Involvement of epigenetically silenced microRNA-181c in gastric carcinogenesis

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Abbreviations: 5-aza-CdR, -2’-deoxycytidine (5-aza-CdR)-treated gastric cancer cell line, KATO-III. On microarray analysis, five miRNAs were found to be upregulated (>3-fold) after 5-aza-CdR treatment compared with untreated cells. Among them, miR-181c and miR-432AS exhibited CpG islands in their upstream sequences on computational analysis, and their upregulation was verified by reverse transcription–polymerase chain reaction (RT–PCR) analysis in particular, miR-181c upregulation was found not only in KATO-III but also in two other gastric and one colorectal cancer cell line with 5-aza-CdR treatment. Decreased expression of miR-181c was observed in 9 of 16 primary gastric carcinoma (GC) cases compared with the corresponding non-cancerous stomach tissues. Hypermethylation signals in the upstream region of miR-181c were observed in some cultured and primary GC cells with negative or low miR-181c expression. Transfection of the precursor miR-181c molecule induced decreased growth of two gastric cancer cell lines, KATO-III and MKN45. As for targets of miR-181c, oncogenic NOTCH4 and KRAS were identified by complementary DNA microarray analysis after precursor miR-181c molecule transfection, computational searches of miRNA target databases and reporter assay using the 3’-untranslated regions of the two genes. These results indicate that miR-181c may be silenced through methylation and play important roles in gastric carcinogenesis through its target genes, such as NOTCH4 and KRAS.

Introduction

MicroRNAs (miRNAs) are non-coding small RNAs, which have important roles in tumorigenesis (1,2). miRNA suppresses the translation and/or stability of target messenger RNAs (mRNAs) by binding to the 3’-untranslated regions (3’-UTRs) of target mRNAs, and it causes cell proliferation, differentiation or apoptosis (1). Expression changes of miRNAs have been reported in a variety of human cancers. For example, let-7 is downregulated in several cancers and negatively regulates expression of the Ras family (3,4). miR-15 and miR-16 are considered to target the anti-apoptotic factor B-cell lymphoma protein 2 in chronic lymphocytic leukemia (5).

Epigenetic modifications of DNA, such as DNA promoter hypermethylation and histone modification, have critical roles in chromatin remodeling and general regulation of gene expression in mammalian development and human diseases (6). DNA methylation of CpG islands in promoter regions has been correlated with silencing of tumor suppressor genes and tumor-related genes and has been recognized as a crucial component of the mechanism underlying tumorigenesis (7).

It has also been reported that DNA methylation-associated silencing of miRNAs occurs in several human tumors (8–11).

Gastric carcinoma (GC) is the second most frequent cause of death from cancer in both sexes in the world (12). To date, many tumor suppressor genes and tumor-related genes associated with DNA methylation have been reported in GCs (13,14). However, the molecular mechanism underlying gastric carcinogenesis remains unclear (15). As for miRNAs, upregulation and downregulation of miRNA expression in GCs have been found (16–18) but it has rarely been determined whether or not DNA methylation is involved in downregulation of miRNA in GC. Thus, we focused on aberrant miRNA expression, particularly that of DNA methylation-related ones, in GC. In this study, we analyzed expression changes of miRNA by miRNA microarray analysis in GC cells with 5-aza-2’-deoxycytidine (5-aza-CdR) treatment and further characterized the roles of miRNAs in gastric carcinogenesis.

Materials and methods

Cell lines and tissue samples

We studied 8 GC cell lines (KATO-III, MKN7, MKN45, MKN74, TGBCi11TKB, AGS, HSC58 and G6CY), 2 colorectal cancer (CRC) cell lines (HCT116 and DKO), 1 human embryonic kidney cell line (293T) and 16 primary gastric cancer patients were randomly obtained from the Affiliated Hospital of School of Medicine, Tokyo Medical and Dental University. Informed consent was obtained from all subjects, and the study was approved by the institutional review committee.

Drug treatment of cells and RNA extraction

For demethylation studies, cells were daily treated with 5 μmol/l 5-aza-CdR (Sigma–Aldrich, St Louis, MO) for 72 h. Total RNA was isolated by using Trizol reagent (Invitrogen, Carlsbad, CA) or a miRCeasy mini kit (Qiagen, Hilden, Germany).

Microarray analysis

miRNA microarray analysis was performed with mirVana miRNA Bioarrays V2 (Ambion, Austin, TX) according to the manufacturer’s instructions. Complementary DNA (cDNA) microarray analysis was conducted by DNA Chip Research (Kanagawa, Japan) with Whole Human Genome oligo DNA arrays (Agilent Technologies, Santa Clara, CA).

End point reverse transcription–polymerase chain reaction procedure

For single-stranded cDNA synthesis, 1 μg (for cell lines) or 2 μg (for tissue samples) of total RNA was reverse transcribed using Superscript II (Invitrogen). The amplification was performed by denaturation at 95°C for 4 min, followed by 21–40 cycles of 1 min each at 95°C, 55–64°C and 72°C and final extension at 72°C for 5 min. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) RNA expression was used as a loading control. The primer sequences and polymerase chain reaction (PCR) product sizes are shown in supplementary Table 1 (available at Carcinogenesis Online).

Quantitative real-time reverse transcription–polymerase chain reaction

Real-time reverse transcription–polymerase chain reaction (RT–PCR) was carried out using a StepOne Real-time PCR System (Applied Biosystems, Foster City, CA), TaqMan Universal PCR Master Mix (Applied Biosystems), a TaqMan Reverse Transcription kit (Applied Biosystems) and TaqMan miRNA assays (Applied Biosystems) according to the manufacturer’s instructions. The expression levels of miRNA were calculated on the amount of target miRNA relative to that of RNU6B as a control to normalize the initial input of total RNA.

Determination of the transcription start site by a 5’-rapid amplification of cDNA ends method

MKN7 total RNA (1 μg) was reverse transcribed into cDNA using SuperScript II reverse transcriptase and a gene specific primer 1 (GSP1). After adding poly-C tail to the 5’-ends of the products with terminal deoxynucleotidyl transferase, cDNAs were amplified by PCR with other specific primers (anchor primers, GSP2 and GSP3) and then the amplified products were sequenced.
The primer sequences are shown in supplementary Table 1 (available at Carcinogenesis Online).

**Methylation analysis**

Bisulfite treatment of DNA was performed with Methylamp (Epigentek, Brooklyn, NY). Bisulfite sequencing and methylation-specific polymerase chain reaction (MSP) analyses were performed as described previously (19).

The primer sequences and PCR product sizes are shown in supplementary Table 1 (available at Carcinogenesis Online).

**Synthetic miRNA transfection**

KATO-III and MKN45 cells were transfected with Precursor Molecule mimicking miR-181c (Pre-miR-181c) (Ambion) or scrambled sequence miRNA (Pre-miR Negative Control #1; Ambion) to give a final concentration of 

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**Fig. 1.** Expression profiling of human miRNAs in GC and CRC cell lines after 5-aza-CdR treatment. (A) A flow chart used for DNA methylation-associated repression of miRNAs in GC cells. (B) RT–PCR analyses of precursor miR-181c and miR-432AS in KATO-III and several human cancer cell lines untreated (U) or treated (A) with 5-aza-CdR (5 μmol/l). Precursor miR-181c expression was also analyzed in KATO-III cells untreated (U) and treated with 0.2 μmol/l of 5-aza-CdR (A0.2), 0.3 μmol/l of TSA (T0.3) or a combination of these two drugs (AT). GAPDH RNA expression was used as a loading control. (C) Quantitative real-time RT–PCR analysis of mature miR-181c expression in GC cell lines, KATO-III and MKN45, and in CRC cell lines, HCT116 and DKO. Bars, SD; **, P < 0.05; ***P < 0.01. The expression levels of 5-aza-CdR-treated cells (A) or DKO were independently calculated relative to those of untreated cells (U), which are normalized to 1. Since the numbers of threshold cycles of miR-181c in KATO-III, MKN45 and HCT116 cells were >35, the expression level in these cells was determined as negative. (D) Quantitative real-time RT–PCR analysis of mature miR-181c expression in three non-cancerous stomach mucosae (NS1-3).
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Fig. 2. Expression of miR-181c in human GC specimens. Quantitative real-time RT–PCR for miR-181c was carried out by using 16 human GC surgical specimens (filled bars) and paired non-cancerous counterparts (open bars). The expression levels of carcinomas were independently calculated relative to those of non-cancerous specimens, which are normalized to 1; bars, SD; **P < 0.01, ***P < 0.001.

10–100 nmol/l by using MicroPorator MP-100 (Digital BioTechnology, Seoul, Korea), according to the manufacturer’s instructions. At 24–72 h after transfection, cells were harvested for western blot or RT–PCR analysis.

Cell proliferation assay
Pre-miR-181c-transfected KATO-III and MKN45 cells were plated at 1 × 10⁴ cells per well on 96-well plates. Cell proliferation was evaluated on days 1, 3 and 4 after transfection by determining the number of cells with cell proliferation reagent water solved tetrazolium 1 (Roche, Mannheim, Germany) according to the manufacturer’s instructions.

miRNA target prediction and western blotting
The predicted targets of miR-181c and their target sites were analyzed using miRBase, TargetScan, PicTar and RegRna. The mRNA expression levels of the predicted targets in transiently transfected cells were analyzed 24 h after transfection by RT–PCR. Western blot analyses were performed as described previously (19). The primary antibodies used were rabbit anti-NOTCH4 (1:400; sc-5594, Santa Cruz Biotechnology, Santa Cruz, CA) and mouse anti-p21 (1:2000; Cell Signaling Technology, Danvers, MA). The secondary antibodies used were alkaline phosphatase-conjugated anti-rabbit IgG and anti-mouse IgG (1:2000; Bio-Rad Laboratories, Hercules, CA). Blots were developed with Immuno-Star AP Substrate (Bio-Rad Laboratories).

Dual luciferase reporter assay
Luciferase constructs were made by ligating oligonucleotides containing the putative target site of the NOTCH2, NOTCH4 or KRAS 3’-UTR into the XbaI site of the pGL4.13 (Luc2/SV40) firefly luciferase reporter vector (Promega, Madison, WI). 293T cells were cotransfected using HiPerFect (Qiagen) with 1 ng of the pGL4.13 vector containing or not containing the 3’-UTR sequences, 1 ng of the pGL4.74 (hRluc/TK) renilla luciferase control vector and 10 nmol/l of Pre-miR-181c. Luciferase activity was measured 24 h after transfection using a Dual-Luciferase Reporter Assay System (Promega). Relative luciferase activity was calculated by normalizing the firefly luminescence as to the renilla luminescence.

Results
Search for miRNAs upregulated by 5-aza-CdR treatment in GC cells
miRNA microarray analysis was performed using a human GC cell line, KATO-III, treated with or without 5-aza-CdR. Five of 328 human miRNAs examined were highly upregulated (>3-fold) in 5-aza-CdR-treated cells (Figure 1A, supplementary Table 2 is available at Carcinogenesis Online, GEO accession No. GSE16006). We next searched the human genome database (NCBI BLAST search) for the presence of CpG islands around these five miRNAs and they were found to be located on/around (upstream 2000 bp) CpG islands. Among them, epigenetic inactivation of miR-9 has been reported in breast cancer (20). When we examined expression changes of the remaining four miRNAs, miR-181c, miR-211, miR-432AS and miR-495, between untreated and 5-aza-CdR-treated KATO-III cells by RT–PCR at the precursor miRNA level, upregulation of two miRNAs (miR-181c and miR-432AS) was confirmed. Thus, we focused on miR-181c and miR-432AS and analyzed the expression changes of these miRNAs between untreated and 5-aza-CdR-treated gastrointestinal cancer cell lines with undetectable expression of miR-181c and/or miR-432AS by RT–PCR at the precursor miRNA level (Figure 1B). Precursor miR-181c expression increased after 5-aza-CdR treatment in three of four GC cell lines, KATO-III, MKN45 and TGBC11TKB, and in a CRC cell line, HCT116 (Figure 1B, upper column), but miR-432AS expression only increased in one of six GC cell lines, KATO-III (Figure 1B, bottom column). We, therefore, further analyzed miR-181c.

To investigate the relationship between miR-181c expression and DNA promoter methylation, we analyzed miR-181c expression in a CRC cell line, HCT116, 5-aza-CdR-treated HCT116 and a DNA methyltransferase 1/3B knock out HCT116 cell line, DKO, and its expression was much higher in DKO cells compared with HCT116 (Figure 1B, upper column). Expression change of miR-181c was also analyzed in KATO-III cells treated with a low dose of 5-aza-CdR (0.2 μmol/l), a histone deacetylase inhibitor, trichostatin A (TSA, 0.3 μmol/l) or a combination of these two drugs. KATO-III cells with low-dose 5-aza-CdR treatment exhibited upregulation of miR-181c, whereas TSA alone did not. miR-181c was synergistically upregulated in KATO-III cells with combined 5-aza-CdR and TSA treatment (Figure 1B, upper column). In addition, with a highly sensitive TaqMan miRNA assay, mature miR-181c was also found to be upregulated by 5-aza-CdR treatment (Figure 1C). On the other hand, three independent non-cancerous stomach mucosae (NS1-3) exhibited higher expression of miR-181c by the TaqMan assay (Figure 1D). These results indicate that miR-181c expression may be downregulated through DNA methylation.

miR-181c expression in human primary GC cases
To determine the miR-181c expression levels in primary GC samples, we examined miR-181c expression using TaqMan RT–PCR in primary GC tissues and corresponding non-cancerous mucosae. A significant reduction of miR-181c expression in GC tissues was observed in 9 of 16 cases (cases 2, 4, 6, 7, 9, 10, 11, 13 and 14) (Figure 2).
Then, we carried out bisulfite sequencing in two upstream regions of transcription start site, BS1 and BS2, which contain CpG islands. As shown in Figure 3C, the CpG islands in a region from \( /C0_{1297} \) to \( /C0_{987} \) (BS2) upstream of the precursor miR-181c sequence were extensively methylated in KATO-III and MKN45 cells, in which miR-181c expression was undetectable. On the other hand, GCIY cells highly expressing miR-181c (Figure 1B) exhibited a pattern of less methylation in the same region (Figure 3C), and no methylation was observed in DKO cells (data not shown), which highly expressed miR-181c. When we analyzed the methylation status of the region after 5-aza-CdR treatment by bisulfite sequencing, demethylation was seen in KATO-III cells (Figure 3C). Thus, the methylation status of these CpG sites was consistent with the miR-181c expression in GC cell lines.

For MSP analyses, we initially tried three primer sets designed in the upstream region of the miR-181c sequence containing CpG islands (Figure 3A), but none of them exhibited complete matches with miR-181c expression. Thus, according to bisulfite sequencing data, we designed MSP primers in the BS2 region (Figure 3C). GC cell lines with undetectable miR-181c expression, KATO-III, MKN45 and GCIY, exhibited only methylation signals, whereas expression-positive cell lines, HSC58 and GCIY, exhibited strong unmethylation and weak methylation signals (Figure 3D), which is consistent with bisulfite sequencing data, such as in GCIY cells (Figure 3C).

We next examined the methylation statuses of the CpG islands in the region from \(-1297 \) to \(-987 \) (BS2) in primary GCs and corresponding non-cancerous tissues by bisulfite sequencing analyses. All the non-cancerous tissues exhibited less methylation patterns (Figure 3E).
GC cases 2 and 9 exhibiting lower miR-181c expression than corresponding non-cancerous tissues revealed a stronger methylation pattern than corresponding non-cancerous tissues, whereas GC cases 3 and 15 with medium miR-181c expression revealed a similar methylation pattern to corresponding non-cancerous tissues (Figure 3E).

We also analyzed the methylation statuses in primary GC tissues by MSP. GC tissues with lower miR-181c expression, cases 2 and 9, revealed only methylation signals, whereas corresponding non-cancerous tissues revealed strong unmethylation and weaker methylation signals. GC and corresponding non-cancerous tissues with medium miR-181c expression, cases 3 and 15, revealed both methylation and unmethylation signals (Figure 3F). These data are consistent with bisulfite sequencing data (Figure 3E).

**Effect of Pre-miR-181c transfection on GC cell proliferation**

To study the function of miR-181c in GC cell lines, we transfected two GC cell lines, KATO-III and MKN45, expressing undetectable miR-181c levels with either Pre-miR Negative Control #1 or Pre-miR-181c. At 4 days after transfection of Pre-miR-181c, a significant reduction of cell growth was observed in Pre-miR-181c-transfected cells compared with negative controls for both cell lines (Figure 4).

**Identification of target genes of miR-181c**

To clarify the cause of the cell growth reduction with Pre-miR-181c treatment, we tried to determine the target genes of miR-181c. miRNA suppresses the translation and/or stability of target mRNAs. Since there is no comprehensive method for determining the targets of miRNA at the protein level, we used cDNA microarray analysis to search for target mRNAs of which the levels might decrease according to exogenous miRNA expression. We transfected KATO-III cells with Pre-miR-181c and carried out cDNA microarray analysis (GEO accession No. GSE16239). Three hundred and sixty genes were upregulated (>1.5-fold) and 578 genes were downregulated (<0.67-fold) by ectopic miR-181c expression. Pathway analysis indicated that some tumorigenesis-related pathways, such as those of NOTCH signaling ($P < 0.021$), Delta-NOTCH NetPath ($P = 0.039$) and G protein signaling ($P = 0.046$), were downregulated by miR-181c. On the other hand, cell cycle G1 to S control reactome ($P = 0.028$), Hedgehog NetPath ($P = 0.038$) and apoptosis ($P = 0.041$) were upregulated by miR-181c (supplementary Table 3A is available at Carcinogenesis Online). In particular, NOTCH homolog 2/4 (NOTCH2 and NOTCH4) and v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (KRAS) were decreased by 0.6-, 0.52- and 0.57-fold, respectively (supplementary Table 3B is available at Carcinogenesis Online).

To alternatively predict the target genes of miR-181c, we performed computational searches of miRNA target databases (miRBase, TargetScan and PicTar) and miRNA binding site searching site RegRna (http://regrna.mbc.nctu.edu.tw/html/prediction.html). NOTCH2 (RegRna), NOTCH4 (miRBase) and KRAS (PicTar) were registered as targets of miR-181c, respectively (Figure 5A, supplementary Table 4 is available at Carcinogenesis Online). Therefore, NOTCH2, NOTCH4 and KRAS were postulated to be targets of miR-181c using two different methods. We validated the microarray results by RT–PCR analysis with...
Pre-miR-181c transfection into KATO-III cells (Figure 5B). Interestingly, p21 (WAF1/Cip1) expression associated with cell cycle G1 to S control reactome was upregulated by miR-181c transfection (Figure 5B). These gene expression changes with miR-181c were seen in MKN45 cells as well (Figure 5B). The downregulation of NOTCH4 and upregulation of p21 (WAF1/Cip1) were also observed at the protein level (Figure 5C). The effects of 5-aza-CdR and/or TSA in KATO-III cells on miRNA targets were also measured by RT–PCR. The downregulation of NOTCH4 and KRAS and upregulation of p21 (WAF1/Cip1) were observed in drug-treated cells (Figure 5D).

The expression of precursor miR-181c and possible target genes, KRAS, NOTCH2 and NOTCH4, was examined by RT–PCR in eight GC cell lines (Table I). The expression patterns of the three genes were mostly opposite to that of miR-181c. Moreover, we compared expression patterns of NOTCH4 with that of miR-181c by RT–PCR in primary tumor samples. Some samples, such as cases 2 and 10, exhibited opposite patterns, but others exhibited ambiguous patterns. It is, however, noteworthy that most GC samples showed higher NOTCH4 expression than non-cancerous stomach mucosae (NS1-3) (data not shown).

**Reporter assay using the 3′-UTR regions**

To determine whether the predicted target sites for miR-181c in the 3′-UTRs of NOTCH2/4 and KRAS mRNAs were responsible for the translational repression, we performed dual luciferase reporter assays with vectors containing the 3′-UTR target sites of NOTCH2/4 or KRAS in KATO-III cells (Figure 5A). Statistically significant repression of luciferase activity was observed in 293T cells cotransfected with Pre-miR-181c and reporter vectors containing the NOTCH4 or KRAS 3′-UTR target sites (Figure 5E). On the other hand, no notable alteration of luciferase activity was detected between the Pre-miR-181c transfectant and the control counterpart in the case of NOTCH2. We made mutants-deleting sequences, which correspond to the miR-181c seed sequence, in the NOTCH4 3′-UTR reporter construct. The luciferase activity was not repressed in 293T...
Expression of miR-181c and possible target genes in GC cell lines

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Fig. 5. continued

Discussion

Although it has been reported that the expression of some miRNAs was decreased in GCs (16,17), the relationship between aberrant expression of miRNAs and DNA methylation was not clear in GCs. In this study, according to the results of microarray analysis and the presence of CpG island around miRNA, we focused on miR-181c. miR-181c upregulation was found in four gastrointestinal cell lines after 5-aza-CdR treatment. The methylation patterns were consistent with miR-181c expression in gastrointestinal cancer cell lines and primary GC tissues. Furthermore, transfection of Pre-miR-181c induced decreased growth in two GC cell lines. These results indicate that miR-181c may be silenced through DNA methylation and that it has a tumor-suppressive function in the stomach.

Ando et al. (21) analyzed methylation levels of miR-124a-1-3 in normal gastric mucosa of healthy volunteers and non-cancerous mucosa and cancer tissues of GC patients with or without Helicobacter pylori infection. They indicated that methylation of these miRNA genes was associated with silencing of miR-124a and Helicobacter pylori infection and involved in the formation of epigenetic field defect in non-cancerous mucosa for GCs. Our data also revealed that there were some methylation signals of miR-181c in non-cancerous mucosa by the bisulfite sequencing and MSP analyses (Figure 3E and F), suggesting epigenetic field defect. It has been reported that combination of 5-aza-CdR and a histone deacetylase inhibitor treatment activates several miRNAs, including miR-512-5p in a GC cell line (22), but they have not reported on the methylation status in the upper regions of miRNA either in GC cell lines or in primary GCs. Therefore, our data are the first report indicating that one of the mechanisms underlying decreased expression of a tumor-suppressive miRNA in GCs is DNA methylation.

Recent studies indicated that some miRNAs were regulated by epigenetic factors in colorectal, lung, bladder, breast and oral cancers, and the miRNA levels were recovered on 5-aza-CdR treatment (8,9,11). Thus, abnormal expression of miRNAs through epigenetic changes may be related to the development of cancer not only in other organs but also in the stomach.

Here, we report decreased expression of miR-181c in GC cell lines and 9 of 16 primary GCs. However, 2 of 16 GC cases exhibited significant upregulation of miR-181c compared with corresponding non-cancerous tissues. Aberrant expression of miR-181c has also been reported in other tumors. In glioblastoma, miR-181c was downregulated compared with the normal brain (23). Upregulation of miR-181 family members (miR-181s) including miR-181c was observed in hepatic stem cell-like hepatocellular carcinomas, whereas downregulation of miR-181s was seen in mature hepatocyte-like ones, suggesting important functions of miR-181s in maintaining an undifferentiated state of hepatic progenitor cells (24). miR-181 has also been shown to be related to differentiation of hematopoietic B cells (25) and myoblasts (26). Therefore, although downregulation of miR-181c was more prevalent than upregulation in the case of GCs, it will be important to analyze more GC samples to clarify the role of miR-181c in gastric carcinogenesis as well as in gastric cell differentiation.

miR-181d is located closely downstream of miR-181c and these two miRNAs belong to the same cluster. However, on miRNA microarray analysis, upregulation of miR-181c was high (3.46-fold), whereas that of miR-181d (1.59-fold) was not in KATO-III cells with 5-aza-CdR treatment. We also analyzed expression changes of miR-181d at the precursor miRNA level after 5-aza-CdR and/or TSA treatment. miR-181d was slightly upregulated but upregulation of miR-181c was stronger than miR-181d (data not shown), suggesting that miR-181d involvement might be smaller in GC cells.

We investigated potential targets of miR-181c. The cDNA microarray results indicated that ectopic expression of miR-181c repressed several oncogenes, such as NOTCH2/4 and KRAS. The mammalian family of Notch receptors consists of four members, NOTCH1 through NOTCH4, and plays important roles in cell fate determination. As for roles of the NOTCH family in tumorigenesis, several studies have indicated that NOTCH signaling can both oncogenic and tumor suppressive (27). NOTCH2 was overexpressed in cancers of the cervix (28), colon, pancreas (29) and so on (27). NOTCH4 was originally identified at a frequent insertion site of the mouse mammary tumor virus in mice and was found to be involved in the development of mammary tumors. Transgenic mice expressing activated Notch4 develop mammary adenocarcinomas (30). Elevated levels of NOTCH4 were detected in seven of eight human breast cancer cell lines (31). We also observed higher NOTCH4 expression in most GC cases. As indicated on reporter assaying, miR-181c repressed the construct with the NOTCH4 3’-UTR but not that with the NOTCH2 3’-UTR, indicating that NOTCH4 may be a more probable target of miR-181c than NOTCH2. To our knowledge, this is the first report that miRNA regulates human NOTCH gene expression. A relationship between NOTCH signaling and GCs has been rarely reported (32). Further studies are necessary to clarify the roles of NOTCH2/4 in gastric carcinogenesis.

Tumor suppressor p21 (WAF1/Cip1) expression increased on ectopic expression of miR-181c in KATO-III cells. Since KATO-III cells lack the p53 gene (33), the increase of p21 in the Pre-miR-181c-transfected KATO-III cells would be p53 independent. Notch1 activation downregulates or upregulates p21 (WAF1/Cip1) expression depending on the cell type (27,34). Thus, the expression change of p21 (WAF1/Cip1) may be induced by NOTCH signaling.

Ectopic expression of miR-181c also repressed expression of KRAS, which is one of the RAS family and is known as a proto-oncogene (35). The reporter assays revealed that miR-181c suppressed the construct containing the KRAS 3’-UTR, indicating direct
regression of KRAS expression. Furthermore, the expression pattern of KRAS was opposite to that of miR-181c in eight GC cell lines. It has been reported that mutations and amplification of KRAS were observed in some GCs, and siRNA-mediated knockdown of KRAS-induced growth inhibition in GC cell lines (35,36). Therefore, reduced expression of miR-181c may play an important role in gastric carcinogenesis through KRAS overexpression.

Our data indicate that NOTCH2/4 and KRAS are possible targets of miR-181c in GCs and that activation of these genes through epigenetic silencing of miR-181c may contribute to the pathogenesis of GCs. It has been reported that oncogenic Ras increases the level and activity of the intracellular form of Notch1 and that Notch1 is necessary to maintain the neoplastic phenotype in Ras-transformed human cells in vitro and in vivo (37). These observations place Notch signaling among key downstream effectors of oncogenic Ras. It is thus possible that KRAS and NOTCH signaling act synergistically as to carcinogenesis in response to decreased miR-181c expression.

In conclusion, these results imply that an inhibitor of DNA methyltransferase induces the expression of some miRNAs in GC cells. Among them, miR-181c may be a tumor suppressive miRNA in GCs and can regulate the expression of target genes that are important in human carcinogenesis. Further studies on epigenetic regulation of miRNA expression are necessary, and regulation of miRNA expression by epigenetic drugs may be a novel therapeutic strategy for gastric and other human cancers.

Supplementary material

Supplementary Tables 1–4 can be found at http://carcin.oxfordjournals.org/

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