Biomarkers, Genomics, Proteomics, and Gene Regulation

Overexpression of IFITM1 Has Clinicopathologic Effects on Gastric Cancer and Is Regulated by an Epigenetic Mechanism

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In an effort to identify novel genes related to the prognosis of gastric cancer, we performed gene expression profiling and found overexpressed levels of human interferon-induced transmembrane protein 1 (IFITM1). We validated the gastric cancer-specific up-regulation of IFITM1 and its association with cancer progression. We also studied its epigenetic regulation and tumorigenesis-related functions. Expression of IFITM1 was evaluated in various human gastric cancer cells and in 35 patient tumor tissues by quantitative RT-PCR and Western blot analyses. The results showed highly up-regulated IFITM1 in cancer cell lines and tissues. Furthermore, IHC studies were performed on 151 patient tissues, and a significant correlation was revealed between higher IFITM1 expression and Lauren's intestinal type ($P = 0.007$) and differentiated adenocarcinoma ($P = 0.025$). Quantitative studies of DNA methylation for 27 CpG sites in the regulatory region showed hypermethylation in cells expressing low levels of IFITM1. Methylation-dependent IFITM1 expression was confirmed further by *in vitro* demethylation using 5-aza-2'-deoxycytidine and luciferase assays. The functional analysis of IFITM1 by silencing of its expression with small-interfering RNA showed decreased migration and invasiveness of cancer cells, whereas its overexpression exhibited the opposite results. In this study, we demonstrated gastric cancer-specific overexpression of IFITM1 regulated by promoter methylation and the role of IFITM1 in cancer prognosis. (Am J Pathol 2012, 181:43–52; <http://dx.doi.org/10.1016/j.ajpath.2012.03.027>)

Gastric cancer is one of the most common human cancers. In 2008, there were approximately 989,600 new cases and 738,000 deaths worldwide.¹ The most important prognostic indicator for patients with gastric cancer is the observation of metastasis to lymph nodes or distant organs since this occurs in 80% to 90% of patients in the United States and Europe.² Although many studies are currently ongoing, reliable molecular biomarkers of gastric cancer that can help predict tumor progression or assist in extending the survival of patients are yet to be established.

Recently developed technologies, such as gene expression profiling, have been applied toward the identification of genes and genetic alterations associated with the development of gastric cancer, potentially serving as biomarkers for the diagnosis and prognosis of patients with cancer.^{3–5} Several novel genes and polymorphisms associated with cancer prognosis have been revealed by recent studies using these methods.^{6–8} Not only genetic alternations but also epigenetic modifications are involved in tumorigenesis, and many researchers have reported the cancer-specific epigenetic regulation in human cancer.^{9–11} For example, methylation of CpGs, especially in the promoter region, has been suggested as a possible factor influencing the activity of gastric cancer-related genes.^{12,13}

From a previous gene expression profiling study of human gastric tumor and normal tissues (GSE30727),

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we identified interferon-induced transmembrane protein 1 (*IFITM1*) as an up-regulated gene in tumors. Additional surveys of public databases [ArrayExpress (<http://www.ebi.ac.uk/arrayexpress>), European Bioinformatics Institute (<http://www.ebi.ac.uk>), and Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/geo>)] also showed its up-regulated expression in diverse cancer cell lines. *IFITM1* (alias Leu13 or 9-27) is a cell surface 17-kDa membrane protein that is encoded on chromosome arm 11p15.5. It is a member of the interferon-induced transmembrane protein family and was initially known to be a leukocyte antigen that is part of a membrane complex involved in the transduction of antiproliferative and homotypic adhesion signals in lymphocytes.^{14–16} In humans, the *IFITM* family consists of at least three other functional genes (*IFITM1*, *IFITM2*, and *IFITM3*), and *IFITM1* has been studied for its involvement in the inhibition of viral replication,¹⁷ promotion of cell invasion,¹⁸ and drug response.¹⁹ Furthermore, recent studies suggest an association between *IFITM1* and several cancers, such as head and neck cancer,¹⁸ serous ovarian cancer,²⁰ leukemia and lymphoma,²¹ and cervical squamous cell carcinoma.²² Although one study indicated that *IFITM1* may modulate the invasiveness of gastric cancer cells,²³ no further results were reported,

and so the role of *IFITM1* and its regulation in gastric cancer is not fully clarified.

In this study, we report the up-regulation of *IFITM1* expression in gastric cancer and the correlation of *IFITM1* expression with clinicopathologic features of patients with gastric cancer. In addition, the promoter CpG methylation analysis revealed that *IFITM1* expression is epigenetically regulated. The *in vitro* functional study showed the effect that *IFITM1* exerts on cell migration and invasion of gastric cancer cells. Here, we propose that *IFITM1* could be a good candidate as a potential target in the therapy of gastric cancer.

Materials and Methods

Cell Cultures and Tissue Samples

All the cell lines were obtained from ATCC (Manassas, VA) or the Korean Cell Line Bank (Seoul, South Korea) and were cultured in the designated media (Thermo Scientific HyClone, Logan, UT) supplemented with 10% fetal bovine serum (Thermo Scientific HyClone) and 1% penicillin-streptomycin (Invitrogen, Carlsbad, CA). HMECs were obtained from Lonza Inc. (Walkersville, MD) and were cultured in

Table 1. Characteristics of the 151 Patients with Gastric Cancer Who Contributed Tissue for These Studies

Characteristic	IFITM1 expression*				P value
	Negative (n = 18)	Mild (n = 66)	Moderate (n = 50)	Marked (n = 17)	
Sex					0.5848
Male	11	46	37	10	
Female	7	20	13	7	
Age (years)					0.3026
<60	10	36	25	5	
≥60	8	30	25	12	
Differentiation					0.0250 [†]
Well	0	16	11	6	
Moderately	4	23	21	7	
Poorly	14	27	18	4	
Lauren's classification					0.0068 [†]
Intestinal	2	37	29	11	
Mixed	5	9	10	4	
Diffuse	11	20	11	2	
LVI					0.2080
Negative	11	47	26	11	
Positive	7	19	24	6	
T classification					0.1264
T1	6	29	16	4	
T2	3	16	7	5	
T3	6	10	11	1	
T4	3	11	16	7	
LN metastasis					0.1141
Negative	8	39	22	5	
Positive	10	27	28	12	
TNM stage [‡]					0.7857
I	6	36	20	7	
II	5	12	10	4	
III	5	11	13	5	
IV	2	7	7	1	

Data are given as number of patients.

*For *IFITM1* expression, negative, mild, moderate, and marked were determined according to the immunostaining intensity, and positive cases were categorized.

[†]*P* < 0.05.

[‡]Sixth edition of the American Joint Cancer Committee/Union Internationale Contre le Cancer.

LN, lymph node; LVI, lymphovascular invasion.

MEGM basal medium (Lonza Inc.) supplemented with supplement reagent and growth factors. All human cell lines were grown in a 5% CO₂-humidified incubator at 37°C.

Thirty-five pairs of normal and gastric carcinoma tissues used in quantitative RT-PCR (RT-qPCR), RT-PCR, and Western blot studies were obtained by endoscopic biopsy from patients after obtaining their informed consent; the protocols were approved by the Institutional Review Board of the National Cancer Center, Goyang Gyeonggi-do, South Korea (see Supplemental Table S1 at <http://ajp.amjpathol.org>). For immunohistochemical studies, tissues from 151 patients who had undergone gastrectomy and were diagnosed as having tubular adenocarcinoma at the Dong-A University Hospital, Busan, South Korea, between January 1, 2005, and October 31, 2005, were investigated (Table 1). All the patients signed an Institutional Review Board-approved written informed consent form.

RT-qPCR and RT-PCR

Total RNA purified using TRIzol reagent (Invitrogen) was reverse transcribed to cDNA using SuperScript III Reverse Transcriptase (Invitrogen) and was subjected to relative quantification of IFITM1 mRNA by quantitative reverse transcription normalized with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or β -2-microglobulin.²⁴ The primers used are listed in Table 2. QuantiFast SYBR green PCR master mix (Qiagen Inc., Valencia, CA) was used in the LightCycler 480 system (Roche Applied Science, Mannheim, Germany). RT-PCR was performed using Taq DNA polymerase (SolGent Co. Ltd., Daejeon, South Korea).

Western Blot Analysis

Immunoblotting was performed using anti-IFITM1 (sc-66827; Santa Cruz Biotechnology, Santa Cruz, CA), anti-FLAG antibody (F7425; Sigma-Aldrich, St. Louis, MO), and anti- β -actin (Abcam Inc., Cambridge, MA). The signal intensity was measured after subtracting the background by using Multi Gauge V3.0 software (Fujifilm, Tokyo, Japan).

Immunohistochemical Analysis of Gastric Carcinoma Tissues

Immunohistochemical detection of IFITM1 was performed on three core cancer tissues and nonneoplastic gastric mucosa specimens (2 mm in diameter) for each individual. Tissues were arranged as arrays of 4.5- μ m sections mounted on silicon-coated glass slides with anti-IFITM1 antibody (sc-66827; Santa Cruz Biotechnology) in the BenchMark XT automated system (Ventana Medical Systems Inc., Tucson, AZ) as described previously.²⁵ Negative or positive controls were determined by immunostaining intensity of gastric carcinoma cells, and positive cases were categorized into mild, moderate, and marked expression. None of the patients had received any preoperative treatments. Outcomes were determined from the date of surgery until death or December 31, 2009, which resulted in follow-up of 0.8 to 59.8 months (mean, 48.9 months). We determined postoperative pathologic stage using the sixth edition of the American Joint Cancer Committee/Union Internationale Contre le Cancer classification system.²⁶

Table 2. Primers Used in this Study

Experiment	Primer ID	Sequence
RT-qPCR	IFITM1-Q-F	5'-ACTAGTAGCCGCCCATAGCC-3'
	IFITM1-Q-R	5'-GCACGTGCACCTTTATTGAATG-3'
	GAPDH-F	5'-TGCACCACCAACTGCTTA-3'
	GAPDH-R	5'-GGATGCAGGGATGATGTTTC-3'
	B2M-F	5'-ACTGAATTACCCCCACTGA-3'
	B2M-R	5'-CCTCCATGATGCTGCTTACA-3'
RT-PCR	TNF- α -F	5'-AAGAATTCAAACCTGGGGCCT-3'
	TNF- α -R	5'-GGCTACATGGGAACAGCCTA-3'
	IL-6-F	5'-GAACCTCTTCTCCACAAGCG-3'
	IL-6-R	5'-TTTTCTGCCAGTGCCTCTTT-3'
	IL-8-F	5'-TAGCAAAATTGAGGCCAAGG-3'
	IL-8-R	5'-GGACTTGTGGATCCTGGCTA-3'
EpiTYPER analysis	IFITM1-01-10-F	5'-AGGAAGAGAGTATGATTTTTGTGTTGAGGGTTTT-3'
	IFITM1-01-10-R	5'-CAGTAATACGACTCACTATAGGGAGAAGGCTCTAATAACCAAAATTTCTACCCCA-3'
	IFITM1-02-10-F	5'-AGGAAGAGAGTTTTTTGTGTATTTTTTGGTTTTGG-3'
	IFITM1-02-10-R	5'-CAGTAATACGACTCACTATAGGGAGAAGGCTAAAAACCTCTCTCTTAACTTCA-3'
	IFITM1-03-10-F	5'-AGGAAGAGAGTTTTGGGTTAATGGATAGTTAGGGAT-3'
	IFITM1-03-10-R	5'-CAGTAATACGACTCACTATAGGGAGAAGGCTCAAAACCAAAAAATACACAAAAA-3'
	IFITM1-04-10-F	5'-AGGAAGAGAGGGTTATGAGGATGTTTGAATAGGG-3'
	IFITM1-04-10-R	5'-CAGTAATACGACTCACTATAGGGAGAAGGCTAACTCACAATAACTTCACCCCA-3'
MSP analysis	IFITM1-Meth-F	5'-GAGATTTTCGTGTTTCGATTATGTC-3'
	IFITM1-Meth-R	5'-ATAAAACCCCAAACTCACCC-3'
	IFITM1-UnMeth-F	5'-AGATTTTTGTGTTTGATTATGTTGT-3'
	IFITM1-UnMeth-R	5'-ATAAAACCCCAAACTCACCAAC-3'
Promoter assay	IFITM1-C1-F(-750)	5'-GGGGTACCGTGGCAGATGTGTGCCCTG-3'
	IFITM1-C2-F(-200)	5'-GGGGTACCTGGCTAATTCACCAATTACAAACAGC-3'
	IFITM1-C1C2-R(-1)	5'-CCAAGCTTCTCTGGCTTTGGGAAGGAAG-3'

B2M, β -2-microglobulin.

Quantitative Methylation and Methylation-Specific PCR Analysis and 5-Aza-dC Treatment

Genomic DNA extracted from the cell lines using the DNeasy kit (Qiagen Inc.) was sodium bisulfite treated using the EZ DNA Methylation-Gold kit (Zymo Research Corp., Orange, CA). Primers for EpiTYPER and methylation-specific PCR (MSP) analysis were designed using EpiDesigner software (Sequenom Inc., San Diego, CA) and are presented in Table 2. The methylation level was analyzed using EpiTYPER software (Sequenom Inc.). For MSP, genomic DNA was bisulfite modified and amplified using HotStarTaq DNA polymerase (Qiagen Inc.) using MSP primers. For demethylation by 5-azacytidine treatment, cells were seeded 24 hours before treatment and were incubated in media containing 20 $\mu\text{mol/L}$ 5-aza-2'-deoxycytidine (5-aza-dC; Sigma-Aldrich) for 3 days; the

medium was changed every 24 hours and harvested for further analysis. For methylation analysis by cloning and sequencing, the promoter CpG site 10–15–contained region was amplified by PCR and was cloned into the pGEM-T easy vector system (Promega Corp., Madison, WI). The sequences of the PCR products were analyzed using a 3730xl DNA analyzer (Applied Biosystems, Foster City, CA).

Promoter Assay Using the Luciferase System

The amplified fragments of the IFITM1 promoter region (construct 1: –200 to –1, and construct 2: –750 to –1 from the start ATG codon; the primer sequences are presented in Table 2) were cloned into the pGL3-basic vector containing the firefly luciferase gene (Promega Corp.). For *in vitro* methylation, the promoter was treated

Figure 1. Up-regulation of IFITM1 in human gastric cancer cell lines and tissues. **A:** Mean $\pm$ SD IFITM1 mRNA expression in two noncancer cell lines (293 and HMEC) and six gastric cancer cell lines (SNU-216, SNU-638, SNU-719, AGS, KATO III, and MKN-28) detected by RT-qPCR. **B:** Western blot analysis of IFITM1 protein in cell lines. β -actin was used as loading control. IB, immunoblotting. **C:** Mean $\pm$ SD IFITM1 mRNA expression is detected in 27 pairs of matched gastric cancer tissues. Asterisks indicate intestinal-type gastric cancer tissues. B2M, β -2-microglobulin. **D:** Western blot analysis of IFITM1 protein levels in eight pairs of matched normal (N) and tumor (T) gastric cancer tissues. **E:** IFITM1, TNF- α , IL-6, and IL-8 mRNA expression detected by RT-PCR in matched normal (N) and tumor (T) stomach tissues.

with insert fragments with SssI CpG methylase (New England Biolabs, Ipswich, MA) and were religated. H23 human lung cancer cells (2×10^5) and each construct ($1 \mu\text{g}$) were co-transfected with 10 ng of pRL-CMV vector and Renilla luciferase as internal control for the transfection efficiency assessment, and 24 hours later, the luciferase activities were measured using the Dual-Luciferase reporter assay system (Promega Corp.).

Silencing of IFITM1 Expression

For the silencing of IFITM1 expression, 1.5×10^5 AGS human gastric cancer cells were transfected using small-interfering RNA (siRNA) specific for human IFITM1 (SI00054117; 5'-ACAGTCTACCATATTATGTTA-3') or non-targeting siRNA (NC) (Qiagen Inc.) in a 6-well plate and were checked every 24 hours by Western blot analysis.

Construction of IFITM1 Expression Clone and Transfection

Human IFITM1 open reading frame isolated by PCR using forward primer 5'-GAATTCACCCCTCCCCAAAGC-CAGAAGATG-3' and reverse primer 5'-GGATCCAGTCAAAGGTTGCAGGCTA-3' (Bioneer Corp., Daejeon, South Korea) was cloned into pFLAG-CMV-2 vector (Sigma-Aldrich) to make pFLAG-CMV2-IFITM1. This construct was transfected into 293 cells using Lipofectamine 2000 (Invitrogen).

In Vitro Migration, Invasion, and Wound-Healing Assay

Transfilter migration and invasion assays were performed using $8.0\text{-}\mu\text{m}$ pore inserts in a 24-well Transwell (Corning Inc., Corning, NY). For this assay, AGS cells were transfected with IFITM1 siRNA or NC siRNA for 2 days, or IFITM1-overexpressing 293 cells were seeded after 1 day of incubation. They were isolated and added to the upper chamber of a Transwell with a noncoated filter in the migration assay. The invasion assay was performed using 10% Matrigel (BD Bioscience, San Jose, CA)-coated filters at 5×10^4 cells per well, and the cells were allowed to incubate for 48 hours. The migrating and invading cells were stained with Diff-Quik (using a staining kit from Sysmex Corp., Kobe, Japan). For wound measuring, a scratch on complete confluence was made, and the percentage of cell-free area at 24 hours was measured relative to the distance at 0 hour (100%) using photographed images. Each experiment was performed in triplicate, and mean values are presented.

Statistical Analysis

The *t*-test was used to evaluate the significance of the difference between two groups. The χ^2 test was conducted to determine the significance of the difference between the covariates. Survival durations were calculated via the Kaplan-Meier method. The log-rank test was used to compare cumulative survival in the patient

Figure 2. Immunohistochemical analysis of IFITM1 expression in well, moderately, and poorly differentiated human gastric adenocarcinomas. IFITM1 (brown) is mostly expressed in cytoplasm with mild (+), moderate (++), and marked (+++) staining intensities. Negative specimens (-) are denoted. IFITM1 was occasionally co-expressed at luminal borders, particularly in well-differentiated adenocarcinomas. Original magnification, $\times 200$.

groups. We applied $P < 0.05$ for the consideration of statistical significance throughout the study. The MedCalc software program (version 11.4; MedCalc Software, Mariakerke, Belgium) was used for the analyses.

Results

Up-Regulation of *IFITM1* in Human Gastric Cancer Cells and Tissues

IFITM1 mRNA and protein expressions in six human gastric cancer cell lines (SNU-216, SNU-638, SNU-719, AGS, KATO III, and MKN-28) and two human noncancer cell lines (293 and HMEC) were analyzed by RT-qPCR and Western blot analysis. We found that the expressions of *IFITM1* mRNA (Figure 1A) and *IFITM1* protein (Figure 1B) were significantly higher in AGS and SNU-638 cell lines than in normal cell lines. The *IFITM1* expressions in terms of protein level in gastric cancer cell lines were correlative to the mRNA level (Figure 1B).

In parallel studies of *IFITM1* mRNA and protein expressions in 27 pairs of gastric cancer tissues and matched normal tissues, *IFITM1* was highly up-regulated in 25 of 27 cancer tissues (92.6%) compared with in their normal counterparts (paired t -test $P < 0.001$) (Figure 1C). The mRNA expression levels of *IFITM1* in 10 intestinal-type (Lauren's classification) tumor tissues showed a much higher elevation (5.7-fold increase in average) than in its normal counterpart. In accordance with the mRNA level difference, the protein levels in the paired patient tissues also showed an elevation in most cancer tissues (five of eight patients; 62.5%) compared with in its normal counterpart ($P = 0.008$, 3.4-fold increase in average) (Figure 1D).

Since *IFITM1* is an inflammation-inducible gene, the higher level of *IFITM1* in some cancer tissues may reflect increased inflammation in these tissues. We, therefore, compared the expression of *IFITM1* with that of other inflammation marker genes, including tumor necrosis factor- α (TNF- α), IL-6, and IL-8. In the 10 patients showing increased *IFITM1* expression (fold change >3 times), IL-6 and IL-8 levels also increased, although TNF- α level was not correlated with *IFITM1* expression (Figure 1E). These results suggest that up-regulation of *IFITM1* expression levels in cancer reflect increased inflammatory responses.

Clinicopathologic and Prognostic Significance of *IFITM1* Expression

One hundred thirty-three of 151 patients (88.1%) showed positive *IFITM1* expression in gastric adenocarcinomas. Mostly, *IFITM1* was expressed in cytoplasm and occasionally was co-expressed at luminal borders, especially in well-differentiated adenocarcinomas (Figure 2; see also Supplemental Figure S1 at <http://ajp.amjpathol.org>). Although detected in infiltrating mononuclear leukocytes, nonneoplastic gastric epithelial cells did not express *IFITM1*. The characteristics of the 151 patients examined are presented in Table 1. Increased *IFITM1*

expression was more frequent in histologically differentiated gastric carcinomas ($P = 0.025$). In particular, the intestinal type (by Lauren's classification) showed a higher proportion of moderate or marked *IFITM1* expression ($P = 0.007$), which is in accordance with mRNA expression analyzed by RT-qPCR. Patients with gastric carcinoma with moderate and marked expression of *IFITM1* were found to have poorer survival than those with its negative and mild expression, even though this difference was not statistically significant ($P = 0.086$) (see Supplemental Figure S2 at <http://ajp.amjpathol.org>).

Figure 3. CpG methylation status of the *IFITM1* gene promoter region in cell lines. **A:** Schematic view of the *IFITM1* gene upstream region from -1000 to 400 of the start codon (ATG). ETS factor (ETSF) and cyclin D binding myb-like transcription factor (DMTF) transcription factor (TF) binding sites are indicated under the scale. Positions of primers for MSP and promoter constructs for luciferase assay are also placed on the map. **B:** Quantitative methylation analysis of CpG sites in *IFITM1* promoter. CpG sites are depicted as a vertical bar in the map (see Supplemental Figure S3 at <http://ajp.amjpathol.org>). **C:** MSP analysis of the *IFITM1* gene promoter region in six cell lines. The methylated (M) and unmethylated (U) DNA was amplified by specific primers for each methylation status. **D:** Mean $\pm$ SD levels of *IFITM1* before and after 5-aza-dC treatment in gastric cancer cell lines SNU-216, SNU-719, and MKN-28. **E:** Promoter activity analysis of the *IFITM1* gene by the measurement of mean $\pm$ SD luciferase activity.

Quantitative Methylation Analysis of IFITM1 and Validation by MSP

DNA methylation levels of IFITM1 were determined in five gastric cancer cell lines and one normal cell line covering 27 CpG sites over the promoter, exon 1, and intron 1 regions (Figure 3A; also see the detailed CpG sites in Supplemental Figure S3 at <http://ajp.amjpathol.org>). In the vicinity of the transcriptional start site, high IFITM1-expressing gastric cancer cell lines (SNU-638 and AGS) showed low methylation compared with the normal cell line (293) and the low IFITM1-expressing gastric cancer cell lines (SNU-216, SNU-719, and MKN-28) (Figure 3B). Although CpG sites 1 to 5 seemed to be unrelated to gene expression, the methylation status of CpG sites 11 to 26 inversely correlated with IFITM1 gene expression, indicating promoter methylation-regulated gene expression.

Based on the results of the quantitative methylation analysis, primers were designed to target four CpG sites (No. 12, 13, 19, and 20). Although there was significant methylation of the four CpG sites in cells expressing low levels of IFITM1 (SNU-216, SNU-719, MKN-28, and 293), there was little methylation of these CpG sites in cells highly expressing IFITM1 (SNU-638 and AGS) (Figure 3C). Therefore, these results suggest that the vicinity of the transcriptional start site region in the IFITM1 promoter, especially CpG sites 12 and 13, may play an important role in the regulation of methylation-related expression of IFITM1. When we searched the transcription factor binding sites of this region using MatInspector analysis software (Genomatix Software GmbH, Munich,

Germany), the predicted binding site of the human proto-oncogene ETS factor and cyclin D binding myb-like transcription factor, were found situated in the exact position of CpG site 12, with core similarity of 1.0 for all and matrix similarity of 0.946 and 0.874, respectively (see Supplemental Figure S3 at <http://ajp.amjpathol.org>).

Analysis of Methylation-Dependent IFITM1 Expression

We examined whether DNA demethylation by 5-aza-dC treatment affected IFITM1 mRNA expression. 5-Aza-dC-treated SNU-216, SNU-719, and MKN-28 gastric cancer cells showed a greater increase in IFITM1 mRNA expression compared with in controls, as judged by RT-qPCR analysis (2.5-, 2.5-, and 30.3-fold increases, respectively) (Figure 3D). These results suggest that DNA methylation suppresses IFITM1 gene expression and that demethylation increases it.

Methylation-dependent IFITM1 expression was further confirmed by *in vitro* methylation studies in which two promoter constructs (1: -750 to -1, and 2: -200 to -1; Figure 3A) were methylated by methyltransferase and ligated to a luciferase reporter gene. Comparison of the luciferase activity of the methylated and unmethylated IFITM1 promoter showed an approximately 28% decrement in the case of construct 2 (Figure 3E), suggesting that the methylation status of the -200 to -1 promoter region (containing CpG 10 to 15) is important for the regulation of IFITM1 expression.

Figure 4. Effect of IFITM1 silencing on gastric cancer cell migration and invasion *in vitro*. **A:** Western blot analysis detecting the silencing of IFITM1 protein expression in AGS cells by non-targeting siRNA (NC) or IFITM1 siRNA transfection. IB, immunoblotting. **B:** IFITM1 siRNA-transfected AGS cells display considerably inhibited wound-healing capabilities compared with NC-transfected AGS cells. **C:** IFITM1 siRNA-transfected AGS cell migration capability is inhibited by approximately 28% compared with NC-transfected AGS cells. Mean $\pm$ SD migrated cell percentages 24 hours after incubation are shown on the right (NC = 100%, $n = 3$). **D:** IFITM1 siRNA treatment dramatically inhibits the invasion of gastric cancer cells by approximately 74% compared with NC-transfected AGS cells. Mean $\pm$ SD invaded cell numbers 48 hours after incubation are shown on the right ($n = 3$). *** $P < 0.001$.

Figure 5. Effect of IFITM1 overexpression on cell migration and invasion. **A:** pFLAG-CMV2-IFITM1-transfected 293 cells effectively produced IFITM1 protein. IB, immunoblotting. **B:** Wound-healing is enhanced by IFITM1 overexpression in 293 cells. **C:** Migration of 293 cells is enhanced by IFITM1 overexpression ($n = 3$). Data are given as mean $\pm$ SD. **D:** *In vitro* invasion assay shows that IFITM1 overexpression dramatically enhances the invasion of 293 cells by approximately 2.5-fold ($n = 3$). Data are given as mean $\pm$ SD. ** $P < 0.01$, **** $P < 0.0001$.

IFITM1 Expression Affects Cell Migration and Invasion

To investigate the role of IFITM1 in the progression of malignancy, we examined the influence of IFITM1 on the migration and invasion of AGS cells. We verified that IFITM1 expression is reduced by siRNA in AGS cells 24 hours after siRNA transfection and remains reduced for 96 hours. Nontargeting siRNA (NC) had no effect on IFITM1 mRNA or protein levels (Figure 4A). At 96 hours, wound healing of IFITM1 siRNA-transfected cells was inhibited (Figure 4B). The cell migration or invasion counted from 10 randomly selected areas per well at 24 hours showed that IFITM1 siRNA also inhibited migration (Figure 4C) and invasion (Figure 4D) remarkably.

We also confirmed that overexpression of IFITM1, conversely, enhances invasion and migration. When the pFLAG-CMV2-IFITM1 construct was transfected into 293 cells (Figure 5A), IFITM1 overexpression enhanced wound healing (Figure 5B), migration (Figure 5C), and invasion of cells (Figure 5D). Taken together, these results suggest that the expression of IFITM1 controls the invasiveness and migration of gastric cancer.

Discussion

A previous study of human gastric cancer using microarray analysis identified differentially expressed genes, including IFITM1. The increased expression of IFITM1 reported for cancers of the cervix,²² colon,²⁷ esophagus,²⁸ and ovary²⁹ strongly suggest the association of IFITM1 with

tumorigenesis. The present data from numerous gastric cancer cell lines and many patient samples confirm that IFITM1 plays a role in gastric cancer tumorigenesis.

An interesting result from this study is that the IFITM1 expression level is markedly different in the intestinal and diffuse types of gastric cancer, with the intestinal type exhibiting higher expression of IFITM1 than the diffuse type (Figure 1C). High expression of IFITM1 was also observed for the intestinal type by Lauren's classification using immunohistochemical analysis in 151 patients with gastric cancer ($P = 0.0070$). Thus, IFITM1 expression clearly influences the development and progression of intestinal-type gastric cancer tumorigenesis. In addition, the appearance of IFITM1 in the inflammatory cells near the tumor regions suggests an inflammatory interaction between the tumor and its microenvironment induced by the inflammatory cytokines, as reported with colorectal cancer.³⁰ Of note, IFITM1 was suggested to be a proinflammatory mediator induced by signaling from the activation of CD147 in macrophages.³¹ We compared the expression of IFITM1 with that of some inflammation markers in gastric cancer tissues. We found that IL-6,³² a potent pleiotropic inflammatory cytokine, and IL-8,³³ a cytokine involved in gastric mucosa inflammation, were also highly expressed along with IFITM1. Although, for reasons we do not yet know, TNF- α , a key molecule in chronic inflammatory diseases,³⁴ was not expressed along with IFITM1 in gastric cancer tissues, we concluded that IFITM1-mediated inflammatory events contribute to tumor promotion and progression. Furthermore, the recent report that IFITM1 was up-regulated after *He-*

licobacter pylori infection³⁵ and the fact that tissue samples used in this study are mostly *H. pylori* positive (up to 90%) suggest that IFITM1-mediated inflammatory events after *H. pylori* infection are a probable scenario for the progression of gastric cancer.

This study also found higher expression of histologically differentiated gastric carcinoma ($P = 0.025$). Although not statistically significant, probably because of the limited number of patients studied, patients with gastric cancer with moderate and marked expression of IFITM1 tended to show reduced survival rates. Taken together, the observations of association between IFITM1 expression and tumor invasion suggest that IFITM1 is a potential marker for poor prognosis in patients with gastric cancer, especially the intestinal type.

These studies attempted to elucidate the mechanisms regulating expression of the *IFITM1* gene, which have not been identified to date. We conducted quantitative methylation analysis of 27 CpG sites in the promoter–exon 1–intron 1 region. This analysis showed that methylation at CpG sites 11 to 26 (–200 to 400) could be involved in the regulation of IFITM1 expression in gastric cancer cell lines. The findings of hypermethylation of promoter CpG sites in low IFITM1–expressing cells, hypomethylation in high IFITM1–expressing cells, recovery of IFITM1 expression on demethylation by 5-aza-dC treatment in gastric cancer cells (SNU-216, SNU-719, and MKN-28), and decreased IFITM1 expression by *in vitro* methylation indicate that IFITM1 expression is epigenetically regulated by DNA methylation, although further methylation studies of a broad range of patients are needed.

In addition, an interesting finding is that ETS and cyclin D binding myb-like transcription factor binding sites were predicted on CpG site 12. This finding suggests that CpG methylation of CpG site 12 may influence gastric cancer tumorigenesis by affecting oncogenic transcription factor binding. The proto-oncogene ETS factor is implicated in the pathogenesis of several types of cancers^{36,37} and in proinflammatory responses.³⁸ Dmp1 encodes a DNA binding protein that receives signals from oncogenic Ras and, thus, functions as a tumor suppressor by activating the Arf-p53 pathway.³⁹ Therefore, a valuable future issue to be addressed is the precise analysis of how and to what extent these transcription factor families cooperate and antagonize each other in promoter CpG methylation.

As a direct approach to clarify the involvement of IFITM1 in invasion and migration, we performed *in vitro* studies of the gain or loss of function studies. The results clearly showed that the suppression or up-regulation of IFITM1 expression significantly affected cancer cell migration and invasion, in agreement with recent reports of head and neck cancers¹⁸ and glioma.⁴⁰ As noted by Fidler,⁴¹ metastasis involves sequential and selective steps so that IFITM1 may be involved in the initial step of invasion. Together with the results of survival analysis by the Kaplan-Meier plot, it is suggested that the migration and invasion of gastric cancer cells influenced by IFITM1 expression contributes to the rapid progression of malignancy.

The finding that IFITM1 expression is highly up-regulated in human gastric cancer and is involved in the

migration and invasion of gastric cancer cells suggests a new target as a prognostic factor for gastric cancer. In addition, the observation that IFITM1 expression is regulated by DNA methylation of its promoter region may be useful to treat cancer. Further investigations are needed to elucidate the molecular mechanisms of IFITM1 and its role in the malignancy of gastric cancer.

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