA was reverse transcribed using specific stem-loop reverse transcription primers, after which they were amplified and detected using polymerase chain reaction (PCR) with specific primers and TaqMan probes. The PCR was run in a 7900HT Fast Real-Time PCR System (Applied Biosystems), and SDS2.2.2 software (Applied Biosystems) was used for comparative delta Ct analysis. 

Real-time reverse transcription–polymerase chain reaction of miRNA

Expression of mature *miR-34b/c* was analyzed using TaqMan MicroRNA Assays (Applied Biosystems, Foster City, CA). Briefly, 5 ng of small RNA or total RNA was reverse transcribed using specific stem-loop reverse transcription primers, after which they were amplified and detected using polymerase chain reaction (PCR) with specific primers and TaqMan probes. The PCR was run in a 7900HT Fast Real-Time PCR System (Applied Biosystems), and SDS2.2.2 software (Applied Biosystems) was used for comparative delta Ct analysis. U6 small RNA (U6snRNA; Applied Biosystems) served as an endogenous control.

Promoter assay

A pGL3 vector harboring the *miR-34b/c* promoter region was prepared as described previously (8). Cells (5 × 10^4 cells per well in 24-well plates) were transfected with 100 ng of one of the reporter plasmids and 2 ng of pRL-TK (Promega, Madison, WI) using Lipofectamine 2000 (Invitrogen, Lucerne). Luciferase activities were then measured 48 h after transfection using a Dual-Luciferase Reporter Assay System (Promega).

Methylation analysis

Genomic DNA (2 μg) was modified with sodium bisulfite using an EpiTect Bisulfite Kit (Qiagen, Hilden, Germany). In *vitro* methylated DNA served as a positive control for methylation, as described previously (8). Genomic DNA from a colon cancer cell line HCT116 harboring genetic disruptions within the *DNMT1* and *DNMT3B* loci (DNMTs KO) served as a negative control (8). Methylation-specific PCR, bisulfite sequencing and bisulfite pyrosequencing were carried out as described previously (8,15). For bisulfite sequencing analysis, amplified PCR products were cloned into pcR2.1-TOPO vector (Invitrogen), and 10–12 clones from each sample were sequenced using an ABI3130x automated sequencer (Applied Biosystems). For bisulfite pyrosequencing, the biotinylated PCR product was purified, made single-stranded and used as a template in a pyrosequencing reaction run according to the manufacturer’s instructions. The PCR products were bound to streptavidin sepharose beads (Amersham Biosciences, Piscataway, NJ), after which beads containing the immobilized PCR product were purified, washed and denatured using a 0.2 M NaOH solution. After addition of 0.3 μM sequencing primer to the purified PCR product, pyrosequencing was carried out using a PSQ96MA system (Biotage, Uppsala, Sweden) and Pyro Q-CpG software (Biotage). Primer sequences and PCR product sizes are listed in supplementary Table 1 (available at Carcinogenesis Online).

Transfection of precursor miRNA

Cells (5 × 10^5 cells) were transfected with 100 pmol of Pre-miR-34b/c Precursor Molecules (Ambion) or Pre-miR* miRNA Molecules Negative Control #1 (Ambion) using a Cell Line Nucleofector kit V (Amaxa, Gaithersburg, MD) with a Nucleofector I electroporation device (Amaxa) according to the manufacturer’s instructions. Total RNA or cell lysate was extracted 48 h after transfection.

Real-time reverse transcription–polymerase chain reaction

Real-time reverse transcription–polymerase chain reaction (RT–PCR) was carried out using TaqMan Gene Expression Assays (MET, Hs00179845_m1; CDK4, Hs00368487_m1; CCNE2, Hs00183039_m1; CCNA2, Hs00153138_m1; 18S ribosomal RNA, Hs99999901_s1; Applied Biosystems) and a 7500 Fast Real-Time PCR System (Applied Biosystems) according to the manufacturer’s instructions. SDS2.2.2 software (Applied Biosystems) was used for comparative delta Ct analysis, and 18S ribosomal RNA (Applied Biosystems) served as an endogenous control.

Gene expression microarray analysis

Total RNA was extracted using TRIZOL (Invitrogen) and then cleaned up using an RNeasy Mini Elute Cleanup kit (Qiagen). Thereafter, one-color microarray-based gene expression analysis was carried out according to the manufacturer’s instructions (Agilent Technologies). Briefly, 700 ng of total RNA was amplified and labeled using a Low RNA Input Linear Amplification kit (Agilent Technologies), after which the synthesized complementary RNA was hybridized to the Whole Human Genome Oligo DNA microarray (G4112F; Agilent technologies). Once hybridized, the array was scanned with an Agilent DNA Microarray Scanner (Agilent technologies), and the microarray data were processed using Feature Extraction software (Agilent technologies). For hierarchical clustering analyses, gene ontology and pathway analyses were carried out using GeneSpring GX version 11 (Agilent technologies).

Western blot analysis

Western blot analysis was carried out as described previously (8). Mouse anti-MET (hepatocyte growth factor receptor) monoclonal antibody (mAb) (25H2; Cell Signaling Technology, Danvers, MA), mouse anti-CDK4 mAb (DCS-35; Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-cyclin E1 mAb (clone EP435E; Millipore, Billerica, MA), rabbit anti-cyclin A2 polyclonal Ab (C-19; Santa Cruz Biotechnology) and mouse anti-actin mAb (Chemicon, Temecula, CA) were all used in accordance with the manufacturer’s instructions. The immunoreactive bands were visualized using peroxidase-conjugated anti-mouse or -rabbit IgG antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) and ECL (GE Healthcare Bio-Sciences KK, Tokyo, Japan).

Detection of miRNA by in situ hybridization assay

In situ detection of miRNA expression was accomplished using formalin-fixed paraffin-embedded tissue sections. Slides were deparaffinized in a xylene series and then rehydrated through an ethanol series. After deparaffinization, the specimens were digested for 30 min using a DIGEST-ALL 3 kit (Invitrogen). The slides were then hybridized overnight with a LNA-modified digoxigenin-labeled probe (Exiqon, Vedbaek, Denmark) in hybridization solution containing 50% formamide, 2× standard saline citrate and 20% dextran sulfate. After stringently washing the slides, Cy3-tyramide signal amplification was carried out, and the signal was detected using a horseradish peroxidase-conjugated antidigoxigenin antibody. Signals were visualized using an AQUA microscope system (Histofix, New Haven, CT).

Statistical analysis

Statistical analyses were carried out using SPSSJ 15.0 (SPSS Japan Inc., Tokyo, Japan). Levels of *miR-34b/c* methylation were compared among groups using analysis of variance or analysis of covariance (for age-adjusted data), followed by post hoc multiple comparison with Sidak correction. Receiver operator characteristic curves were constructed based on the levels of *miR-34b/c* methylation. After categorizing the methylation levels into four quartiles, odds ratios (ORs) for cancer risk or multiple cancer risk were calculated using logistic regression models. Values of *P* < 0.05 (two-sided) were considered significant.

Results

Screening for epigenetically silenced miRNAs in GC cells

To screen for epigenetically silenced miRNAs, we initially carried out miRNA microarray analyses using two GC cell lines (MKN74 and AGS), with or without DAC treatment. In addition, because previous studies have shown that treatment with a combination of DAC and PBA induces stronger reexpression of miRNAs than either agent alone (6,14), GC cells treated with DAC and PBA were also subjected to microarray analysis. We found that the combination treatment induced greater numbers of miRNAs than treatment with DAC alone. Of the 470 miRNAs analyzed in AGS cells, 48 were upregulated (>5-fold) by DAC alone, whereas 135 were upregulated by the combination treatment (supplementary Tables 2 and 3 and Figure 1 are available at Carcinogenesis Online). In MKN74 cells, DAC induced upregulation (>5-fold) of 53 miRNAs, whereas the combination treatment upregulated 150 miRNAs (supplementary Tables 4 and 5 and Figure 1 are available at Carcinogenesis Online). The majority of the miRNAs showing the strongest upregulation were located in the
miRNA cluster on chromosome 19 (C19MC) (supplementary Table 6 is available at Carcinogenesis Online). That this finding is consistent with recent reports validates our drug treatment protocol as well as the findings of the microarray analysis (14,19).

Analysis of miR-34b/c expression in GC cells
Among the miRNAs detected in the microarray analysis, we focused on miR-34b and miR-34c because miR-34s have been strongly implicated in cancer. All three members of miR-34 family are directly regulated by p53 (20,21) and exhibit tumor suppressive effects in human cancer (8,22). Our microarray analysis revealed that both miR-34b and miR-34c are upregulated by DAC in MKN74 and AGS cells, whereas miR-34a was abundantly expressed in both cell lines, with or without DAC treatment. Moreover, the upregulation of miR-34b and miR-34c was further enhanced by the combined treatment with DAC and PBA in both cell types.

We next used TaqMan RT–PCR to analyze the expression of miR-34b/c in a panel of GC cell lines and in normal stomach tissue. Both miR-34b and miR-34c were downregulated in all GC cell lines, as compared with normal stomach tissue (Figure 1A), but DAC treatment restored expression of miR-34b and miR-34c in the GC cells (Figure 1B). We also confirmed that combined treatment with DAC and PBA synergistically upregulated expression of miR-34b and miR-34c in MKN74 and AGS cells (Figure 1C). Thus, miR-34b and miR-34c appears to be epigenetically silenced in GC.

DNA methylation of the miR-34b/c CpG island in GC cells
It was previously reported that mature miR-34b and miR-34c are processed from a single primary transcript, and a CpG island in the proximal upstream region of miR-34b contains promoter activity (Figure 2A) (8,20). To confirm that finding, we carried out luciferase assays using a reporter construct containing the miR-34b/c CpG island and observed high levels of luciferase activity following transient transfection of two GC cell lines (MKN74 and AGS) with the reporter vector (supplementary Figure 2 is available at Carcinogenesis Online). We next asked whether DNA methylation in this region is responsible for the silencing of miR-34b/c in GC cells. Methylation-specific PCR analysis showed that the CpG island was completely methylated in the majority of GC cell lines tested (Figure 2B). In addition, bisulfite pyrosequencing revealed high levels of DNA methylation in the GC cell lines, whereas only limited methylation was found in normal stomach from a healthy individual (Figure 2C). We also carried out bisulfite sequencing in selected samples, which confirmed that the CpG sites in this region are extensively methylated in GC cell lines (Figure 2D). In contrast, the majority of CpG sites are unmethylated in normal stomach (Figure 2D).

Fig. 1. Analysis of miR-34b/c expression in GC cell lines. (A) TaqMan RT–PCR results for miR-34b and miR-34c in a panel of GC cell lines and normal stomach tissue. Results are normalized to internal U6 snoRNA expression. Shown are the means of three replications; error bars represent standard deviations. (B) TaqMan RT–PCR results for miR-34b and miR-34c in the indicated GC cell lines, with (++) or without (−) DAC treatment. (C) TaqMan RT–PCR results for miR-34b and miR-34c in the indicated GC cell lines, with DAC alone or DAC plus PBA or without treatment (mock).
To test whether miR-34b and miR-34c serve as tumor suppressors in GC, we transfected GC cell lines (MKN74, AGS and SNU638) with miR precursor molecules or a negative control and then carried out 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide assays. We observed that 48 h after transfection, ectopic expression of miR-34b and miR-34c had suppressed the growth of all three cell lines (Figure 3A). Real-time RT–PCR analysis revealed that expression of hepatocyte growth factor receptor (MET), cyclin-dependent kinase 4 (CDK4) and cyclin E2 (CCNE2), three well-known targets of miR-34s, was suppressed in the transfected cells (8) (Figure 3B). In addition, western blot analysis revealed significant suppression of MET protein (Figure 3C).

To further clarify the effect of the miRNAs, we used oligonucleotide microarrays to assess global changes in the gene expression profile induced by miR-34b/c in SNU638 cells. We found that 1113 probe sets were downregulated (>1.5-fold) by ectopic miR-34b expression and 3202 were downregulated by ectopic miR-34c expression. And because of the high homology between miR-34b and miR-34c, there was significant overlap between the genes downregulated by the two miRNAs (supplementary Figure 3 and Table 7 are available at Carcinogenesis Online). Our microarray analysis also revealed that a number of cell cycle-related genes were downregulated by miR-34b/c, and gene ontology analysis showed that ‘cell cycle phase’ genes and ‘mitosis’ genes were the most enriched among the downregulated genes (supplementary Table 8 is available at Carcinogenesis Online). We next searched for significant pathways affected by miR-34b/c using the list of downregulated genes. We found that a number of cell cycle-related processes (e.g. M phase, mitosis and the G2/M transition checkpoint) were affected by miR-34b/c in GC cells (supplementary Figure 4 is available at Carcinogenesis Online), and downregulation of a representative gene (CCNA2) was confirmed by real-time RT–PCR and western blot analysis (Figure 3B and C).

Finally, using real-time RT–PCR, we observed that cell cycle-related genes (CDK4, CCNE2 and CCNA2) were also downregulated in AGS cells treated with DAC plus PBA, suggesting reexpression of endogenous miRNAs can exert effects similar to those seen with transfection of exogenous miR precursor molecules (Figure 3D).

Analysis of miR-34b/c methylation and expression in primary GC
We next analyzed the methylation of the miR-34b/c CpG island in a panel of tumor specimens from GC patients. Using bisulfite methylation analysis of the miR-34b/c CpG island in GC cells. (A) Diagram of the miR-34b/c CpG island. The regions analyzed using methylation-specific PCR (MSP), bisulfite sequencing and bisulfite pyrosequencing are indicated by bars below the CpG sites. (B) MSP analysis of the miR-34b/c CpG island in a set of GC cell lines. In vitro methylated DNA (IVD) and DNMT KO cells served as positive and negative controls, respectively. Bands in the ‘M’ lanes are PCR products obtained with methylation-specific primers and those in the ‘U’ lanes are products obtained with unmethylated-specific primers. (C) Bisulfite pyrosequencing analysis of miR-34b/c in GC cell lines. A representative result in AGS cells is shown above, and the percent methylation at seven CpG sites is indicated on the top. Shown below are the summarized results of bisulfite pyrosequencing in GC cell lines and normal stomach tissue. (D) Bisulfite sequencing of the miR-34b/c CpG island in the indicated GC cell lines and normal stomach tissue. Open and filled circles represent unmethylated and methylated CpG sites, respectively.
pyrosequencing, we detected elevated levels of miR-34b/c methylation (>15.0%) in 83 of 118 (70.3%) primary GCs tested. In contrast, only limited (15.0%) methylation was detected in normal gastric mucosa from H. pylori-negative healthy individuals, indicating that methylation of the miR-34b/c region is a tumor-predominant phenomenon (Figure 4A). We confirmed these results with bisulfite sequencing in selected specimens. Tumor tissue showed a mixture of entirely and partially methylated alleles, as well as unmethylated alleles that probably reflected contamination of the sample by normal cells (Figure 4B). No significant correlation between miR-34b/c methylation and clinicopathological characteristics or between p53 mutation and miR-34b/c methylation were observed (data not shown).

We then performed TaqMan RT–PCR to assess the expression of miR-34b/c in normal gastric mucosa from healthy individuals (n = 7) and primary GC tissues harboring miR-34b/c methylation (n = 14). We found substantial downregulation of miR-34b/c expression in the tumor tissues, as compared with normal gastric mucosa (Figure 4C). We also used in situ hybridization to assess the spatial distribution of the miRNAs in GC tissues. Using a specific probe, we observed expression of miR-34b/c in a colorectal cancer cell line treated with DAC but not in untreated cells (data not shown). In primary GC specimens, miR-34b/c expression was downregulated in tumor tissues but was expressed in adjacent non-cancerous tissues (a representative result in supplementary Figure 5, available at Carcinogenesis Online).

Elevated methylation of miR-34b/c in non-cancerous gastric mucosa

One recent study showed hypermethylation of several miRNA genes in non-cancerous gastric mucosa from GC patients, which suggests the possible involvement of miRNA gene methylation in an epigenetic field defect. We therefore analyzed samples of endoscopically obtained non-cancerous gastric mucosa from 109 GC patients (32 patients with synchronous or metachronous multiple GC and 77 with single GC) and samples of normal gastric mucosa from 85 healthy individuals (78 individuals with H. pylori and 7 without). Biopsy specimens were obtained from the gastric body and antrum of each individual, and the average methylation level of the two specimens was determined. Among the healthy individuals, the mean levels of miR-34b/c methylation in H. pylori-positive and negative gastric mucosa were 20.7% and 7.8%, respectively, suggesting that methylation of miR-34b/c is associated with H. pylori infection (Figure 4D, Table I). In cancer patients, the mean methylation level in non-cancerous gastric mucosa was 22.7%, which is about the same as in the H. pylori-positive healthy individuals. On the other hand, we found that the methylation levels were significantly higher in patients with multiple GC than in those...
with single GC (27.3 versus 20.8%; P < 0.001) (Figure 4D, Table I). Because there were age differences between these two groups, we also calculated the age-adjusted levels of miR-34b/c methylation and compared them between these groups using analysis of covariance with post hoc multiple comparisons. We found the same tendency in both the crude and the age-adjusted models, indicating that age-related differences in methylation did not account for the results (supplementary Table 9 is available at Carcinogenesis Online).
Paired non-cancerous gastric mucosae and GC tissues were available from several patients (26 with a single GC and 12 with multiple GCs), which enabled us to compare the methylation levels between the two tissues. Interestingly, although the methylation levels of non-cancerous and cancerous tissues did not significantly differ in patients with a single GC (22.1 versus 22.8%; \( P = 0.710 \)), cancer tissues showed significantly higher levels of methylation than their non-cancerous counterparts in patients with multiple GCs (32.3 versus 41.4%; \( P = 0.011 \)) (Figure 4E).

To assess the association between miR-34b/c methylation and GC, we categorized the gastric mucosa specimens into four quartiles of methylation (Table II). As compared with individuals who had the least miR-34b/c methylation (\( \leq 17.5\% \)), having the highest methylation (\( > 25.4\% \)) was not significantly associated with GC (age-adjusted OR 2.1; \( P = 0.125 \); 95% confidence interval 0.8–5.4), though it was strongly associated with multiple GC (age-adjusted OR 27.7: 95% confidence interval 3.3–228.9) (Table II). Moreover, when we calculated the ORs for multiple GC adjusted for age, gender, H. pylori status and gastritis grade, we found them to be even more significant (Table II), which suggests hypermethylation of miR-34b/c in non-cancerous gastric mucosae is an independent additive risk for multiple GC.

We also generated a receiver operator characteristic curve to assess the clinical utility of DNA methylation for the prediction of GC (Figure 4F and G). Although miR-34b/c methylation failed to distinguish between non-cancerous gastric mucosa from GC patients and normal gastric mucosa from healthy individuals (area under the curve = 0.639) (Figure 4F), it was highly discriminative between gastric mucosa from patients with multiple GC and mucosa from patients with single GC or from healthy individuals (area under the curve = 0.843) (Figure 4G). The most discriminating cutoff of miR-34b/c methylation for multiple GC was 23.1% (sensitivity 90.6% and specificity 72.8%). This suggests that methylation of miR-34b/c may be a useful marker with which to screen individuals at a high risk of GC.

Discussion

Dysregulation of miRNA expression is commonly observed in wide variety of cancers, and epigenetic mechanisms have been shown to be key mediators underlying the downregulation of miRNA expression. To screen for epigenetically silenced miRNA genes in GC, we first performed a microarray analysis to identify miRNAs upregulated by demethylation and histone deacetylase inhibition. Consistent with recent reports, these treatments significantly upregulated expression of the C19MC in GC cell lines. The C19MC is composed of 46 miRNA genes, forming a cluster spanning ~100 kb on chr19q13.41. These miRNA genes are interspersed among Alu repeats and, with the exception of the placenta, are silenced in human tissues (23).

Our microarray analysis also identified a number of miRNAs whose silence is reportedly associated with DNA methylation in cancer. For example, miR-127 is the first miRNA gene known to be activated by epigenetic drug treatment in cancer cells (6). In addition, methylation of miR-9 and miR-148a has been observed in human metastatic cancer cell lines (24), and miR-203 is epigenetically silenced in hematopoietic malignancies, which leads to enhanced expression of ABL1 and BCR-ABL1 (25). We found that these miRNA genes are also hypermethylated in cultured and primary GCs (data not shown), though further study is needed to clarify their role in gastric carcinogenesis.

MiR-34s have been strongly implicated in cancer. For example, miR-34a reportedly acts as a tumor suppressor in colon cancer and neuroblastoma (22,26). All three miR-34 family members are directly regulated by p53, and ectopic expression of miR-34s induces cell cycle arrest and/or apoptosis in human cancer cells (20,21,27–30). Conversely, expression of miR-34s is frequently downregulated in human malignancies, including lung, colon and ovarian cancer (8,20,22,31). Genes-encoding miR-34a and miR-34b/c are located in 1p36.23 and 11q23.1, respectively, and both are targets of epigenetic silencing in cancer (8,32). In particular, we and others recently showed that silencing of miR-34b/c is associated with CpG island hypermethylation in colon and oral cancers (8,33). In addition, Lujambio et al. (24) identified miR-34b/c methylation by screening cell lines derived from metastatic colon cancer, melanoma and head and neck cancer and Conroy et al. (31) recently reported downregulation of miR-34b/c in metastatic ovarian cancer, which suggests inactivation of miR-34b/c may be associated with cancer metastasis.

In the present study, we found that miR-34b and miR-34c are significantly upregulated by epigenetic drug treatment in GC cells, whereas miR-34a is abundantly expressed without treatment, which was consistent with our earlier observation in colon cancer cells (8). Apparently, the gene-encoding miR-34b/c is epigenetically silenced in a majority of GC cell lines, and silencing is associated with hypermethylation of the neighboring CpG island. In addition, our functional study suggests that miR-34b/c may be a useful therapeutic target in GC as their ectopic expression significantly downregulated their target genes (e.g. CDK4 and MET) and suppressed GC cell proliferation. Microarray analysis revealed that miR-34b/c induces dramatic changes in the gene expression profiles in GC cells and that cell cycle-related genes are the most significantly affected, which is consistent with earlier observations in colon and lung cancer cells (8,20,21). Methylation of miR-34b/c was observed in 70% of primary GC specimens, though no correlation between miR-34b/c methylation and p53 mutation was found. The high rate of miR-34b/c methylation in GC and our functional analysis suggest that they act as tumor suppressors in response to gastric tumorigenesis. Thus, reactivation of miR-34b/c could be an effective therapeutic strategy for the treatment of GC.

H. pylori is a major carcinogenic factor in the stomach, and a number of studies have shown that it induces aberrant DNA methylation in gastric epithelial cells (34–37). Recently, Ando et al. (13) reported that miR-124a family genes (miR-124a-1, -2 and -3) are frequently methylated in primary GC and in normal gastric mucosa from healthy individuals with \textit{H. pylori} infections. Among \textit{H. pylori}-negative individuals, methylation levels are significantly higher in non-cancerous gastric mucosae from GC patients than gastric mucosae from healthy individuals, which suggest methylation of miRNA genes contributes to a field defect contributing to the pathogenesis of GC (13). We also observed that miR-34b/c methylation is significantly associated with \textit{H. pylori} infection among healthy individuals.
individuals. Levels of miR-34b/c methylation in non-cancerous gastric mucosa from patients with single GC were similar to those in gastric mucosa from H. pylori-positive healthy individuals. It is noteworthy, however, that non-cancerous gastric mucosa from patients with multiple GC showed even higher methylation levels, suggesting that miR-34b/c methylation may be a useful marker predictive of the risk of GC recurrence.

In summary, we have shown that a novel miRNA gene is often epigenetically silenced in GC. Taken together, the high rate of miR-34b/c methylation and the results of our functional study suggest that they are novel tumor suppressor genes in GC. In normal stomachs of healthy individuals, moderate levels of miR-34b/c methylation are associated with H. pylori infection. Moreover, the higher methylation levels seen in non-cancerous gastric mucosa from patients with multiple GC strongly suggest that methylation is involved in an epigenetic field defect contributing to GC development. Our results therefore suggest that methylation of miR-34b/c could serve as a useful tumor marker and that restoration of its expression could be an effective anticancer therapy.

Supplementary material
Supplementary Figures 1–5 and Tables 1–9 can be found at http://carcin.oxfordjournals.org/

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