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 *ß-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).

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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% CO_2. 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

Table I. Primer sequences and PCR conditions.

Method	Gene	Forward primer	Reverse primer	Annealing temp. (˚C)
RT-PCR	*RELN*	5'-ACCAGTGGGCAGTCGATGACATCAT-3'	5'-CTTCATTAGCCAACATCAACCACAC-3'	55
	GAPDH	5'-CGGAGTCAACGGATTTGGTCGTAT-3'	5'-AGCCTTCTCCATGGTGGTGAAGAC-3'	67
COBRA and bisulfite-sequencing		5'-GGTTTTAAGAAGGTGTGGAG-3'	5'-TCCCCATCCCCTTCCAAC-3'	63

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, Minneapolis, MN), the RNeasy Mini kit (Qiagen) or the AllPrep DNA/RNA Mini 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. Quantitative real-time PCR experiments were performed 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.

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 *Afl*II, 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 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

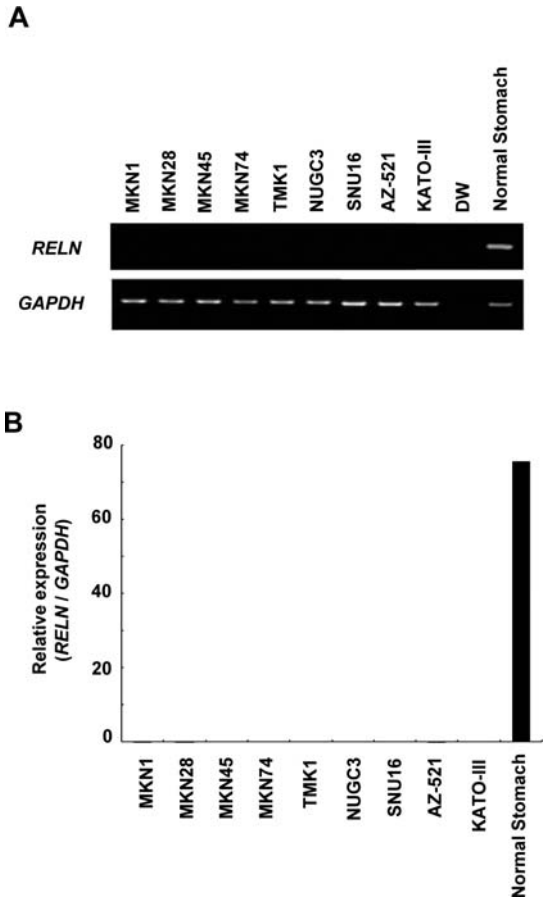

Figure 1. Expression of *RELN* mRNA in nine GC cell lines and in normal stomach. *RELN* mRNA expression was examined in the indicated GC cell lines and in normal stomach by conventional RT-PCR (A) and by quantitative real-time RT-PCR (B). *GAPDH* was used as an internal control. DW in (A) is a deionized water control.

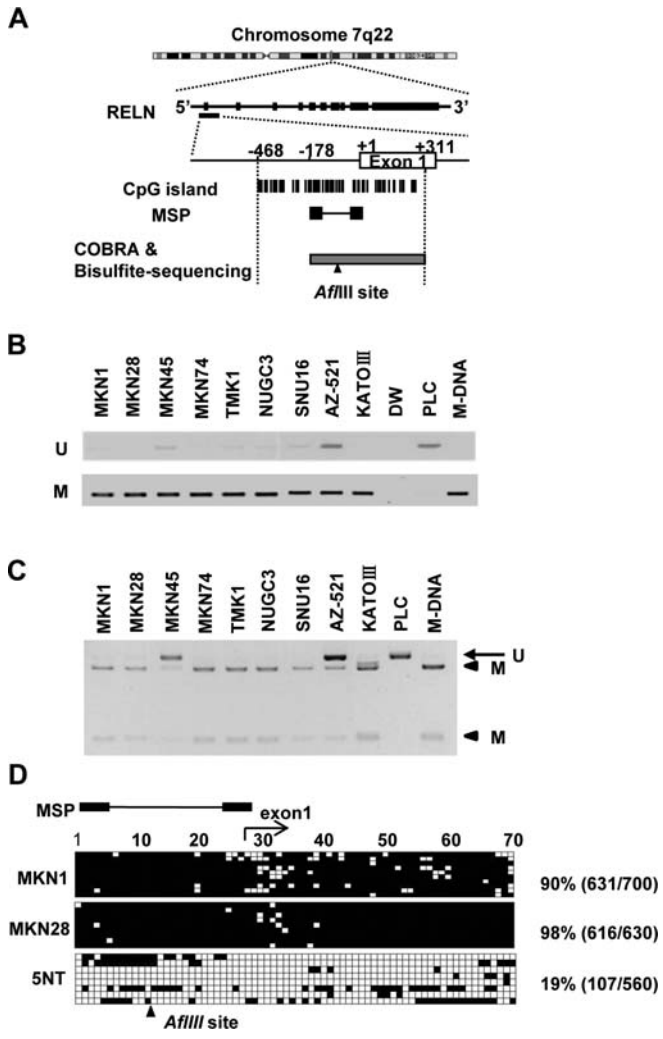

Figure 2. Analysis of *RELN* methylation. (A) Schematic map of the CpG island extending into exon 1 of *RELN*. Exon 1 is indicated by an open box, and the transcription start site is marked at +1. CpG sites are indicated by vertical ticks. The regions selected for MSP and for COBRA and bisulfite-sequencing are indicated. The restriction site for *Afl*III is indicated by the black arrowhead. (B) MSP analysis of *RELN* in the nine indicated GC cell lines and in normal peripheral lymphocytes (PLC). Parallel amplification reactions were performed using primers specific for unmethylated (U) or methylated (M) DNA. M-DNA indicates CpGenome universal methylated DNA. PLC and M-DNA were used as controls for unmethylated and methylated DNA, respectively. DW is a deionized water control. (C) COBRA of *RELN* in the nine GC cell lines. The arrow and arrowheads indicate undigested products (U, unmethylated DNA) and digested fragments (M, methylated DNA), respectively. (D) Bisulfite-sequencing of two GC cell lines (MKN1 and MKN28) and a non-tumor gastric epithelial tissue (5NT). All 70 CpG sites were sequenced. Each square indicates CpG dinucleotides: open squares, unmethylated; solid squares, methylated. Percentages indicate the fraction of methylated CpG dinucleotides. The regions selected for MSP and the restriction site for *Afl*III are indicated.

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 χ^2 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 be 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).

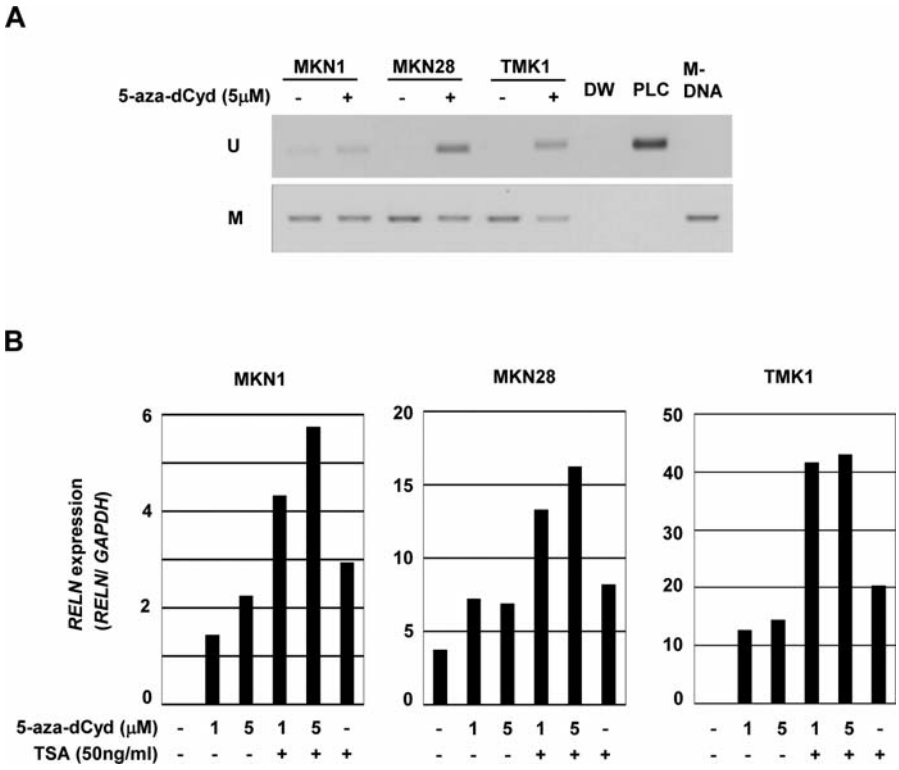

Figure 3. Effect of 5-aza-dCyd and TSA treatment on *RELN* expression. (A) Demethylation of *RELN* in MKN1, MKN28 and TMK1 cells, before and after exposure to 5 μM of 5-aza-dCyd at for 4 days, was analyzed by MSP as described in the legend to Fig. 2. PLC and M-DNA are as in Fig. 2. (B) Expression of *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 μM) for 4 days and/or TSA (50 ng/ml) for 24 h.

We first assessed the methylation status of a subdomain of the *RELN* CpG island (Fig. 2A) by MSP in the nine GC cell lines lacking *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 *RELN* in these GC cell lines, we next assayed DNA methylation levels of a subdomain of the *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 *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 *RELN* expression but not in a non-tumor gastric epithelial tissue (5NT) (Fig. 2D). Taken together, these data show that the *RELN* CpG island is frequently hypermethylated in GC cells.

Role of DNA methylation and histone deacetylation in the silencing of RELN expression in GC cells. To investigate the role of methylation of the *RELN* CpG island in silencing of the *RELN* gene, we assayed the effect of demethylation of the *RELN* CpG island on the expression of *RELN* mRNA. Three of the GC cell lines that lack *RELN* expression (MKN1, MKN28 and TMK1) were therefore treated with 5-aza-dCyd, a methyltransferase inhibitor, following which RELN mRNA expression was assayed by quantitative real-time RT-PCR. Demethylation of the *RELN* CpG island was observed by PCR analysis (Fig. 3A) and was accompanied by restoration of the expression of *RELN* mRNA in all three cell lines (Fig. 3B). These data implied that methylation of the *RELN* CpG island silences *RELN* mRNA expression.

We additionally observed elevated expression of *RELN* mRNA after treatment with the histone deacetylase inhibitor, TSA, and TSA treatment also enhanced expression of *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 *RELN*.

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

To clarify the relationship between the level of the RELN protein in GC tumors and various clinicopathological para-

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

	RELN protein expression[a]		
Tissue	2+	1+	-
Non-tumor	25 (100%)	0	0
Tumor	0	15 (60%)	10 (40%)

[a]2+, strong; 1+, weak; -, absent.

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

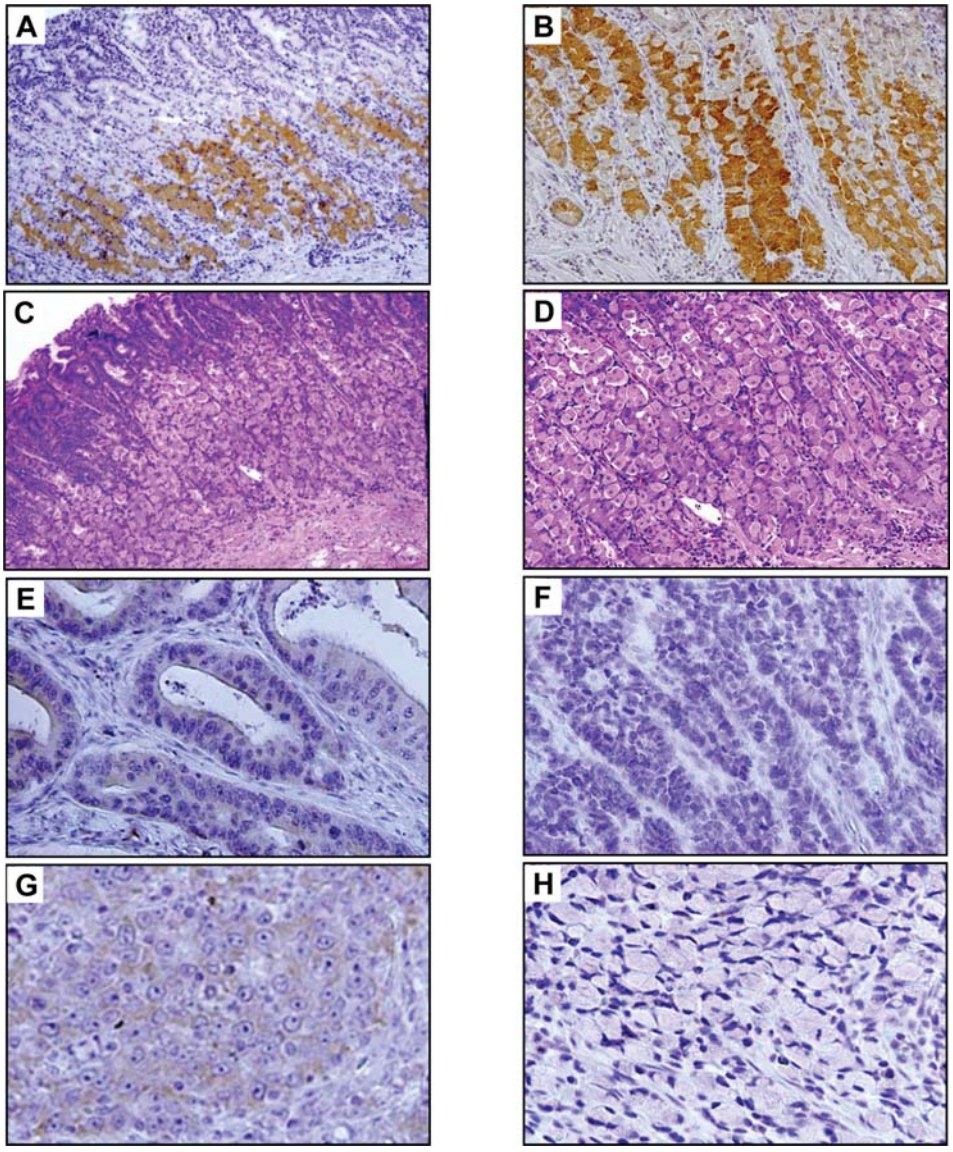

Figure 4. Representative immunostaining of RELN in non-tumor gastric epithelia and in primary GC tumors. (A and B) Immunostaining (brown color) of RELN in non-tumor gastric epithelia at low (A, x40) and high (B, x200) magnification. (C and D) Hematoxylin and eosin staining of the same specimens shown 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.

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

Features	n	RELN expression[a]		P-value[c]
		1+ (n=15)	- (n=10)	
Age				
≤70	12	7	5	0.89
>70	13	8	5	
Gender				
Male	17	12	5	0.13
Female	8	3	5	
Histological type[b]				
Intestinal type	13	9	4	0.28
Diffuse type	12	6	5	
Stage (TNM)				
Stage I/II	17	13	4	0.01
Stage III/IV	8	2	6	
Lymph node metastasis				
(-)	12	9	3	0.14
(+)	13	6	7	

[a]1+, weak; -, absent. [b]Lauren's histological classification. [c]$\chi 2$ test.

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

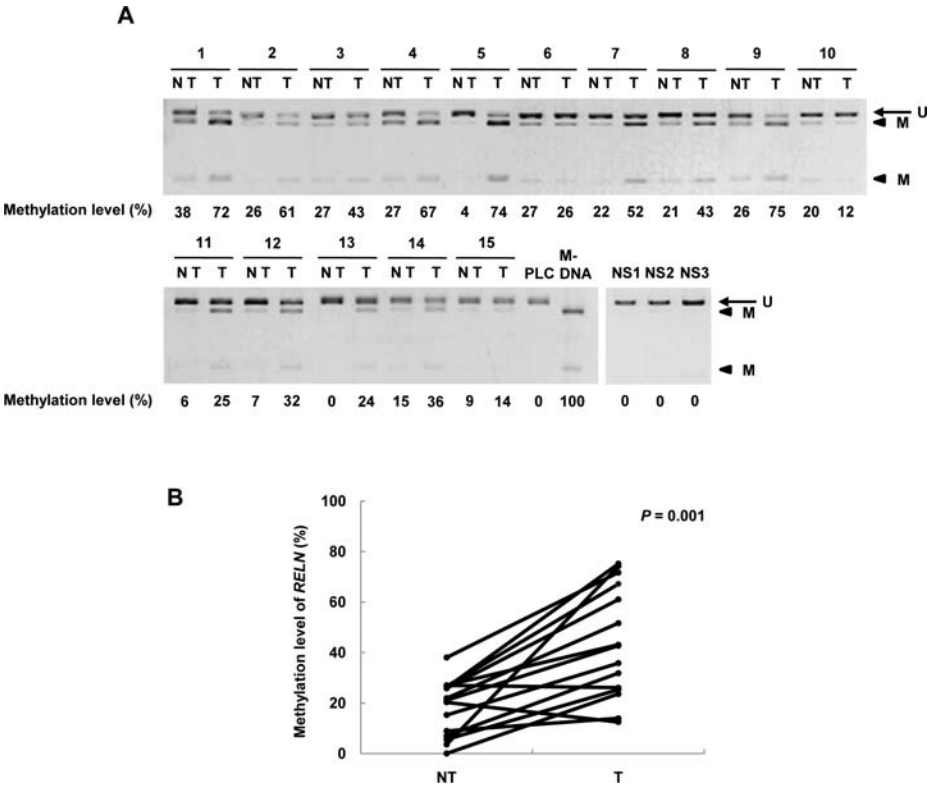

Figure 5. Analysis of *RELN* methylation in paired tumor and non-tumor tissues. (A) COBRA of *RELN* in paired tumor (T) and non-tumor (NT) tissues from 15 patients with primary GC and from three normal gastric epithelia (NS). The arrow and arrowheads indicate undigested products (U, unmethylated DNA) 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 pancreatic cancers in which the *RELN* promoter was hypermethylated (14). Although the functional role of RELN in tumorigenesis remains largely unknown, knockdown of *RELN* in pancreatic cancer cells by small interfering RNA resulted in enhanced cell mobility, invasiveness and colony-forming ability (14). These findings suggest the possibility that the loss of RELN might enhance cell metastasis. Clearly further studies are required to fully understand the role of RELN in tumorigenesis.

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

1. Ferlay J, Bray F, Pisani P and Parkin DM: GLOBOCAN 2000: Cancer Incidence, Mortality, and Prevalence Worldwide. Version 1.0. Vol. 5. IARC Press, Lyon.
2. Baylin SB, Herman JG, Graff JR, Vertino PM and Issa JP: Alterations in DNA methylation: a fundamental aspect of neoplasia. Adv Cancer Res 72: 141-196, 1998.
3. Ushijima T and Sasako M: Focus on gastric cancer. Cancer Cell 5: 121-125, 2004.
4. D'Arcangelo G, Miao GG, Chen SC, Soares HD, Morgan JI and Curran T: A protein related to extracellular matrix proteins deleted in the mouse mutant reeler. Nature 374: 719-723, 1995.
5. Curran T and D'Arcangelo G: Role of reelin in the control of brain development. Brain Res Brain Res Rev 26: 285-294, 1998.
6. Tissir F and Goffinet AM: Reelin and brain development. Nat Rev Neurosci 4: 496-505, 2003.
7. Trommsdorff M, Gotthardt M, Hiesberger T, Shelton J, Stockinger W, Nimpf J, Hammer RE, Richardson JA and Herz J: Reeler/Disabled-like disruption of neuronal migration in knockout mice lacking the VLDL receptor and ApoE receptor 2. Cell 97: 689-701, 1999.
8. Howell BW, Hawkes R, Soriano P and Cooper JA: Neuronal position in the developing brain is regulated by mouse disabled-1. Nature 389: 733-737, 1997.
9. Takada H, Imoto I, Tsuda H, Nakanishi Y, Sakakura C, Mitsufuji S, Hirohashi S and Inazawa J: Genomic loss and epigenetic silencing of very-low-density lipoprotein receptor involved in gastric carcinogenesis. Oncogene 25: 6554-6562, 2006.
10. De Silva U, Arcangelo GD, Braden VV, Chen J, Miao GG, Curran T and Green ED: The human reelin gene: isolation, sequencing, and mapping on chromosome 7. Genome Res 7: 157-164, 1997.
11. Chen Y, Sharma RP, Costa RH, Costa E and Grayson DR: On the epigenetic regulation of the human reelin promoter. Nucleic Acids Res 30: 2930-2939, 2002.
12. Abdolmaleky HM, Cheng KH, Russo A, Smith CL, Faraone SV, Wilcox M, Shafa R, Glatt SJ, Nguyen G, Ponte JF, Thiagalingam S and Tsuang MT: Hypermethylation of the reelin (RELN) promoter in the brain of schizophrenic patients: a preliminary report. Am J Med Genet B Neuropsychiatr Genet 134: 60-66, 2005.
13. Grayson DR, Jia X, Chen Y, Sharma RP, Mitchell CP, Guidotti A and Costa E: Reelin promoter hypermethylation in schizophrenia. Proc Natl Acad Sci USA 102: 9341-9346, 2005.
14. Sato N, Fukushima N, Chang R, Matsubayashi H and Goggins M: Differential and epigenetic gene expression profiling identifies frequent disruption of the RELN pathway in pancreatic cancers. Gastroenterology 130: 548-565, 2006.
15. Hojo H: Establishiment of cultured cell lines of human stomach cancer - origin and their morphological characteristics (in Japanese). Niigata Igakukai Zasshi 91: 737-752, 1977.
16. Motoyama T, Hojo H and Watanabe H: Comparison of seven cell lines derived from human gastric carcinomas. Acta Pathol Jpn 36: 65-83, 1986.
17. Ochiai A, Yasui W and Tahara E: Growth-promoting effect of gastrin on human gastric carcinoma cell line TMK-1. Jpn J Cancer Res 76: 1064-1071, 1985.
18. Akiyama S, Amo H, Watanabe T, Matsuyama M, Sakamoto J, Imaizumi M, Ichihashi H, Kondo T and Takagi H: Characteristics of three human gastric cancer cell lines, NU-GC-2, NU-GC-3 and NU-GC-4. Jpn J Surg 18: 438-446, 1988.
19. Park JG, Frucht H, La Rocca RV, Bliss DP Jr, Kurita Y, Chen TR, Henslee JG, Trepel JB, Jensen RT, Johnson BE, Bang YJ, Kim JP and Gazdar AF: Characteristics of cell lines established from human gastric carcinoma. Cancer Res 50: 2773-2780, 1990.
20. Sekiguchi M, Sakakibara K and Fujii G: Establishment of cultured cell lines derived from a human gastric carcinoma. Jpn J Exp Med 48: 61-68, 1978.
21. Takada H, Imoto I, Tsuda H, Sonoda I, Ichikura T, Mochizuki H, Okanoue T and Inazawa J: Screening of DNA copy-number aberrations in gastric cancer cell lines by array-based comparative genomic hybridization. Cancer Sci 96: 100-110, 2005.
22. Price AB: The Sydney System: histological division. J Gastroenterol Hepatol 6: 209-222, 1991.
23. Xiong Z and Laird PW: COBRA: a sensitive and quantitative DNA methylation assay. Nucleic Acids Res 25: 2532-2534, 1997.
24. Lauren P: The two histological main types of gastric carcinoma: diffuse and so-called intestinal-type carcinoma. an attempt at a histo-clinical classification. Acta Pathol Microbiol Scand 64: 31-49, 1965.

25. Japanese Gastric Cancer Association: Japanese Classification of Gastric Carcinoma - 2nd English edition. Gastric Cancer 1: 10-24, 1998.
26. Asaka M, Kimura T, Kudo M, Takeda H, Mitani S, Miyazaki T, Miki K and Graham DY: Relationship of Helicobacter pylori to serum pepsinogens in an asymptomatic Japanese population. Gastroenterology 102: 760-766, 1992.
27. El-Zimaity H: Gastritis and gastric atrophy. Curr Opin Gastroenterol 24: 682-686, 2008.
28. Wang Q, Lu J, Yang C, Wang X, Cheng L, Hu G, Sun Y, Zhang X, Wu M and Liu Z: CASK and its target gene Reelin were co-upregulated in human esophageal carcinoma. Cancer Lett 179: 71-77, 2002.
29. Perrone G, Vincenzi B, Zagami M, Santini D, Panteri R, Flammia G, Verzı A, Lepanto D, Morini S, Russo A, Bazan V, Tomasino RM, Morello V, Tonini G and Rabitti C: Reelin expression in human prostate cancer: a marker of tumor aggressiveness based on correlation with grade. Mod Pathol 20: 344-351, 2007.