

Gastrokine 1 Expression in the Human Gastric Mucosa Is Closely Associated with the Degree of Gastritis and DNA Methylation

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Purpose: Gastrokine 1 plays an important role in gastric mucosal defense. Additionally, the Gastrokine 1-miR-185-DNMT1 axis has been shown to suppress gastric carcinogenesis through regulation of epigenetic alteration. Here, we investigated the effects of Gastrokine 1 on DNA methylation and gastritis.

Materials and Methods: Expression of Gastrokine 1, DNMT1, EZH2, and c-Myc proteins, and the presence of *Helicobacter pylori* CagA protein were determined in 55 non-neoplastic gastric mucosal tissue samples by western blot analysis. The CpG island methylation phenotype was also examined using six markers (*p16*, *hMLH1*, *CDH1*, *MINT1*, *MINT2* and *MINT31*) by methylation-specific polymerase chain reaction. Histological gastritis was assessed according to the updated Sydney classification system.

Results: Reduced Gastrokine 1 expression was found in 20 of the 55 (36.4%) gastric mucosal tissue samples and was closely associated with miR-185 expression. The Gastrokine 1 expression level was inversely correlated with that of DNMT1, EZH2, and c-Myc, and closely associated with the degree of gastritis. The *H. pylori* CagA protein was detected in 26 of the 55 (47.3%) gastric mucosal tissues and was positively associated with the expression of DNMT1, EZH2, and c-Myc. In addition, 30 (54.5%) and 23 (41.9%) of the gastric mucosal tissues could be classified as CpG island methylation phenotype-low and CpG island methylation phenotype-high, respectively. Reduced expression of Gastrokine 1 and miR-185, and increased expression of DNMT1, EZH2, and c-Myc were detected in the CpG island methylation phenotype-high gastric mucosa.

Conclusions: Gastrokine 1 has a crucial role in gastric inflammation and DNA methylation in gastric mucosa.

Key Words: Gastrokine 1; DNA methylation; microRNA-185; CagA protein; Gastric mucosa

Introduction

DNA methylation is an important epigenetic alteration process that occurs during malignant transformations. It includes global hypermethylation and also the hypermethylation of CpG islands

confined to the regulatory regions of most human genes.¹ Aberrant promoter methylation of cancer-related genes is detected in gastric tumors, suggesting a connection to the induction/promotion of gastric cancer.^{2,3} The three most-studied genes for promoter hypermethylation in gastric carcinoma are *p16*, *E-cadherin*, and *hMLH1*.⁴ Of these genes, *p16* (*CDKN2a/INK4a*) is an important tumor suppressor gene, and its protein is considered to be a negative regulator of G1 phase progression.⁵ In addition, E-cadherin (*CDH1*) is a transmembrane glycoprotein expressed on epithelial cells, and is also responsible for calcium-dependent homotypic cell adhesion.⁶ Cell-cell and cell-matrix interactions play a crucial role in neoplastic transformation and metastasis.^{7,8} Hypermethylation of

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the DNA mismatch repair gene, *hMLH1*, on chromosome 3p21 has been found in gastric tumors.⁹ DNA methylation is regulated by a family of DNA methyltransferases (DNMTs), including DNMT1, DNMT2, DNMT3a, DNMT3b, and DNMT31. DNMT1 has been implicated in S-phase replication foci and acts primarily as a maintenance methyltransferase.¹⁰ Enhancer of zeste homolog 2 (EZH2), a polycomb group protein, is a human homolog of the *Drosophila* protein. Overexpression of EZH2 is associated with poor prognosis or increased cancer risk in a variety of epithelial cell-derived digestive tumors, including esophageal cancer, gastric cancer, and colorectal cancer.¹¹ In gastric cancer, high EZH2 expression is correlated with p53 expression and is significantly associated with distant metastases.¹² Since aberrant promoter CpG island methylation is often present in non-neoplastic tissues with *Helicobacter pylori*-related gastritis,¹³ it is likely that gastric inflammation induces aberrant DNA methylation and gastric carcinogenesis.

Gastrokine 1 (GKN1) has been isolated from the gastric mucosal cells of several mammalian species, including the rat.¹⁴ GKN1 protects the antral mucosa and promotes healing by facilitating restitution and proliferation after injury.¹⁵ In addition, GKN1 also protects the intestinal mucosal barrier by acting on specific tight junction proteins and stabilizing peri-junctional actin.¹⁶ In our previous studies, we observed frequent loss of GKN1 expression in gastric cancers and identified tumor suppressor activities of GKN1 by inhibiting cell proliferation through regulation of epigenetic alteration.¹⁷

Here, we hypothesized that GKN1 is a candidate biomarker for gastritis and hypermethylation of tumor suppressor genes in the gastric mucosa. We examined the methylation status of the *p16*, *CDHI*, *MINT1*, *MINT2*, *MINT31*, and *hMLH1* genes and the expression level of GKN1, EZH2, miR-185, c-Myc, and DNMT1 in 55 non-neoplastic gastric mucosal tissue samples. Our results implicate GKN1 as a candidate biomarker for determining the degree of gastritis and methylation status in gastric mucosa.

Materials and Methods

1. Samples

This study examined a total of 55 patients with sporadic gastric cancer who underwent a gastrectomy between 2011 and 2012 at Seoul St. Mary Hospital, The Catholic University of Korea. Patients with a history of familial gastric cancer were excluded. The clinicopathologic characteristics of the study population are listed in Table 1. Remote non-neoplastic mucosal tissue (≥ 5 cm distance) from the patient was used in this study. In addition, gastric mucosal

Table 1. Patient clinicopathological characteristics

Parameter	Value
Age (yr)	58.2 (38.0~77.0)
Gender	
Male	37 (67.3)
Female	18 (32.7)
Depth of invasion	
Early gastric cancer	42 (76.4)
Advanced gastric cancer	13 (23.6)
TNM stage	
I	40 (72.7)
II	9 (16.4)
III	6 (10.9)
IV	0 (0.0)
No. of metastatic lymph node	
N0 (=0)	39 (70.9)
N1 ($\geq 1, \leq 2$)	8 (14.5)
N2 ($\geq 3, \leq 6$)	5 (9.1)
N3 (>6)	3 (5.5)
Site	
Fundus & cardia	2 (3.6)
Body	22 (40.0)
Antrum & pylorus	31 (56.4)
Lauren	
Diffuse	12 (21.8)
Intestinal	23 (41.8)
Mixed	20 (36.4)
Differentiation	
Well	10 (18.2)
Moderate	18 (32.7)
Poor	27 (49.1)
Total	55 (100.0)

Values are presented as median (interquartile range) or number (%).

tissues adjacent to each frozen specimen were also fixed in formalin and stained with hematoxylin-eosin. Histological assessment was performed independently by two pathologists. According to the updated Sydney system,¹⁸ a gastritis score of 0 (absent), 1 (mild), 2 (moderate), or 3 (marked) was assigned for each of the following parameters: activity (granulocytic infiltration), inflammation (lymphocytic and plasma cell infiltration), intestinal metaplasia and glandular atrophy. When the specimens were grade 2 or more, histological findings of granulocyte infiltration and mononuclear cell infiltration were diagnosed as inflammation positive. Atrophy was defined as the loss of appropriate glands; periodic acid Schiff staining was used to identify intestinal metaplasia. The study design was approved by the Institutional Review Board of The Catholic University of Korea, College of Medicine (CUMC09U089).

Table 2. Primer sequences and annealing temperature for methylation-specific polymerase chain reaction

GENE		Primer sequences	Length (bp)	Annealing temperature (°C)
<i>CDH1</i>	M	F: 5'-TTA GGT TAG AGG GTT ATC GCG T-3' R: 5'-TAA CTA AAA ATT CAC CTA CCG AC-3'	116	57
	U	F: 5'-TAA TTT TAG GTT AGAA GGG TTA TTG T-3' R: 5'-CAC AAC CAA TCA ACA ACA CA-3'	97	57
<i>hMLH1</i>	M	F: 5'-GAT AGC GAT TTT TAA CGC-3' R: 5'-TCT ATA AAT TAC TAA ATC TCC TCG-3'	93	53
	U	F: 5'-AGA GTG GAT AGT GAT TTT TAA TGT-3' R: 5'-ACT CTA TAA ATT ACT AAA TCT CTT CA-3'	100	53
<i>MINT1</i>	M	F: 5'-AAT TTT TTT ATA TAT ATT TTC GAA GC-3' R: 5'-AAA AAC CTC AAC CCC GCG-3'	96	52
	U	F: 5'-AAT TTT TTT ATA TAT ATT TTT GAA GTG T-3' R: 5'-AAC AAA AAA CCT CAA CCC CAC A-3'	100	54
<i>MINT2</i>	M	F: 5'-TTG TTA AAG TGT TGA GTT CGT C-3' R: 5'-AAT AAC GACGAT TCC GTA CG-3'	90	55
	U	F: 5'-GAT TTT GTT AAA GTG TTG AGT TTG TT-3' R: 5'-CAA AAT AAT AAC AAC AAT TCC ATA GA-3'	100	52
<i>MINT31</i>	M	F: 5'-TGT TGG GGA AGT GTT TTT CGG C-3' R: 5'-CGA AAA CGA AAC GCC GCG-3'	84	65
	U	F: 5'-TAG ATG TTG GGG AAG TGT TTT TTG GT-3' R: 5'-TAA ATA CCC AAA AAC AAA ACA CCA CA-3'	96	63
<i>p16</i>	M	F: 5'-TTA TTA GAG GGT GGG GCG GAT CGC-3' R: 5'-GAC CCC GAA CCG CGA CCG TAA-3'	150	60
	U	F: 5'-TTA TTA GAG GGT GGG GTG GAT TGT-3' R: 5'-CAA CCC CAA ACC ACA ACC ATA A-3'	151	60

M = specific primer sequences to methylated DNA; U = specific primer sequences to unmethylated DNA; F = forward sequence; R = reverse sequence.

2. Measurement of GKN1, CagA, EZH2, DNMT1, and c-Myc expression

Expression of GKN1, CagA, EZH2, DNMT1, and c-Myc protein was examined in the 55 gastric mucosal tissues by western blot analysis. Each tissue sample was ground to very fine powder in liquid nitrogen using a mortar and pestle and then suspended in ice-cold Nonidet p-40 lysis buffer supplemented with a 1X protease inhibitor mix (Roche Molecular Biochemicals, Mannheim, Germany). The cell lysates were separated by 10% polyacrylamide gel electrophoresis and blotted onto a Hybond-polyvinylidene difluoride transfer membrane (Amersham Pharmacia Biotech, Piscataway, NJ, USA), which was subsequently probed with antibodies against GKN1, CagA, EZH2, DNMT1, c-Myc, or GAPDH. The protein bands were detected using enhanced chemiluminescence western blotting detection reagents (Amersham Pharmacia Biotech). Expression levels of the proteins in the western blots analysis were defined by densitometry. A ratio <0.8 was considered indicative of reduced protein expression.

3. Measurement of miR-185 expression

Total RNA from the 55 frozen gastric mucosal tissues was extracted and quantified. Reverse transcription polymerase chain reaction was performed using a SYBR Green Q-PCR Master Mix (Bio-Rad, Hercules, CA, USA), according to the manufacturer's protocol. miR-185 expression was quantified by quantitative real-time reverse transcription polymerase chain reaction (QPCR) and normalized to human U6 snRNA. The following primer sequences were used: adaptor for miR-185 RT: 5'-GTCGTATC-CAGTGCAGGGTCCGAGGTAT TCGCACTGGATACGACT-CAGGAA-3', miR-185 forward: 5'-CAATGGAGAGAAAGG CAGTTCC-3', reverse: 5'-AATCCATGAGAGATCCCTACCG-3' and U6 forward: 5'-ATTGGAACGATACAGAGAAGATT-3', reverse: 5'-GGAACGCTTCACGAATTT-3'. Data are reported as relative quantities according to an internal calibrator using the $2^{-\Delta\Delta CT}$ method.¹⁹ The standard curve method was used for quantification of the relative amounts of gene expression products. This method provides unit-less normalized expression values that can be used

for direct comparison of the relative amount of RNA in different samples. Reduced expression of miR-185 was defined as a ratio < 0.5 in QPCR. For statistical analysis, the expression levels of the proteins and miR-185 in HFE-145 non-neoplastic gastric mucosal epithelial cells were used as a control.

4. Methylation-specific PCR (MSP)

The methylation status of the promoter region of the six CpG island methylation phenotype (CIMP) panel markers (*p16*, *MINT1*, *MINT2*, *MINT31*, *CDH1*, and *hMLH1*) was determined using sodium bisulfite treatment of the DNA followed by MSP, as described in the literature with minor modifications.²⁰ Briefly, DNA was incubated in 0.2 M NaOH at 42°C for 30 minutes in a total volume of 50 μ l. After adding 350 μ l of 3.6 M sodium bisulfite containing 1 mM hydroquinone at pH 5.0, the samples were incubated for 16 hours at 55°C in the dark. The modified DNA was recovered with 5 μ l glassmilk (BIO 101, Irvine, CA, USA) and 800 μ l of 6 M NaCl. The glassmilk with the modified DNA was washed three times with 70% ethanol at room temperature, treated with 0.3 M NaOH and 90% ethanol, and washed twice with 90% ethanol. The DNA was finally eluted from the dried pellet with 30 μ l of 1 mM Tris-HCl (pH 8.0) for 15 minutes at 55°C. Five microliters of the bisulfite-modified DNA was subjected to *methylation-specific PCR* (MSP) using two sets of primers for the methylated and unmethylated six CIMP panel markers. PCR was performed in a total volume of 30 μ l, which contained 5 μ l of the template DNA, 0.5 μ M of each primer, 0.2 μ M of each dNTP, 1.5 mM MgCl₂, 0.4 units Ampli Taq gold polymerase (Perkin-Elmer, Waltham, MA, USA), and 3 μ l of 10 \times buffer. The reaction solution was initially denatured for 1 minute at 95°C. Amplification was carried out for 40 cycles of 30 s at 95°C, 30 s at 52 to 65°C, and 30 s at 72°C, followed by a final 5 minutes extension at 72°C. Each PCR product was loaded directly onto 2% agarose gels, stained with ethidium bromide, and visualized under ultraviolet illumination. Sequences of the primers and annealing temperatures are described in Table 2. CIMP status was classified as CIMP-negative if none of the analyzed genes were hypermethylated, CIMP-low (L) if $< 50\%$ of genes were hypermethylated, and CIMP-high (H) if $\geq 50\%$ of genes were hypermethylated.

5. Statistical analyses

Pearson and Student's t-tests were used to analyze the correlation between expression of GKN1, miR-185, DNMT1, and EZH2, and the methylation status of the CIMP panel of markers. A

P-value < 0.05 was considered to be the limit of statistical significance. To further evaluate the diagnostic usefulness of the markers based on dichotomous classification, receiver operating characteristic (ROC) curve analysis was used. A ROC curve is a plot of the true-positive fraction versus the false-positive fraction evaluated for all possible cutoff point values.

Results

1. Association between GKN1 expression and the degree of gastritis

Based on histological analysis of the non-neoplastic gastric mucosal tissues, granulocytic and mononuclear cell infiltrations, atrophy, and intestinal metaplasia were found in 11 (20%), 42 (76.4%), 18 (32.7%), and 20 (36.4%) of the 55 cases, respectively. Reduced expression of GKN1 and the presence of *H. pylori* CagA protein were found in 20 (36.4%) and 26 (47.3%) of the 55 gastric mucosal samples, respectively (Fig. 1A). When we compared the expression level of GKN1 to the degree of gastritis, GKN1 expression was significantly reduced in the cases with mononuclear cell infiltration ($P=0.0332$), atrophy ($P<0.0001$), and intestinal metaplasia ($P=0.0079$) (Fig. 1B). However, altered GKN1 expression did not significantly correlate with granulocytic infiltration ($P=0.4889$). Interestingly, GKN1 predicted atrophy and intestinal metaplasia risk with an area under the ROC curve (AUC) value of 0.865 and 0.973, respectively (Fig. 1C).

2. GKN1, miR-185, DNMT1, EZH2, and c-Myc expression

Next, we examined the expression levels of the GKN1, miR-185, DNMT1, EZH2, and c-Myc expression in the 55 non-neoplastic gastric mucosal tissue samples. In agreement with our previous data,¹⁷ expression levels of miR-185 were lower in the cases with reduced GKN1 expression, whereas expression levels of DNMT1, EZH2, and c-Myc proteins were higher in the gastric mucosal tissues with reduced GKN1 expression (Fig. 2A). Statistically, GKN1 protein expression showed a positive correlation with miR-185 expression ($P=0.0001$) and an inverse relationship with expression of DNMT1 ($P<0.0001$), EZH2 ($P<0.0001$), and c-Myc ($P<0.0001$). In addition, *H. pylori* CagA protein was inversely associated with miR-185 ($P=0.0014$) expression and positively associated with expression of DNMT1 ($P=0.0002$), EZH2 ($P=0.0016$), and c-Myc ($P=0.0366$) (Fig. 2B). In each case, GKN1 protein expression was closely related to the expression of miR-185,

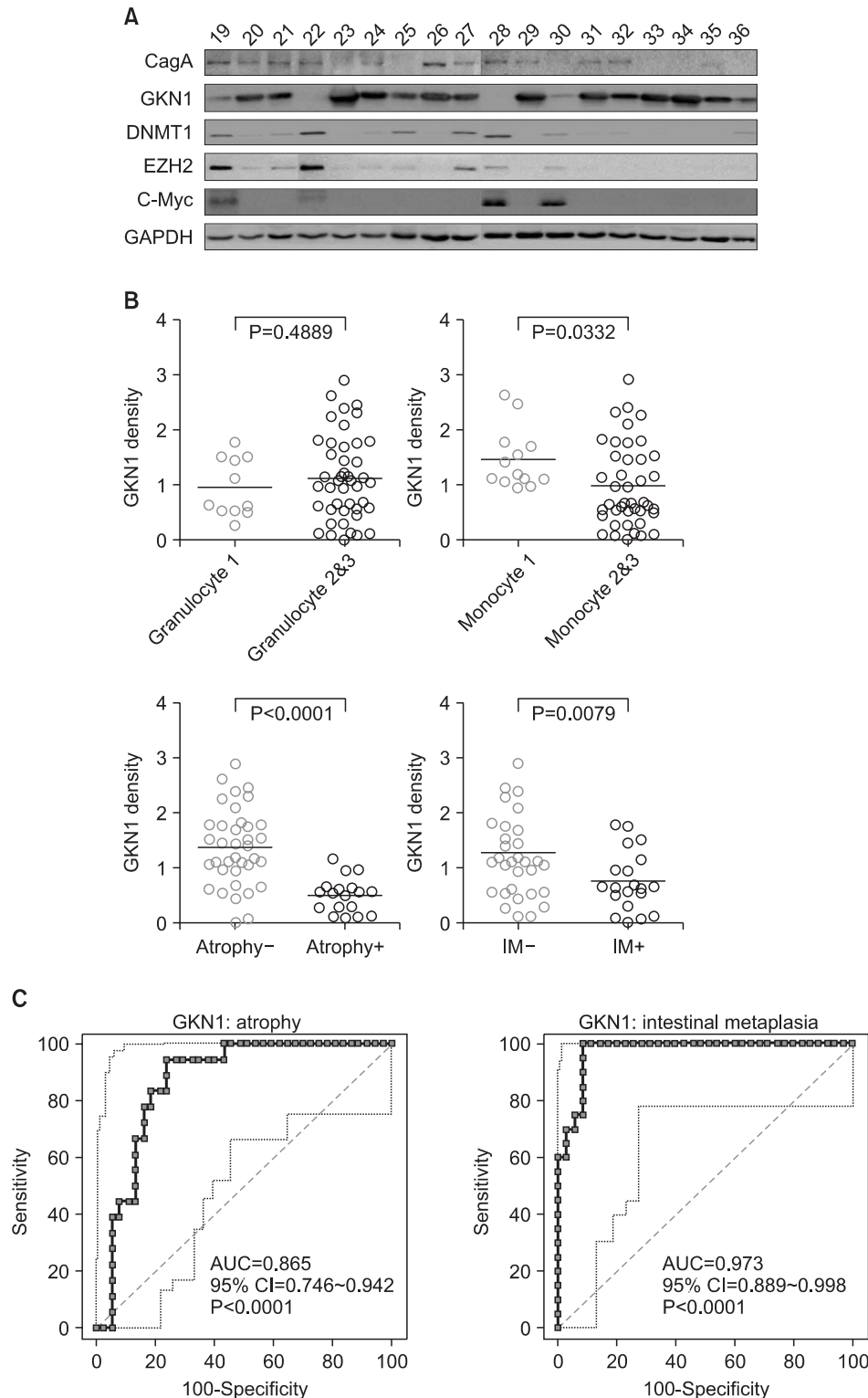


Fig. 1. GKN1 protein is a representative marker of the degree of gastritis. (A) Western blotting analysis with GKN1, CagA, DNMT1, EZH2, and c-Myc in gastric mucosal tissues. (B) Based on scatterplot analysis, GKN1 expression was closely associated with mononuclear cell infiltration (Student's t-test, $P=0.0332$), atrophy ($P<0.0001$), and IM ($P=0.0079$), but not with granulocytic infiltration ($P=0.4889$). (C) ROC curve analysis using GKN1 to distinguish patients with atrophy and intestinal metaplasia from human gastric mucosal tissues. GKN1 yielded an AUC value of 0.865 (95% confidence interval [CI], 0.746~0.942; $P<0.0001$) and 0.973 (95% CI, 0.889~0.998; $P<0.0001$) in distinguishing atrophy and intestinal metaplasia, respectively. GKN1 = Gastrokine 1; DNMT1 = DNA methyltransferase 1; EZH2 = enhancer of zeste homolog 2; ROC = receiver operating characteristic; AUC = area under the ROC curve; IM = intestinal metaplasia.

DNMT1, EZH2, and c-Myc ($P<0.0001$) (Fig. 2C).

3. Methylation status of CIMP panel markers

Bisulfite-modified DNA methylation analysis of the selected six genes was performed in the 55 non-neoplastic gastric mucosal

tissues, which yielded high quality DNA. Based on MSP, the highest methylation frequency was detected for *CDHI* in 45 (81.8%) of gastric mucosal tissue samples. The methylation frequencies of the other five genes were 56.4% for *CDKN2A* (31 cases), 58.1% for *MINT1* (32 cases), 36.4% for *MINT2* (20 cases), 5.4% for *MINT31*

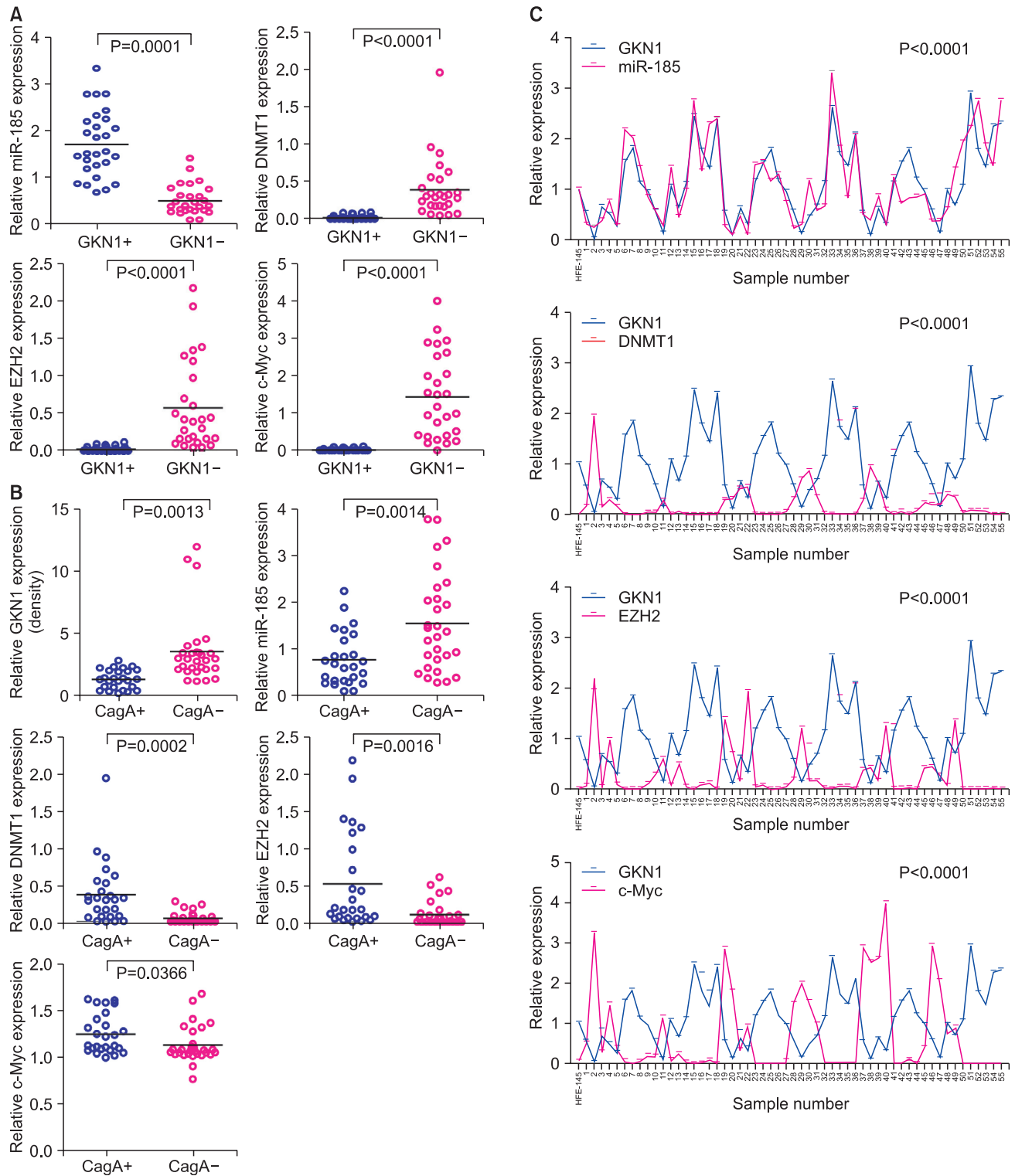


Fig. 2. Expression of GKN1 is positively related to miR-185 expression and inversely related to expression of DNMT1, EZH2, and c-Myc. (A) Based on scatterplot analysis, expression of miR-185 (Pearson test, $P=0.0001$) was higher, while expression of DNMT1 ($P<0.0001$), EZH2 ($P<0.0001$), and c-Myc ($P<0.0001$) was lower in GKN1-positive (GKN1+) gastric mucosa. (B) Scatterplot analysis showed that expression of GKN1 (Pearson test, $P=0.0013$) and miR-185 ($P=0.0014$) was reduced and expression of DNMT1 ($P=0.0002$), EZH2 ($P=0.0016$), and c-Myc ($P=0.0366$) was increased in CagA-positive (CagA+) gastric mucosa. (C) Expression of GKN1 was positively correlated to miR-185 and negatively correlated to DNMT1, EZH2, and c-Myc expression (Pearson test, $P<0.0001$). The value of miR-185 was obtained by quantitative real-time RT-PCR. GKN1 = Gastrokine 1; DNMT 1 = DNA methyltransferase 1; EZH2 = enhancer of zeste homolog 2; RT-PCR = reverse transcription polymerase chain reaction.

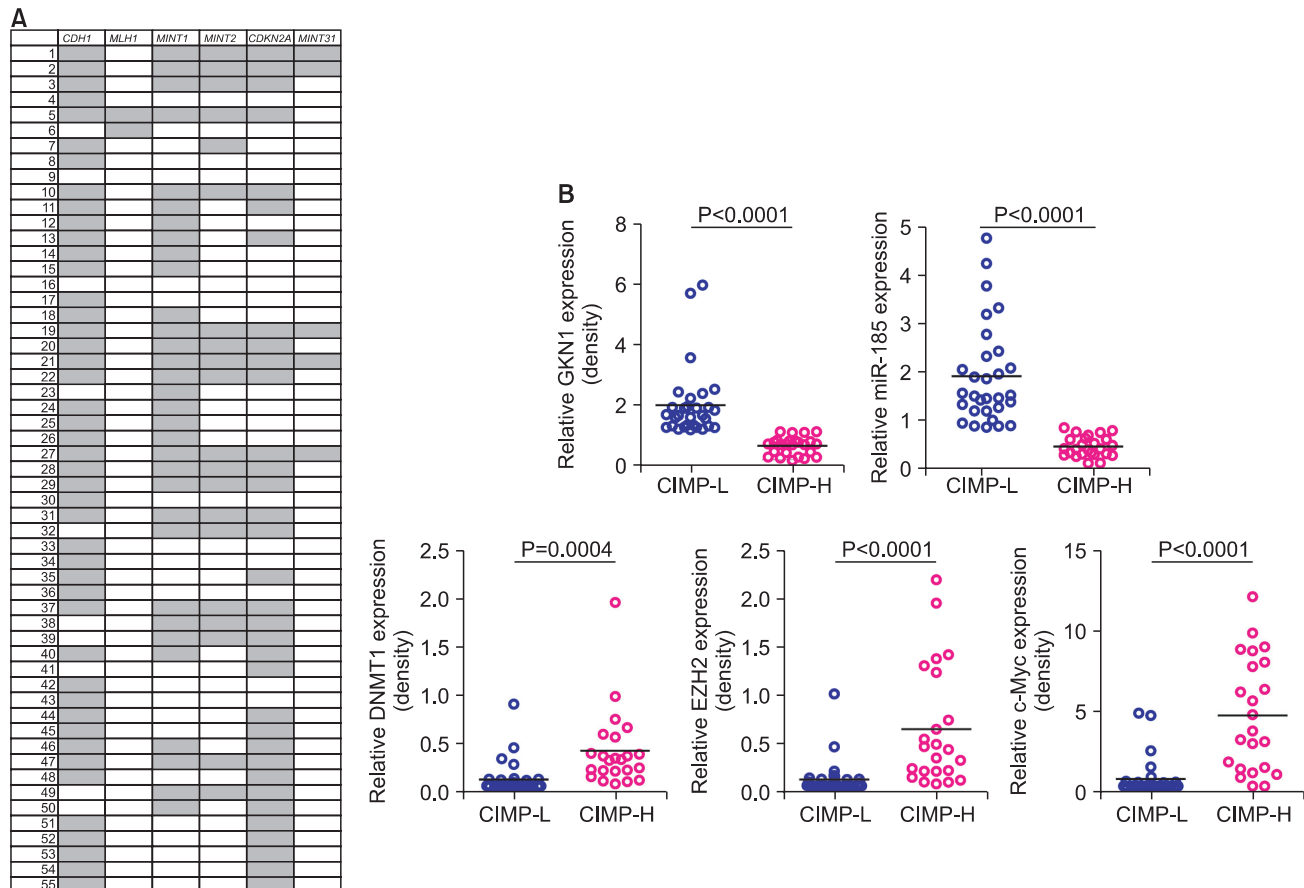


Fig. 3. Expression levels of the epigenetic regulators were closely associated with methylation status in gastric mucosa. (A) In the chart showing methylation in the CIMP panel (*p16*, *MINT1*, *MINT2*, *MINT31*, *CDH1*, and *MLH1*), the gray-colored boxes indicate hypermethylation. (B) Scatterplot analysis with CIMP-L and CIMP-H revealed that methylation status was closely associated with expression levels of GKN1 (Student's t-test, $P < 0.0001$), miR-185 ($P < 0.0001$), DNMT1 ($P = 0.0004$), EZH2 ($P < 0.0001$), and c-Myc ($P < 0.0001$). CIMP = CpG island methylation phenotype; H = high; L = low; GKN1 = Gastrokine 1; DNMT1 = DNA methyltransferase 1; EZH2 = enhancer of zeste homolog 2.

(3 cases), and 3.6% for *MLH1* (2 cases). Among the included cases, at least one gene was methylated in 53 (96.4%) cases and no methylation in any of the examined genes was observed in two cases (Fig. 3A). When we classified the methylation status into CIMP-L and CIMP-H, 30 (54.5%) and 23 (41.9%) cases were CIMP-L and CIMP-H, respectively. In addition, when we compared the methylation status to the expression of GKN1, miR-185, DNMT1, EZH2, and c-Myc, the GKN1 and miR-185 expression was reduced in the CIMP-H cases, whereas expression of DNMT1, EZH2, and c-Myc were up-regulated (Fig. 3B). Statistically, there was a close relationship between the methylation status and expression of GKN1 ($P < 0.0001$), miR-185 ($P < 0.0001$), DNMT1 ($P = 0.0004$), EZH2 ($P < 0.0001$), and c-Myc ($P < 0.0001$).

Discussion

Inflammation has been recognized to be the key risk factor in

the development of many types of cancers. In particular, promoter hypermethylation in tumor-related genes is frequently detected in premalignant gastric lesions.¹³ *H. pylori* infection and the resultant chronic inflammation in the gastric mucosa is a major step in the initiation and development of gastric cancer.²¹ In addition, aberrant DNA methylation is one of the major inactivating mechanisms of tumor suppressor genes and is strongly associated with *H. pylori* infection.^{22,23} Previously, we demonstrated that GKN1 suppresses gastric carcinogenesis through the regulation of epigenetic alteration in a miR-185 dependent manner.¹⁷ However, the precise relationship between gastritis and GKN1 has not been evaluated. Here, we investigated whether altered GKN1 expression is associated with the severity of gastritis and DNA methylation in non-neoplastic gastric mucosa.

Chronic inflammation of the stomach initiates histopathologic progression of chronic gastritis to gastric atrophy, intestinal metaplasia, dysplasia and finally gastric cancer.²⁴ Interleukin (IL)-1 β

activates the transcription factor nuclear factor- κ B (NF- κ B) pathway in myeloid cells by binding to its receptor, IL-1RI.²⁵ A number of reports have demonstrated that NF- κ B is key in linking inflammation to cancer.²⁶ Previously, we reported that GKN1 expression suppresses activation and nuclear translocation of NF- κ B by inhibiting the degradation and phosphorylation of I κ B and inactivating IKK α / β .²⁷ Here, to investigate whether GKN1 is involved in gastric mucosal inflammation, we examined the expression level of GKN1 in 55 non-neoplastic gastric mucosal samples from patients with sporadic gastric cancer and compared GKN1 expression with the degree and characteristics of gastritis. The reduction or loss of GKN1 expression was found in 20 of 55 (36.4%) gastric mucosal tissues and was inversely associated with DNMT1, EZH2, and c-Myc expression (Fig. 1, 2). In addition, *H. pylori* CagA protein was detected in 26 of 55 (47.3%) gastric mucosae and its expression was negatively associated with GKN1 and miR-185 (Fig. 2). GKN1 expression was significantly reduced in gastric mucosa with mononuclear cell infiltration, atrophy, and intestinal metaplasia (Fig. 1B). When we analyzed the ability of GKN1 to discriminate patients with gastritis using an ROC curve, GKN1 predicted gastric mucosal atrophy and intestinal metaplasia risk with an AUC value of 0.865 and 0.973, respectively (Fig. 1C). Thus, our data implicates GKN1 as playing an important role in gastric mucosal inflammation, and suggests GKN1 as a candidate biomarker for atrophy and intestinal metaplasia in the gastric mucosa. Since subjects with normal and healthy gastric mucosa have no significant cancer risk, the ability to differentiate between individuals with healthy and diseased gastric mucosa is clinically relevant for identifying patients possibly at risk. Further studies are necessary to clarify these initial observations and to develop a diagnostic and screening tool using GKN1 as a cost-effective gastritis marker to assess gastric juice and biopsy tissue samples.

Epigenetic alterations, such as CpG island methylation of the promoter region in cancer-associated genes, are regarded as one of the major mechanisms of the development and progression of gastric carcinoma.¹² The fraction of DNA molecules with aberrant methylation in gastric mucosae has been correlated with gastric cancer risk,^{28,29} indicating that methylation in non-cancerous tissues is related to gastric carcinogenesis. Previously, we observed the frequent loss of GKN1 expression in gastric cancers and tumor suppressor activities of GKN1 by inhibiting cell proliferation through epigenetic regulation.¹⁷ Thus, to determine whether GKN1 also plays a pivotal role in DNA methylation in non-neoplastic gastric mucosa, we analyzed the expression level of miR-185, DNMT1,

EZH2, and c-Myc and the expression of CIMP using six CIMP panel markers, including *p16*, *MINT1*, *MINT2*, *MINT31*, *E-CDH*, and *hMLH1* in gastric mucosae. At least one gene was methylated in 53 (96.4%) cases. Of these, 30 (54.5%) and 23 (41.9%) cases were CIMP-L and CIMP-H, respectively (Fig. 3A). The reduction or loss of GKN1 and miR-185 expression was inversely correlated with DNMT1, EZH2, and c-Myc expression (Fig. 1), and closely associated with the methylation status of the CIMP markers (Fig. 3B). Interestingly, expression of DNMT1 (P=0.0002), EZH2 (P=0.0016), and c-Myc (P=0.0366) was positively associated with *H. pylori* CagA protein (Fig. 2) and methylation status (Fig. 3B). These results strongly indicate that GKN1 may play an important role in epigenetic regulation in the gastric mucosal epithelium, and further support that GKN1 functions as a hypomethylating agent by achieving its biological target effect as a DNMT1 inhibitor.¹⁷

In conclusion, GKN1 expression was significantly reduced in gastric mucosa with mononuclear cell infiltration, atrophy, and intestinal metaplasia; and was inversely correlated with expression of DNMT1, EZH2, c-Myc proteins and the methylation status of CIMP markers in non-neoplastic gastric mucosa. These observations provide evidence that GKN1 may be a predictive and diagnostic biomarker for the degree of gastritis and the methylation status in non-neoplastic gastric mucosa. In addition, our data also further support that GKN1 plays an important role in the multi-step process of gastric carcinogenesis.³⁰ Further studies with larger sample sizes are necessary to rationalize and optimize the use of GKN1 as a diagnostic biomarker for gastritis and methylation status in non-neoplastic gastric mucosa.

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