Epigenetic silencing of RELN in gastric cancer

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Abstract. RELN (Reelin) is an extracellular glycoprotein that plays a critical role in neuronal migration. Here we show that the RELN gene is frequently silenced in gastric cancers (GCs) by aberrant promoter hypermethylation. Although RELN was strongly expressed in non-tumor gastric epithelia, its expression was weak, or absent, in GC cell lines and primary GC tumors. Absence of RELN expression significantly correlated with a more advanced stage of GC. Methylation of the RELN promoter was frequently found in GC cell lines and in primary GC tumors. These findings suggest that disruption of the RELN pathway may be involved in gastric carcinogenesis.

Introduction

Gastric cancer (GC) is the second most common cause of cancer-associated death worldwide (1). The molecular basis of GC involves several genetic changes including oncogenic activation of β-catenin and KRAS, amplification of ERBB2 and MET, inactivation of tumor suppressor genes, such as p53, APC, CDH1 (E-cadherin) and CDKN2A (p16), and microsatellite instability.

Epigenetic alterations, as well as genetic alterations, are involved in the development and progression of cancer. DNA methylation of CpG islands in the 5′ region of tumor suppressor genes is known to inhibit transcriptional initiation and thereby silence these genes (2). Several tumor suppressor genes, including CDKN2A, CDH1, hMLH1 and RUNX3, have been reported to be inactivated by promoter methylation in GC (3).

RELN (Reelin) is an extracellular 420-kDa glycoprotein that plays a critical role in the regulation of neuronal migration during brain development (4,5). The Reln gene was isolated from reeler mice that have an autosomal recessive mutation in the Reln gene, which results in widespread disruption of laminated regions of the brain (4). Secreted RELN binds to two cell surface receptors termed the very low density lipoprotein receptor (VLDLR) and apolipoprotein E receptor (ApoER2), which transmit the extracellular RELN signal to intracellular signaling processes through Disabled-1 (DAB1), an intra-cellular adaptor protein that activates the tyrosine kinase (6,7). These signaling components are essential for RELN signaling since knockout mice lacking Vldlr, ApoER2 or Dab1 mimic the phenotype of mice lacking Reln (7,8).

The recent observation that the VLDLR gene is frequently silenced by promoter hypermethylation in GC suggested that disruption of the RELN pathway may be involved in gastric carcinogenesis (9). The RELN gene harbors a long CpG-rich promoter region (10) and expression of RELN is regulated by the methylation status of the promoter (11). Thus, the RELN promoter is hypermethylated in schizophrenia (12,13) and RELN is silenced in pancreatic adenocarcinomas by aberrant promoter hypermethylation (14). These studies prompted us to investigate the methylation and expression status of RELN in GC.

Materials and methods

Cell lines and primary tumors. Nine human GC cell lines were used in this study: MKN1, MKN28, MKN45, MKN74 (15,16), TMK1 (17), NUGC3 (18), SNU16 (19), KATO-III (20) and AZ-521 (21). All the cell lines were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum and 100 U/ml penicillin/100 μg/ml streptomycin at 37°C in a humidified atmosphere of 5% CO2. For immunohistochemistry, primary GC samples were obtained from 25 patients who underwent surgery at the Hospital of Kyoto Prefectural University of Medicine (Kyoto, Japan). Surgical specimens were fixed in formalin and embedded in paraffin using standard procedures. For methylation analysis, paired GC tissues and non-tumor gastric epithelial tissues were obtained during upper gastrointestinal endoscopic inspection from an additional 15 patients who underwent biopsy for
diagnostic purposes at the Hospital of Kyoto Prefectural University of Medicine. Normal gastric epithelial tissues were obtained from three *Helicobacter pylori*-negative healthy volunteers who underwent endoscopy. All biopsy specimens were immediately frozen in liquid nitrogen and stored at -80°C until required. *H. pylori* infection status was examined with a rapid urease test (PyloriTek Test kit; Serim Research Corp., Elkhart, IN, USA), with hematoxylin-eosin staining or with a serum IgG antibody test (SBS, Kanagawa, Japan). A patient was defined as *H. pylori*-positive if one or more of these tests gave a positive result. Atrophic gastritis was diagnosed by endoscopy (22). None of the patients had undergone radiation therapy, chemotherapy or immunotherapy prior to the operation. Genomic DNA and total RNA were isolated from the GC cell lines and primary GC tumors using the DNeasy Tissue kit (Qiagen). Prior to the study, informed consent was obtained and the study was approved by the ethics committee.

Reverse transcription-polymerase chain reaction (RT-PCR). Single-stranded cDNAs were generated from total cellular RNA using the QuantiTect Reverse Transcription kit (Qiagen) according to the manufacturer's protocol. Conventional PCR of RELN was performed using the Ex Taq DNA polymerase (Takara, Otsu, Japan). The PCR products were separated on 3% agarose gels and stained with ethidium bromide.

Methylation analysis. The methylation status of the 5' CpG island of RELN was examined by methylation-specific PCR (MSP) as described previously (14). Methylation of RELN was further analyzed by bisulfite PCR followed by restriction enzyme digestion [combined bisulfite and restriction analysis (COBRA)] (23) and bisulfite sequencing analysis. For COBRA, genomic DNA (2 μg) was treated with sodium bisulfite using an EZ DNA Methylation kit (Zymo Research, Orange, CA) and subjected to PCR using primers (Table I) designed to amplify a region from -178 to +311 bp relative to the transcription start site of RELN. The PCR products were digested with *AflII*, which recognizes sequences unique to the methylated alleles but cannot recognize unmethylated alleles, and the digested products were electrophoresed on 3% agarose gels and stained with ethidium bromide. The gel images were saved as TIFF files. Methylation levels were calculated as the ratio of the gray scale value of the methylated band to that of the combined methylated and unmethylated bands. The gray scale value was obtained by scanning the gel with Adobe Photoshop CS3 Extended software (Adobe Systems Incorporated, San Jose, CA, USA). For bisulfite-sequencing, the PCR products were cloned using the TOPO XL PCR Cloning kit (Invitrogen, Carlsbad, CA) and then sequenced. DNA derived from normal peripheral blood lymphocytes and CpGenome universal methylated DNA (Chemicon, Billerica, MA) served as controls for unmethylated and methylated DNA, respectively.

Drug treatment. Cells were treated with 1 or 5 μM of 5-aza-2'-deoxycytidine (5-aza-dCyd; Sigma-Aldrich, St. Louis, MO) for 4 days or with 50 ng/ml of trichostatin A (TSA; Wako, Osaka, Japan) for 1 day. For assay of drug synergy, the cells were cultured in the presence of 1 or 5 μM of 5-aza-dCyd for 4 days, and were then treated for an additional 24 h with 50 ng/ml of TSA.

Immunohistochemistry. Immunohistochemical staining of the RELN protein was performed on formalin-fixed, paraffin-embedded sections from 25 primary GCs, consisting of paired tumor and surrounding non-tumor tissues, using a mouse monoclonal antibody against RELN (clone E-5; Santa Cruz Biotechnology, Santa Cruz, CA). Deparaffinized sections were microwaved in 10 mM citrate buffer (pH 6.0) for 20 min. After blocking of endogenous peroxidase with 3% hydrogen peroxide, the sections were incubated overnight for 20 min at room temperature with the LightCycler system using FastStart DNA Master Plus SYBR Green I (Roche Diagnostics, Penzberg, Germany) according to the manufacturer's protocol. The primer sequences and PCR conditions are shown in Table I. GAPDH was used as an internal control. Human stomach total RNA (Clontech Laboratories, Mountain View, CA) was used as a control for RT-PCR.

Table I. Primer sequences and PCR conditions.

<table>
<thead>
<tr>
<th>Method</th>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Annealing temp. (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RT-PCR</td>
<td>RELN</td>
<td>5'-ACCAGTGCCAGTCGATGACATCAT-3'</td>
<td>5'-CTTCATTAGCCAACATCAACCACAC-3'</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td>GAPDH</td>
<td>5'-CGGAGTCAACGGATTGGTCGTAT-3'</td>
<td>5'-AGCCTTCTCCATGTTGGTGAAG-3'</td>
<td>67</td>
</tr>
<tr>
<td>COBRA and bisulfite-sequencing</td>
<td>5'-GGTTTTAAGAAGGTTGGAG-3'</td>
<td>5'-TCCCCATCCCCCTCCAAC-3'</td>
<td>63</td>
<td></td>
</tr>
</tbody>
</table>

Drug treatment. Cells were treated with 1 or 5 μM of 5-aza-2'-deoxycytidine (5-aza-dCyd; Sigma-Aldrich, St. Louis, MO) for 4 days or with 50 ng/ml of trichostatin A (TSA; Wako, Osaka, Japan) for 1 day. For assay of drug synergy, the cells were cultured in the presence of 1 or 5 μM of 5-aza-dCyd for 4 days, and were then treated for an additional 24 h with 50 ng/ml of TSA.

Immunohistochemistry. Immunohistochemical staining of the RELN protein was performed on formalin-fixed, paraffin-embedded sections from 25 primary GCs, consisting of paired tumor and surrounding non-tumor tissues, using a mouse monoclonal antibody against RELN (clone E-5; Santa Cruz Biotechnology, Santa Cruz, CA). Deparaffinized sections were microwaved in 10 mM citrate buffer (pH 6.0) for 20 min. After blocking of endogenous peroxidase with 3% hydrogen peroxide, the sections were incubated overnight at 4°C with the anti-RELN antibody (1:50). The sections were then incubated for 20 min at room temperature with peroxidase-labeled polymer-conjugated goat anti-mouse immunoglobulin [Histofine Simple Stain Max-PO (Multi); Nichirei, Tokyo, Japan], followed by 3,3'-diaminobenzidine tetrahydrochloride as the chromogen. The sections were then lightly counterstained with hematoxylin. Negative controls were evaluated in the absence of the primary antibody. Immunoreactivity was scored according to the intensity of staining as follows: 0, absent; 1+, weak; 2+, strong. GCs were classified into intestinal or diffuse type according to
Lauren's histological classification (24). Tumor stages were classified according to the TNM (tumor-node-metastasis) classification of the Japanese Classification of Gastric Cancer (25).

Statistical analyses. The χ² test, Fisher's exact probability test, and the Wilcoxon signed-rank test were performed using SPSS 15.0 software (SPSS, Inc., Chicago, IL). P-values of <0.05 were considered significant.

Results

Loss of expression of RELN mRNA in GC cell lines. To determine the potential role of RELN in GC, we first analyzed the expression of RELN mRNA in nine human GC cell lines by conventional RT-PCR (Fig. 1A) and by quantitative real-time RT-PCR (Fig. 1B). RELN expression was not detected in any of the nine cell lines, whereas its expression was detected in normal stomach.

Methylation of the RELN promoter in GC cell lines. Aberrant methylation of DNA in 5' regulatory regions harboring CpG-rich regions (CpG islands) is strongly associated with transcriptional silencing (2). We therefore examined whether the lack of expression of the RELN gene in the nine GC cell lines might be due to aberrant methylation of the RELN promoter. For analysis of the methylation status of the RELN promoter, we identified a CpG island within a 799-bp sequence, that stretches from -468 to +311 bp relative to the transcription start site and extends into exon 1 of RELN, by means of the genome database of the European Bioinformatics Institute (http://www.ebi.ac.uk/emboss/cpgplot/) (Fig. 2A). This region corresponds to a part of the promoter of RELN (11).
We first assessed the methylation status of a subdomain of the \textit{RELN} CpG island (Fig. 2A) by MSP in the nine GC cell lines lacking \textit{RELN} expression. Seven of the nine cell lines displayed exclusively methylated products, whereas the MKN45 and AZ-521 cells yielded both methylated and unmethylated products (Fig. 2B).

To confirm and quantify the methylation status of \textit{RELN} in these GC cell lines, we next assayed DNA methylation levels of a subdomain of the \textit{RELN} CpG island (Fig. 2A) by the COBRA technique which involves bisulfite PCR followed by restriction enzyme digestion. Consistent with the results of MSP, the \textit{RELN} CpG island was hypermethylated in all of the nine GC cell lines and the MKN45 and AZ-521 cells were partly unmethylated (Fig. 2C). Further analysis of the PCR products by bisulfite-sequencing showed that the CpG island is hypermethylated in two representative GC cell lines (MKN1 and MKN28) that lack \textit{RELN} expression but not in a non-tumor gastric epithelial tissue (5NT) (Fig. 2D). Taken together, these data show that the \textit{RELN} CpG island is frequently hypermethylated in GC cells.

Figure 3. Effect of 5-aza-dCyd and TSA treatment on \textit{RELN} expression. (A) Demethylation of \textit{RELN} in MKN1, MKN28 and TMK1 cells, before and after exposure to 5 \( \mu \)M of 5-aza-dCyd at for 4 days, was analyzed by MSP as described in the legend to Fig. 2. PLC and M-DNA are as in Fig. 2. (B) Expression of \textit{RELN} determined by quantitative real-time RT-PCR in the MKN1, MKN28 and TMK1 cell lines with or without treatment with 5-aza-dCyd (1 or 5 \( \mu \)M) for 4 days and/or TSA (50 ng/ml) for 24 h.

We additionally observed elevated expression of \textit{RELN} mRNA after treatment with the histone deacetylase inhibitor, TSA, and TSA treatment also enhanced expression of \textit{RELN} mRNA by 5-aza-dCyd in all three cell lines (Fig. 3B). These findings suggested that histone deacetylation may also contribute to transcriptional silencing of \textit{RELN}.

Defective expression of the \textit{RELN} protein in primary GC tumors. To determine whether the silencing of \textit{RELN} that was observed in the GC cell lines was relevant for primary human carcinomas, we compared the expression of the \textit{RELN} protein in 25 primary GC samples with that of the respective non-tumor tissue by immunohistochemistry. The results of the immunostaining of \textit{RELN} are summarized in Table II, and representative images are shown in Fig. 4. The \textit{RELN} protein was strongly expressed in the cytoplasm of all non-tumor gastric epithelia (Fig. 4A-D). In contrast, expression of \textit{RELN} was weak or absent in GC tumors (Fig. 4E-H). For all of the GCs, the expression of \textit{RELN} was lower in the tumors than in their non-tumor counterparts (Table II). When \textit{RELN} was detected in GC cells, it also localized in the cytoplasm.

To clarify the relationship between the level of the \textit{RELN} protein in GC tumors and various clinicopathological para-
meters, we correlated RELN expression with available clinical data from the 25 patients. For this analysis the patients were divided into two groups based on whether the expression of the RELN protein was weak or absent (Table III). Absence of RELN expression significantly correlated with advanced tumor stage (stage III or IV).

Methylation of RELN in primary GC tumors. To determine whether the methylation of the RELN CpG island observed in GC cell lines also occurs in primary human carcinomas, we assessed the methylation status of RELN in paired tumor and non-tumor tissues from an additional 15 patients with primary GC and in three normal gastric epithelial tissues by COBRA. Twelve of the 15 patients were positive for *H. pylori*. All 15 of the non-tumor gastric mucosal samples were from patients that had endoscopically documented atrophic gastritis. Methylation of RELN was observed in all 15 GC tumors and in 12 of the 15 non-tumor gastric epithelial tissues, but not in any of the three normal gastric epithelial tissues (Fig. 5A). Although methylation of RELN was found in both GC tumors

Table II. Expression levels of RELN protein in paired tumor tissues and non-tumor tissues from 25 patients with GC.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>2+</th>
<th>1+</th>
<th>-</th>
</tr>
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<tbody>
<tr>
<td>Non-tumor</td>
<td>25 (100%)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Tumor</td>
<td>0</td>
<td>15 (60%)</td>
<td>10 (40%)</td>
</tr>
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*a2+, strong; 1+, weak; -, absent.*

Figure 4. Representative immunostaining of RELN in non-tumor gastric epithelia and in primary GC tumors. (A and B) Immunostaining (brown color) of RELN in non-tumor gastric epithelia at low (A, x40) and high (B, x200) magnification. (C and D) Hematoxylin and eosin staining of the same specimens shown in (A) and (B), respectively. (E-H) Immunostaining of RELN in primary GC tumors: intestinal type (E and F) and diffuse type (G and H). Expression of RELN was weak (E and G) or absent (F and H) in primary GC tumors. Original magnification x200.
and in non-tumor tissues, the level of RELN methylation was significantly higher in 13 of the 15 tumors when compared with their non-tumor tissue counterparts (Wilcoxon signed-rank test, P=0.001) (Fig. 5B). These findings are consistent with the results of immunohistochemistry that indicate that the expression of RELN is reduced in tumors compared with their non-tumor counterparts.

Discussion

This is the first report that RELN is silenced in GC by aberrant promoter hypermethylation and we have demonstrated this silencing by a number of approaches. Thus, all nine GC cell lines assayed lacked the expression of RELN mRNA. Furthermore, methylation assays of GC cells by MSP, COBRA, bisulfite-sequencing and 5-aza-dCyd and TSA drug treatment, indicated that a CpG island in the 5' promoter region of RELN, is hypermethylated. Methylation of the RELN CpG island was found not only in GC cell lines but also in all 15 primary GCs examined. Moreover, the expression of the RELN protein was weak or absent in primary GC tumors and RELN expression level was significantly reduced in tumors compared with their non-tumor counterparts. Our data further suggest that silencing of RELN is associated with the progression of GC since the absence, rather than the weak expression, of RELN significantly correlated with a more advanced stage of primary GC. The combined results indicate that methylation

Table III. Relationship between levels of expression of RELN protein and clinicopathological features in 25 primary GCs.

<table>
<thead>
<tr>
<th>Features</th>
<th>n</th>
<th>RELN expression</th>
<th>P-value^c</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1+ (n=15)</td>
<td>- (n=10)</td>
</tr>
<tr>
<td>Age ≤70</td>
<td>12</td>
<td>7</td>
<td>5</td>
</tr>
<tr>
<td>&gt;70</td>
<td>13</td>
<td>8</td>
<td>5</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>17</td>
<td>12</td>
<td>5</td>
</tr>
<tr>
<td>Female</td>
<td>8</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>Histological type^b</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intestinal type</td>
<td>13</td>
<td>9</td>
<td>4</td>
</tr>
<tr>
<td>Diffuse type</td>
<td>12</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>Stage (TNM)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stage I/II</td>
<td>17</td>
<td>13</td>
<td>4</td>
</tr>
<tr>
<td>Stage III/IV</td>
<td>8</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>Lymph node metastasis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(-)</td>
<td>12</td>
<td>9</td>
<td>3</td>
</tr>
<tr>
<td>(+)</td>
<td>13</td>
<td>6</td>
<td>7</td>
</tr>
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</table>

^a1+, weak; -, absent. ^bLauren's histological classification. ^c^2 test.

Figure 5. Analysis of RELN methylation in paired tumor and non-tumor tissues. (A) COBRA of RELN in paired tumor (T) and non-tumor (NT) tissues from 15 patients with primary GC and from three normal gastric epithelia (NS). The arrow and arrowheads indicate undigested products (U, unmethylated DNA) and digested fragments (M, methylated DNA), respectively. Methylation levels of RELN were determined as described in Materials and methods and are expressed as a percentage of the methylated DNA (M-DNA) positive control value. The value obtained for normal peripheral lymphocytes (PLC) was used as the baseline (0%). (B) Plot of the methylation levels of RELN in paired tumors (T) and non-tumor tissues (NT) from 15 patients with primary GCs. The level of RELN methylation was significantly higher in tumors when compared with their non-tumor tissue counterparts (Wilcoxon signed-rank test, P=0.001).
of the RELN promoter contributes to the silencing of the RELN gene and that silencing of the RELN gene plays a role in GC tumor progression. However, the number of primary GC samples examined in this study was relatively small. Furthermore, it was not possible to directly determine the correlation between levels of expression and methylation of RELN in primary GCs due to the small amount of total RNA that was obtained from the biopsy samples. Therefore, further studies using a larger number of primary samples are required to clarify the exact relationship between methylation and RELN expression in primary GCs.

Interestingly, methylation of RELN was also observed in most of the non-tumor gastric epithelial tissues examined, although levels of RELN methylation in non-tumor gastric epithelial tissues were lower than those observed in GC tumors. All of the non-tumor gastric mucosa showed atrophic gastritis, and most of the patients from whom the samples were taken were infected with H. pylori, the major cause of chronic gastritis which leads to atrophic changes in the gastric mucosa (26). Most GCs arise on a background of atrophic gastritis (27). These findings suggest that methylation of RELN occurs in precancerous atrophic gastritis and is enhanced during the development and progression of GC.

Our immunohistochemical analyses, combined with the histological examinations, suggested that RELN is expressed mainly in the pepsinogen-secreting chief cells of gastric epithelia. The physiological function of RELN in the stomach is currently unclear but is an important issue for future studies.

To date, varying levels of RELN expression have been reported in cancers. High expression of RELN was reported in 87.5% of esophageal cancers (28) and in 39% of prostate cancers (29). Conversely, the expression of RELN was reported to be only focal or completely absent in 72% of cancers (29). Conversely, the expression of RELN was reported in 87.5% of esophageal cancers (28) and in 39% of prostate cancers (29). High expression of RELN was reported in 87.5% of esophageal cancers (28) and in 39% of prostate cancers (29). High expression of RELN was reported in 87.5% of esophageal cancers (28) and in 39% of prostate cancers (29). High expression of RELN was reported in 87.5% of esophageal cancers (28) and in 39% of prostate cancers (29). High expression of RELN was reported in 87.5% of esophageal cancers (28) and in 39% of prostate cancers (29). High expression of RELN was reported in 87.5% of esophageal cancers (28) and in 39% of prostate cancers (29).

In conclusion, the expression of RELN is lost or highly reduced by aberrant promoter hypermethylation in GCs. Although the exact mechanism by which RELN contributes to tumorigenicity remains to be elucidated, the data presented in this study, together with the recent finding that the VLDLR gene, whose product functions as the receptor for RELN, is also epigenetically silenced in GC (9), clearly suggest that disruption of the RELN pathway is involved in gastric carcinogenesis.

References