S, Jass J, Haile R, Laird P: CpG island methylator phenotype underlies sporadic microsatellite instability and is tightly associated with BRAF mutation in colorectal cancer. Nat Genet 2006, 38:787
10. Shen L, Toyota M, Kondo Y, Lin E, Zhang L, Guo Y, Hernandez N, Chen X, Ahmed S, Konishi K, Hamilton S, Issa J: Integrated genetic and epigenetic analysis identifies three different subclasses of colon cancer. Proc Natl Acad Sci U S A 2007, 104:18654
11. Samowitz W, Albertsen H, Herrick J, Levin T, Sweeney C, Murtaugh M, Wolff R, Slattery M: Evaluation of a large, population-based sample supports a CpG island methylator phenotype in colon cancer. Gastroenterology 2005, 129:837–845
12. Ogino S, Kawasaki T, Kirkner GJ, Loda M, Fuchs CS: CpG island methylator phenotype-low (CIMP-low) in colorectal cancer: possible associations with male sex and KRAS mutations. J Mol Diagn 2006, 8:582–588
13. Hinoue T, Weisenberger DJ, Lange CP, Shen H, Byun HM, Van Den Berg D, Malik S, Pan F, Noushmehr H, van Dijk CM, Tollenaar RA, Laird PW: Genome-scale analysis of aberrant DNA methylation in colorectal cancer. Genome Res 2012, 22:271–282
14. Spring K, Zhao Z, Karamatic R, Walsh M, Whitehall VLJ, Pike T, Simms L, Young J, James M, Montgomery G, Appleyard M, Hewett D, Togashi K, Jass J, Leggett B: High prevalence of sessile serrated adenomas with BRAF mutations: a prospective study of patients undergoing colonoscopy. Gastroenterology 2006, 131:1400
15. Leggett B, Whitehall V: Role of the serrated pathway in colorectal cancer pathogenesis. Gastroenterology 2010, 138:2088–2100
16. Nagasaka T, Koi M, Kloor M, Gebert J, Vilkin A, Nishida N, Shin S, Sasamoto H, Tanaka N, Matsubara N, Boland CR, Goel A: Mutations in both KRAS and BRAF may contribute to the methylator phenotype in colon cancer. Gastroenterology 2008, 134:1950
17. Goel A, Nagasaka T, Arnold C, Inoue T, Hamilton C, Niedzwiecki D, Compton C, Mayer R, Goldberg R, Bertagnolli M, Boland CR: The CpG island methylator phenotype and chromosomal instability are inversely correlated in sporadic colorectal cancer. Gastroenterology 2007, 132:127
18. Kudo S, Tamura S, Nakajima T, Yamano H, Kusaka H, Watanabe H: Diagnosis of colorectal tumorous lesions by magnifying endoscopy. Gastrointest Endosc 1996, 44:8–14
19. Kudo S, Lambert R, Allen J, Fujii H, Fujii T, Kashida H, Matsuda T, Mori M, Saito H, Shimoda T, Tanaka S, Watanabe H, Sung J, Feld A, Inadomi J, O'Brien M, Lieberman D, Ransohoff D, Soetikno R, Triadafilopoulos G, Zauber A, Teixeira C, Rey J, Jaramillo E, Rubio C, Van Gossum A, Jung M, Vieth M, Jass J, Hurlstone P: Nonpolypoid neoplastic lesions of the colorectal mucosa. Gastrointest Endosc 2008, 68:S3–S47
20. Kimura T, Yamamoto E, Yamano HO, Suzuki H, Kamimae S, Nojima M, Sawada T, Ashida M, Yoshikawa K, Takagi R, Kato R, Harada T, Suzuki R, Maruyama R, Kai M, Imai K, Shinomura Y, Sugai T, Toyota M: A novel pit pattern identifies the precursor of colorectal cancer derived from sessile serrated adenoma. Am J Gastroenterol 2012, 107:460–469
21. Akino K, Toyota M, Suzuki H, Mita H, Sasaki Y, Ohe-Toyota M, Issa JP, Hinoda Y, Imai K, Tokino T: The Ras effector RASSF2 is a novel tumor-suppressor gene in human colorectal cancer. Gastroenterology 2005, 129:156–169
22. Torlakovic E, Skovlund E, Snover D, Torlakovic G, Nesland J: Morphologic reappraisal of serrated colorectal polyps. Am J Surg Pathol 2003, 27:65–81
23. Goto Y, Shinjo K, Kondo Y, Shen L, Toyota M, Suzuki H, Gao W, An B, Fujii M, Murakami H, Osada H, Taniguchi T, Usami N, Kondo M, Hasegawa Y, Shimokata K, Matsuo K, Hida T, Fujimoto N, Kishimoto T, Issa JP, Sekido Y: Epigenetic profiles distinguish malignant pleural mesothelioma from lung adenocarcinoma. Cancer Res 2009, 69: 9073–9082
24. Toyota M, Suzuki H, Sasaki Y, Maruyama R, Imai K, Shinomura Y, Tokino T: Epigenetic silencing of microRNA-34b/c and B-cell translocation gene 4 is associated with CpG island methylation in colorectal cancer. Cancer Res 2008, 68:4123–4132
25. Parsons DW, Wang TL, Samuels Y, Bardelli A, Cummins JM, DeLong L, Silliman N, Ptak J, Szabo S, Willson JK, Markowitz S, Kinzler KW, Vogelstein B, Lengauer C, Velculescu VE: Colorectal cancer: mutations in a signalling pathway. Nature 2005, 436:792
26. Sugai T, Habano W, Nakamura S, Uesugi N, Sasou S, Itoh C: A unique method for mutation analysis of tumor suppressor genes in colorectal carcinomas using a crypt isolation technique. Arch Pathol Lab Med 2000, 124:382–386
27. Sugai T, Habano W, Endoh M, Konishi Y, Akasaka R, Toyota M, Yamano H, Koeda K, Wakabayashi G, Suzuki K: Molecular analysis of gastric differentiated-type intramucosal and submucosal cancers. Int J Cancer 2010, 127:2500–2509
28. Boland CR, Thibodeau SN, Hamilton SR, Sidransky D, Eshleman JR, Burt RW, Meltzer SJ, Rodriguez Bigas MA, Fodde R, Ranzani GN, Srivastava S: A National Cancer Institute Workshop on Microsatellite Instability for cancer detection and familial predisposition: development of international criteria for the determination of microsatellite instability in colorectal cancer. Cancer Res 1998, 58:5248–5257
29. Igarashi S, Suzuki H, Niinuma T, Shimizu H, Nojima M, Iwaki H, Nobuoka T, Nishida T, Miyazaki Y, Takamaru H, Yamamoto E, Yamamoto H, Tokino T, Hasegawa T, Hirata K, Imai K, Toyota M, Shinomura Y: A novel correlation between LINE-1 hypomethylation and the malignancy of gastrointestinal stromal tumors. Clin Cancer Res 2010, 16:5114–5123
30. Suzuki H, Igarashi S, Nojima M, Maruyama R, Yamamoto E, Kai M, Akashi H, Watanabe Y, Yamamoto H, Sasaki Y, Itoh F, Imai K, Sugai T, Shen L, Issa JP, Shinomura Y, Tokino T, Toyota M: IGFBP7 is a p53-responsive gene specifically silenced in colorectal cancer with CpG island methylator phenotype. Carcinogenesis 2010, 31:342–349
31. Suzuki H, Watkins DN, Jair KW, Schuebel KE, Markowitz SD, Chen WD, Pretlow TP, Yang B, Akiyama Y, Van Engeland M, Toyota M, Tokino T, Hinoda Y, Imai K, Herman JG, Baylin SB: Epigenetic inactivation of SFRP genes allows constitutive WNT signaling in colorectal cancer. Nat Genet 2004, 36:417–422
32. Hinoue T, Weisenberger DJ, Pan F, Campan M, Kim M, Young J, Whitehall VL, Leggett BA, Laird PW: Analysis of the association between CIMP and BRAF in colorectal cancer by DNA methylation profiling. PLoS One 2009, 4:e8357
33. Ogino S, Kawasaki T, Kirkner GJ, Ohnishi M, Fuchs CS: 18q Loss of heterozygosity in microsatellite stable colorectal cancer is correlated with CpG island methylator phenotype-negative (CIMP-0) and inversely with CIMP-low and CIMP-high. BMC Cancer 2007, 7:72
34. Cheng YW, Pincas H, Bacolod MD, Schemmann G, Giardina SF, Huang J, Barral S, Idrees K, Khan SA, Zeng Z, Rosenberg S, Notterman DA, Ott J, Paty P, Barany F: CpG island methylator phenotype associates with low-degree chromosomal abnormalities in colorectal cancer. Clin Cancer Res 2008, 14:6005–6013
35. Fang F, Turcan S, Rimner A, Kaufman A, Giri D, Morris LG, Shen R, Seshan V, Mo Q, Heguy A, Baylin SB, Ahuja N, Viale A, Massague J, Norton L, Vahdat LT, Moynahan ME, Chan TA: Breast cancer methylomes establish an epigenomic foundation for metastasis. Sci Transl Med 2011, 3:75ra25
36. Jover R, Nguyen T, Prez-Carbonell L, Zapater P, Pay A, Alenda C, Rojas E, Cubiella J, Balaguer F, Morillas J, Clofent J, Bujanda L, Re J, Bessa X, Xicola R, Nicols-Prez D, Castells A, Andreu M, Llor X, Boland CR, Goel A: 5-Fluorouracil adjuvant chemotherapy does not increase survival in patients with CpG island methylator phenotype colorectal cancer. Gastroenterology 2011, 140:1174–1181
37. Noushmehr H, Weisenberger DJ, Diefes K, Phillips HS, Pujara K, Berman BP, Pan F, Pelloski CE, Sulman EP, Bhat KP, Verhaak RG, Hoadley KA, Hayes DN, Perou CM, Schmidt HK, Ding L, Wilson RK, Van Den Berg D, Shen H, Bengtsson H, Neuvial P, Cope LM, Buckley J, Herman JG, Baylin SB, Laird PW, Aldape K; Cancer Genome Atlas Research Network: Identification of a CpG island methylator phenotype that defines a distinct subgroup of glioma. Cancer Cell 2010, 17:510–522
38. Jones S, Chen W, Parmigiani G, Diehl F, Beerenwinkel N, Antal T, Traulsen A, Nowak M, Siegel C, Velculescu V, Kinzler K, Vogelstein B, Willis J, Markowitz S: Comparative lesion sequencing provides insights into tumor evolution. Proc Natl Acad Sci U S A 2008, 105:4283–4288
39. Yagi K, Takahashi H, Akagi K, Matsusaka K, Seto Y, Aburatani H, Nakajima A, Kaneda A: Intermediate methylation epigenotype and its correlation to KRAS mutation in conventional colorectal adenoma. Am J Pathol 2012, 180:616–625
40. Jass JR, Baker K, Zlobec I, Higuchi T, Barker M, Buchanan D, Young J: Advanced colorectal polyps with the molecular and morphological features of serrated polyps and adenomas: concept of a "fusion" pathway to colorectal cancer. Histopathology 2006, 49:121–131

41. Pino M, Chung D: The chromosomal instability pathway in colon cancer. Gastroenterology 2010, 138:2059–2072
42. Ogino S, Kawasaki T, Kirkner G, Suemoto Y, Meyerhardt J, Fuchs C: Molecular correlates with MGMT promoter methylation and silencing support CpG island methylator phenotype-low (CIMP-low) in colorectal cancer. Gut 2007, 56:1564–1571
43. Ogino S, Nosho K, Irahara N, Shima K, Baba Y, Kirkner G, Meyerhardt J, Fuchs C: Prognostic significance and molecular associations of 18q loss of heterozygosity: a cohort study of microsatellite stable colorectal cancers. J Clin Oncol 2009, 27:4591–4598
44. He Y, Van't Veer LJ, Mikolajewska-Hanclich I, van Velthuysen ML, Zeestraten EC, Nagtegaal ID, van de Velde CJ, Marijnen CA: PIK3CA mutations predict local recurrences in rectal cancer patients. Clin Cancer Res 2009, 15:6956–6962
45. Baba Y, Nosho K, Shima K, Hayashi M, Meyerhardt JA, Chan AT, Giovannucci E, Fuchs CS, Ogino S: Phosphorylated AKT expression is associated with PIK3CA mutation, low stage, and favorable outcome in 717 colorectal cancers. Cancer 2011, 117:1399–1408
46. Whitehall VLJ, Rickman C, Bond C, Ramsnes I, Greco S, Umapathy A, McKeone D, Faleiro R, Buttenshaw R, Worthley D, Nayler S, Zhao Z, Montgomery G, Mallitt K, Jass J, Matsubara N, Notohara K, Ishii T, Leggett B: Oncogenic PIK3CA mutations in colorectal cancers and polyps. Int J Cancer 2012, 131:813–820
47. Corcoran RB, Dias Santagata D, Bergethon K, Iafrate AJ, Settleman J, Engelman JA: BRAF gene amplification can promote acquired resistance to MEK inhibitors in cancer cells harboring the BRAF V600E mutation. Sci Signal 2010, 3:ra84
48. Little AS, Balmanno K, Sale MJ, Newman S, Dry JR, Hampson M, Edwards PA, Smith PD, Cook SJ: Amplification of the driving oncogene, KRAS or BRAF, underpins acquired resistance to MEK1/2 inhibitors in colorectal cancer cells [Erratum appears in Sci Signal 2011, 4: er2]. Sci Signal 2011, 4:ra17
49. Yamauchi M, Morikawa T, Kuchiba A, Imamura Y, Qian Z, Nishihara R, Liao X, Waldron L, Hoshida Y, Huttenhower C, Chan A, Giovannucci E, Fuchs C, Ogino S: Assessment of colorectal cancer molecular features along bowel subsites challenges the conception of distinct dichotomy of proximal versus distal colorectum. Gut 2012, 61:847–854
50. Yamauchi M, Lochhead P, Morikawa T, Huttenhower C, Chan A, Giovannucci E, Fuchs C, Ogino S: Colorectal cancer: a tale of two sides or a continuum? Gut 2012, 61:794–797

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Research Article

Cancer Prevention Research

Aberrant Methylation of *RASGRF1* Is Associated with an Epigenetic Field Defect and Increased Risk of Gastric Cancer

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Abstract

Aberrant DNA methylation is implicated in the epigenetic field defect seen in gastric cancer. Our aim in this study was to identify predictive biomarkers by screening for DNA methylation in noncancerous background gastric mucosa from patients with gastric cancer. Using methylated-CpG island amplification coupled with CpG island microarray (MCAM) analysis, we identified 224 genes that were methylated in the noncancerous gastric mucosa of patients with gastric cancer. Among them, *RASGRF1* methylation was significantly elevated in gastric mucosa from patients with either intestinal or diffuse type gastric cancer, as compared with mucosa from healthy individuals (8.3% vs. 22.4%, $P < 0.001$; 8.3% vs. 19.4%, $P < 0.001$). *RASGRF1* methylation was independent of mucosal atrophy and could be used to distinguish both serum pepsinogen test-positive [sensitivity, 70.0%; specificity, 86.7%; area under the receiver operator characteristic (ROC) curve, AUC, 0.763] and -negative patients with gastric cancer (sensitivity, 72.2%; specificity, 87.0%; AUC, 0.844) from healthy individuals. Ectopic expression of *RASGRF1* suppressed colony formation and Matrigel invasion by gastric cancer cells, suggesting it may be involved in gastric tumorigenesis. Collectively, our data suggest that *RASGRF1* methylation is significantly involved in an epigenetic field defect in the stomach, and that it could be a useful biomarker to identify individuals at high risk for gastric cancer. *Cancer Prev Res; 5(10); 1203–12. ©2012 AACR.*

Introduction

Gastric cancer is a major cause of cancer-related mortality, worldwide. *Helicobacter pylori* (*H. pylori*) plays an important role in gastric carcinogenesis, although the majority of the individuals with *H. pylori* infection do not develop gastric cancer (1). Histologically, gastric cancers are divided into 2 subgroups, intestinal and diffuse, which are thought to develop through separate pathologic pathways (2). Etiologic analysis has shown that individuals with *H. pylori*–related gastritis, severe atrophy, and intestinal metaplasia are at high risk of developing intestinal type gastric cancers, which are often associated with metachronous gastric cancer development. On the other hand, individuals with *H. pylori*–related pangastritis and enlarged-fold gastritis, which are lesions without mucosal atrophy or intestinal metaplasia, are at increased risk of developing diffuse type gastric cancers (3). Surveillance of these high-risk patients using reliable and accurate predictive markers is important for reducing the incidence of gastric cancer and its mortality.

Aberrant DNA methylation is one of the most common molecular alterations found in neoplasias; CpG island hypermethylation is associated with the silencing of tumor suppressor genes and other tumor-related genes, whereas global hypomethylation is thought to induce oncogene activation or chromosomal instability (4). The list of genes aberrantly methylated in gastric cancer is growing and now includes genes involved in cell-cycle regulation, apoptosis, immune function, cell signaling, and tumor invasion and metastasis (3, 5). In addition, aberrant DNA methylation is frequently observed in noncancerous gastric mucosa in *H. pylori*–infected patients, suggesting aberrant DNA methylation is an early step during gastric carcinogenesis (6, 7). We previously showed hypomethylation of LINE-1 repetitive elements and hypermethylation of *CDH1* in enlarged-fold gastritis, which is an indicator of a high risk for diffuse type gastric cancer (8). More recently, we and others reported

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frequent hypermethylation of tumor suppressive miRNA genes in the noncancerous gastric mucosa of patients with gastric cancer (9, 10). Taken together, these results suggest that aberrant methylation in the gastric mucosa could be a useful biomarker for evaluating gastric cancer risk.

Our aim in the present study was to identify predictive biomarkers by screening for DNA methylation in the noncancerous background gastric mucosa in cases of gastric cancer. For this purpose, we carried out high-throughput CpG island methylation profiling in a set of noncancerous gastric mucosa specimens from patients with gastric cancer and from cancer-free individuals. We identified a number of aberrantly methylated genes in the gastric mucosa of the patients with gastric cancer, including *RASGRF1*, which was frequently methylated in cases of both intestinal and diffuse type gastric cancer. We provide evidence that *RASGRF1* is a novel target for epigenetic silencing in gastric cancer, and that its methylation in the gastric mucosa is strongly associated with an elevated risk for both types of gastric cancer.

Materials and Methods

Study population and cell lines

A total of 130 primary gastric cancer specimens were obtained through surgical resection or endoscopic biopsy. Samples of noncancerous gastric mucosa were obtained through endoscopic biopsy from 91 patients with gastric cancer and 69 healthy individuals. From each patient, biopsy specimens of noncancerous gastric mucosa were taken from the gastric body and antrum. *H. pylori* infection was assessed using a rapid urease test, a serum antibody test, or a urea breath test. If any one of these assays was positive, the patients were considered to be *H. pylori*–positive. The updated Sydney system and serum pepsinogen test was used to estimate the degree of gastritis (11). The serum pepsinogen test was carried out by assessing the serum pepsinogen I (PGI) and pepsinogen II (PGII) levels; the criteria for positivity were PGI $\leq$ 70 ng/mL and a PGI/PGII ratio $\leq$3.0 (12). Informed consent was obtained from all patients before the collection of specimens. Approval of this study was obtained from the Institutional Review Board of Akita Red Cross Hospital (Akita, Japan) and Sapporo Medical University (Sapporo, Japan).

Gastric cancer cell lines (MKN7, SH101, SNU1, SNU638, JRST, KatoIII, AZ521, AGS, and NCI-N87) were obtained and cultured as described previously (9, 13). SH101 and HSC43 cells were kindly provided by Dr. Kazuyoshi Yanagihara, Yasuda Women's University (14, 15). In some instances, cells were treated with 2 μmol/L 5-aza-2′-deoxycytidine (5-aza-dC; SIGMA) for 72 hours, replacing the drug and medium every 24 hours. Genomic DNA was extracted using the standard phenol–chloroform procedure. Total RNA was extracted using TRIZOL reagent (Invitrogen), and then treated with a DNA-free Kit (Ambion).

Methylated CpG island amplification coupled with CpG island microarray analysis

Methylated CpG island amplification (MCA) was conducted as described previously (16, 17). Briefly, 500 ng of genomic DNA was digested with the methylation-sensitive restriction endonuclease *Sma*I (New England Biolabs), after which it was digested with the methylation-insensitive restriction endonuclease *Xma*I. The adaptors were prepared by addition of the oligonucleotides RMCA12 (5′-CCGGGCAGAAAG-3′) and RMCA24 (5′-CCACCGCCATCCGAGCCTTTCTGC-3′). After the ligation of the digested DNA to the adaptors, PCR amplification was carried out. Using a BioPrime Plus Array CGH Genomic Labeling System (Invitrogen), MCA amplicons from gastric cancers and samples of *H. pylori*–positive noncancerous gastric mucosa were labeled with Alexa Fluor 647, and those from pooled samples of a mixture of *H. pylori*–negative normal gastric mucosa were labeled with Alexa Fluor 555. Labeled MCA amplicons were then hybridized to a custom human CpG island microarray containing 15,134 probes covering 6,157 unique genes (G4497A; Agilent Technologies; ref. 18). After washing, the array was scanned using an Agilent DNA Microarray Scanner (Agilent Technologies), and the data were processed using Feature Extraction software ver. 10.7 (Agilent Technologies). The data were then analyzed using GeneSpring GX ver. 11 (Agilent Technologies) after which unsupervised hierarchical clustering analysis were carried out using JMP ver. 8 (SAS Institute). The microarray data in this study have been submitted to the Gene Expression Omnibus (GEO) and accession number is GSE39175.

Methylation analysis

Genomic DNA (1 μg) was modified with sodium bisulfite using an EpiTect Bisulfite Kit (QIAGEN) after which methylation-specific PCR (MSP), bisulfite sequencing, and pyrosequencing were carried out as described previously (9). Briefly, for bisulfite pyrosequencing, the biotinylated PCR product was purified, made single-stranded, and used as a template in a pyrosequencing reaction run according to the manufacturer's instructions. The pyrosequencing reaction was carried out using a PSQ96 system with a PyroGold Reagent Kit (QIAGEN), and the results were analyzed using Q-CpG software (QIAGEN). For bisulfite sequencing, amplified PCR products were cloned into pCR2.1-TOPO vector (Invitrogen), and 12 to 14 clones from each sample were sequenced using an ABI3130x automated sequencer (Applied Biosystems). Primer sequences and PCR product sizes are listed in Supplementary Table S1.

RT-PCR of *RASGRF1*

Single-stranded cDNA was prepared using SuperScript III reverse transcriptase (Invitrogen), after which the integrity of the cDNA was confirmed by amplifying glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). Primer sequences and PCR product sizes are shown in Supplementary Table S1. Quantitative reverse transcription-PCR (RT-PCR) was carried out using TaqMan Gene Expression Assays (*RASGRF1*, Hs00182314_m1; *GAPDH*, Hs99999905_m1; Applied Biosystems) and a 7500 Fast Real-Time PCR System

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(Applied Biosystems). SDS ver. 1.4 software (Applied Biosystems) was used for comparative ΔC_t analysis.

Construction of a *RASGRF1* expression vector

A full-length *RASGRF1* cDNA was amplified by PCR using cDNA derived from *RASGRF1*-expressing AZ521 cells as a template and then cloned into pcDNA3.2/V5/GW/D-TOPO (Invitrogen). The sequence was then verified. Primer sequences and PCR product sizes are shown in Supplementary Table S1.

Western blot analysis

Western blot analysis was carried out as described previously (9). Mouse anti-V5 monoclonal antibody (mAb; Invitrogen), rabbit anti-RASGRF1 polyclonal Ab (sc-863, Santa Cruz Biotechnology), and mouse antiactin mAb (Chemicon) were used as instructed by the manufacturers. The immunoreactive bands were visualized using enhanced chemiluminescence (Amersham Biosciences).

Colony formation assays

Colony formation assays were carried out as described previously (13). Briefly, cells (1×10^5 cells) were transfected with 4 μg of *RASGRF1* expression vector or empty pcDNA3.1 using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Cells were then plated on 60-mm culture dishes and selected for 10 days in 0.4 mg/mL G418. Colonies were stained with Giemsa and counted using the NIH IMAGE software.

Matrigel invasion assays

Cell invasion was assessed using Matrigel invasion assays as described previously (19). Gastric cancer cells (2×10^6 cells) were transfected with 5 μg of *RASGRF1* expression vector or a negative control using a Cell Line Nucleofector kit V (Lonza) with a Nucleofector I electroporation device (Lonza) according to the manufacturer's instructions. After incubation for 24 hours, 1×10^5 transfectants suspended in 500 μL of serum-free RPMI-1640 medium were added to the tops of BD BioCoat Matrigel Invasion Chambers (BD Biosciences) prehydrated with PBS, and 700 μL of RPMI-1640 media supplemented with 10% FBS were added to the lower wells of the chambers. After incubation for 22 hours, the invading cells were stained with 1% toluidine and then counted in 3 randomly selected microscopic fields per membrane.

Statistical analysis

To compare differences in continuous variables between groups, *t* tests or ANOVA with *posthoc* Tukey tests were conducted. Fisher exact test or χ^2 test was used for analysis of categorical data. Receiver operator characteristic (ROC) curves were constructed on the basis of the levels of methylation. Values of $P < 0.05$ (2-sided) were considered statistically significant. Statistical analyses were carried out using SPSS statistics 18 (IBM Corporation) and GraphPad Prism ver. 5.0.2 (GraphPad Software).

Results

Identification of *RASGRF1* methylation in background gastric mucosa in gastric cancer

To screen for methylation changes early during gastric carcinogenesis, we compared the methylation status of noncancerous background gastric mucosa from patients with gastric cancer with that in samples of gastric mucosa from healthy individuals. To accomplish this, we carried out methylated-CpG island amplification coupled with CpG island microarray (MCAM) analysis using a set of *H. pylori*–positive gastric mucosa specimens from otherwise healthy individuals (gastric antrum, $n = 11$; gastric body, $n = 15$), noncancerous gastric mucosa from patients with gastric cancer (gastric antrum, $n = 10$; gastric body, $n = 10$), gastric cancer tissues ($n = 10$), and gastric cancer cell lines (AGS, HSC43, KatoIII, MKN74, and NUGC4). The gastric mucosa specimens from the healthy individuals were divided into 2 histologic groups: antrum-predominant gastritis ($n = 14$), which has a low risk for gastric cancer and pangastritis or corpus-predominant gastritis ($n = 12$), which has a high risk for gastric cancer. We thus aimed to identify genes that showed greater methylation in noncancerous antral gastric mucosa from patients with gastric cancer than in mucosa from healthy individuals with antrum-predominant gastritis. MCAM analysis revealed 889 unique genes that were hypermethylated in the background gastric mucosa in intestinal type gastric cancer, as compared with antrum-predominant gastritis (Fig. 1A, Supplementary Table S2). In addition, we identified 478 unique genes that were methylated in the background mucosa in diffuse type gastric cancer (Fig. 1A, Supplementary Table S3). Subsequent Venn diagram analysis identified 224 genes that were methylated in the background gastric mucosae of patients with either type of gastric cancer but not in healthy individuals (Fig. 1A, Supplementary Table S4).

Unsupervised hierarchical clustering analysis using MCAM data for the 224 selected genes revealed that the genes could be categorized into 3 subclasses (Fig. 1B, Supplementary Table S4): group 1 genes (81 unique genes) were methylated in the majority of antral gastric mucosae from patients with gastric cancer and in gastric cancer tissues; group 2 genes (35 unique genes) were prevalently methylated in patients with gastric cancer and in otherwise healthy individuals with pangastritis or corpus-predominant gastritis, suggesting the methylation was inflammation-related and less cancer-specific; and group 3 genes (108 unique genes), which gave an elevated signal in the antrum of patients with gastric cancer and patients with pangastritis or corpus-predominant gastritis, but overall, the levels of methylation were relatively low in all of the specimens tested. These results suggest that genes predictive of gastric cancer risk are likely enriched in group 1. Among the group 1 genes, we selected 11 (*RASGRF1*, *SOX5*, *GALNT14*, *RGS20*, *RPIB9*, *SYT5*, *WNT3*, *BASP1*, *ITGA4*, *KCNV1*, and *PAX5*) that gave the highest microarray signals in the gastric cancer tissues and background gastric mucosa. Using MSP, we tested their methylation status in a small set of clinical specimens and found that 3 genes (*RASGRF1*, *GALNT14*,

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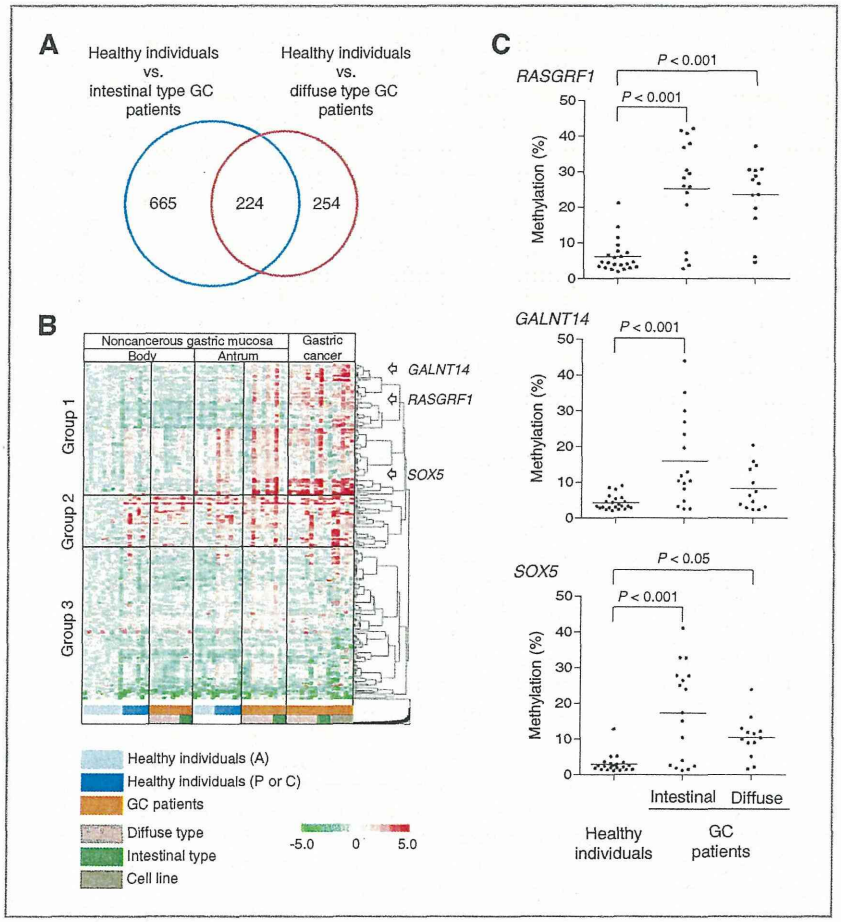

Figure 1. Identification of genes methylated in the background gastric mucosa in gastric cancer. A, MCAM analysis identified 889 and 478 genes that were differentially methylated (>1.5-fold) between antrum-predominant gastritis from otherwise healthy individuals and noncancerous gastric mucosa from patients with intestinal or diffuse type gastric cancer, respectively. Venn diagram analysis revealed 224 genes that were methylated in the background gastric mucosa in both gastric cancer types. B, unsupervised hierarchical clustering analysis of the MCAM data obtained from patients with antrum-predominant gastritis (A), pangastritis, or corpus-predominant gastritis (P or C), noncancerous mucosae from patients with gastric cancer, gastric cancer tissues, and gastric cancer cell lines using the selected 224-gene set. Each row represents a single probe and each column represents a sample. Three genes (*RASGRF1*, *GALNT14*, and *SOX5*) were selected from among the group 1 genes. C, summarized results of bisulfite pyrosequencing of *RASGRF1*, *GALNT14*, and *SOX5* in a set of gastric mucosa specimens from healthy individuals ($n = 22$) and noncancerous gastric mucosae from patients with intestinal type gastric cancer ($n = 16$) or diffuse type gastric cancer ($n = 13$).

and *SOX5*) strongly discriminated between healthy individuals and patients with gastric cancer (Fig. 1B, Supplementary Fig. S1). We therefore used quantitative bisulfite pyrosequencing to assess their methylation levels in a set of antral mucosae from *H. pylori*–positive healthy individuals ($n = 22$) and noncancerous gastric mucosae from the antrum of patients with intestinal ($n = 16$) or diffuse ($n = 13$) type gastric cancer (Fig. 1C, Supplementary Fig. S2). Consistent with the MCAM and MSP data, the levels of methylation of these 3 genes were elevated in the background gastric mucosae from patients with gastric cancer, although methylation of *GALNT14* in diffuse type patients with gastric cancer was less pronounced. In contrast, methylation of *RASGRF1* was significantly elevated in patients with either type of gastric cancer, suggesting it could be a useful biomarker for predicting gastric cancer risk.

Analysis of *RASGRF1* methylation and expression in gastric cancer

On the basis of the results summarized earlier, we selected *RASGRF1* for further analysis. The promoter region and exon 1 of *RASGRF1* are embedded within a typical CpG island (Supplementary Fig. S3). Bisulfite pyrosequencing revealed that *RASGRF1* was highly methylated in a majority of the gastric cancer cell lines tested (Fig. 2A), and that expression of *RASGRF1* mRNA was absent in those cells. Treatment with a DNA methyltransferase inhibitor, 5-aza-dC, restored *RASGRF1* mRNA expression in multiple cell lines, suggesting *RASGRF1* is epigenetically silenced in gastric cancer cells (Fig. 2A). In contrast, methylation levels were low in MKN74 and AZ521 cells, in which *RASGRF1* mRNA was abundantly expressed (Fig. 2A and data not shown). Elevated levels (>15%) of *RASGRF1* methylation were also frequently detected in both types of primary gastric cancer (intestinal type, 31 of 62, 50.0%; diffuse type, 41 of 68, 60.3%; Fig. 2B, Supplementary Table S5). We also found that *RASGRF1* methylation correlates inversely with higher pathologic T (pT) categories and distant metastasis (Supplementary Table S5). When the methylation status of *RASGRF1* was further assessed in selected specimens, we observed that its CpG island is densely methylated in gastric cancer cell lines, primary tumors, and background

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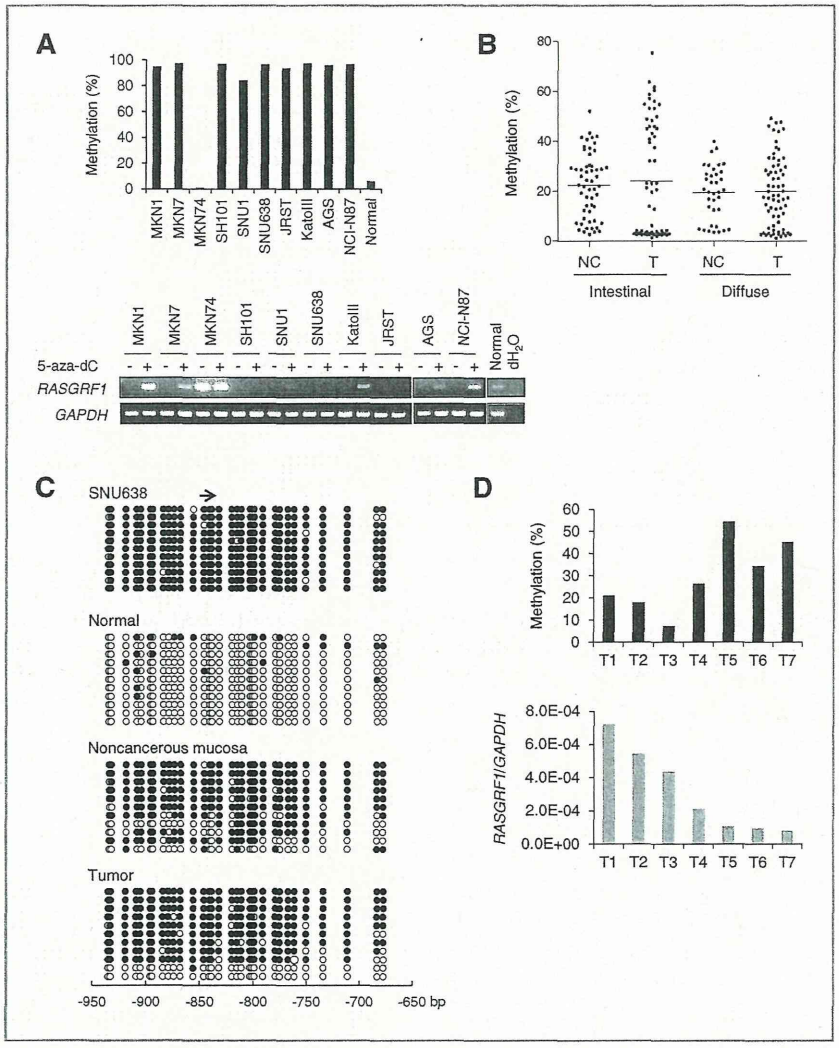

Figure 2. Analysis of *RAGRF1* methylation and expression in gastric cancer. A, bisulfite pyrosequencing of *RASGRF1* in the indicated gastric cancer cell lines and a sample of normal gastric mucosa (top). RT-PCR analysis of *RASGRF1* in gastric cancer cell lines with (+) or without (−) 5-aza-dC treatment, and in normal stomach (bottom). RT-PCR analysis of *GAPDH* was carried out with all samples to ensure the cDNA quality; dH_2O indicates no RNA added. B, summary of bisulfite pyrosequencing in primary tumors (T) from patients with intestinal type gastric cancer ($n = 62$) and diffuse type gastric cancer ($n = 68$). Noncancerous gastric mucosae (NC) from patients with intestinal type gastric cancer ($n = 55$) and diffuse type gastric cancer ($n = 36$) are also shown. C, representative bisulfite sequencing of the *RASGRF1* promoter in a gastric cancer cell line (SNU638), normal gastric mucosa, primary samples of noncancerous gastric mucosa, and a gastric cancer tumor. Open and filled circles represent unmethylated and methylated CpG sites, respectively. The region analyzed by bisulfite pyrosequencing is indicated by an arrow on the top. D, methylation and expression of *RASGRF1* in a set of primary gastric cancer tissues. Methylation levels were determined by bisulfite pyrosequencing (top) and expression was assessed by quantitative RT-PCR (bottom).

noncancerous gastric mucosa, whereas normal gastric mucosa from a healthy individual showed only limited methylation (representative results in Fig. 2C). To test whether methylation of *RASGRF1* is associated with its downregulation in primary gastric cancer, we carried out bisulfite pyrosequencing and quantitative RT-PCR with a set of tissue specimens, which revealed an inverse relationship between methylation and expression (Fig. 2D).

Increased *RASGRF1* methylation in noncancerous gastric mucosae from gastric cancer patients

The elevated levels of *RASGRF1* methylation in the background gastric mucosa of patients with gastric cancer suggest its involvement in an epigenetic field defect. We therefore next assessed its clinical usefulness as a predictive biomarker of gastric cancer risk by comparing the levels of *RASGRF1* methylation in a set of gastric mucosa specimens from healthy individuals (*H. pylori*–negative, $n = 12$; *H. pylori*–positive, $n = 50$) and noncancerous gastric mucosa specimens from patients with gastric cancer (intestinal type, $n = 55$; diffuse type, $n = 36$). All specimens were collected from the gastric antrum, and the clinicopathologic features of the samples are shown in Table 1. In healthy individuals, the levels of *RASGRF1* methylation did not significantly differ between *H. pylori*–negative and -positive gastric mucosae (5.4% and 9.0%, average 8.3%), suggesting that severe *RASGRF1* methylation is not induced by *H. pylori* infection alone (Fig. 3A). In contrast, methylation levels were significantly elevated in noncancerous mucosae from intestinal and diffuse type patients with gastric cancer (22.5% and 19.4%, average 21.3%), indicating that *RASGRF1* methylation in noncancerous

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Table 1. Clinicopathologic features of the patients in this study

		Healthy individuals ($n = 69$)	Patients with cancer		
			Noncancerous mucosa ($n = 91$)	Gastric cancer tissues ($n = 130$)	Total ($n = 290$)
Age	Mean	60.0	70.1	66.7	66.2
	SD	12.8	9.0	11.4	11.7
Sex	Male	50 (72.5%)	65 (71.4%)	87 (66.9%)	
	Female	17 (24.6%)	26 (28.6%)	36 (27.7%)	
	Unknown	2 (2.9%)	0 (0.0%)	7 (5.4%)	
H. pylori	Positive	50 (72.5%)	41 (45%)	24 (18.5%)	115 (39.7%)
	Negative	12 (17.4%)	14 (15.4%)	5 (3.8%)	31 (10.7%)
	Unknown	7 (10.1%)	36 (39.6%)	101 (77.7%)	144 (49.7%)
Histology	Intestinal type		55 (60.4%)	62 (47.7%)	
	Diffuse type		36 (39.4%)	68 (52.3%)	
PG	Positive	15 (21.7%)	20 (22.0%)	9 (6.9%)	44 (15.2%)
	Negative	23 (33.3%)	18 (19.8%)	11 (8.5%)	52 (17.9%)
	Unknown	31 (44.9%)	53 (58.2%)	110 (84.6%)	194 (66.9%)

gastric mucosae may be associated with gastric cancer risk (Fig. 3A).

We also generated a ROC curve to assess the clinical use of *RASGRF1* methylation for prediction of gastric cancer. *RASGRF1* methylation was highly discriminative between noncancerous gastric mucosa from patients with gastric cancer and gastric mucosa from healthy individuals (Fig. 3B and Table 2). Earlier studies showed that severe gastric mucosal atrophy induced by *H. pylori* infection is a hallmark of gastric cancer risk, and that

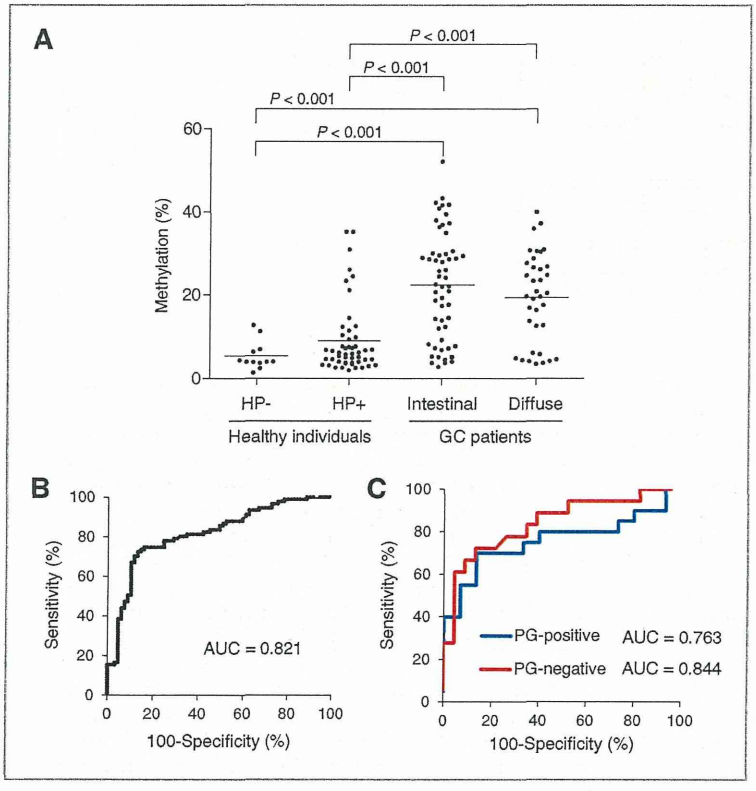

Figure 3. Analysis of *RASGRF1* methylation in gastric mucosae from healthy individuals and patients with gastric cancer. A, summarized results of bisulfite pyrosequencing in normal gastric mucosae from healthy individuals with ($n = 50$) or without *H. pylori* (HP) infection ($n = 12$), and noncancerous gastric mucosae from patients with intestinal type gastric cancer ($n = 55$) or diffuse type gastric cancer ($n = 36$). B, ROC curve analysis of *RASGRF1* methylation. The area under the ROC curve (AUC) for each site conveys its use (in terms of sensitivity and specificity) for distinguishing between noncancerous gastric mucosae from patients with gastric cancer and normal stomach from healthy individuals. C, ROC curve analysis distinguishing between noncancerous gastric mucosae from serum pepsinogen (PG) test-positive (blue) or -negative (red) patients with gastric cancer and normal stomach from healthy individuals.

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its evaluation based on serum pepsinogen tests is a useful means of assessing cancer risk (12, 20). To evaluate the relationship between *RASGRF1* methylation and mucosal atrophy, we divided the samples based on the pepsinogen test results and then generated respective ROC curves. Notably, *RASGRF1* methylation was highly discriminative between healthy individuals and patients with gastric cancer irrespective of the pepsinogen test results (Fig. 3C and Table 2), which suggests that the strong association between *RASGRF1* methylation and gastric cancer is independent of gastric mucosal atrophy. This could make *RASGRF1* methylation a powerful biomarker with which to identify individuals at high risk for gastric cancer.

Functional analysis of *RASGRF1* in gastric cancer cells

Finally, we tested whether *RASGRF1* serves as a tumor suppressor in gastric cancer. Gastric cancer cell lines were transfected with a *RASGRF1* expression vector or a negative control, after which colony formation assays were carried out with the transfectants. Western blot analysis confirmed that the transfectants expressed exogenous RASGRF1 (Supplementary Fig. S4). Moreover, introduction of *RASGRF1* markedly suppressed colony formation by the cell lines tested (Fig. 4A and B). When we then carried out Matrigel invasion assays to test the effect of *RASGRF1* expression on gastric cancer cell invasion; we observed marked inhibition of cell invasion by SNU638 cells expressing *RASGRF1* (Fig. 4C). These results suggest that *RASGRF1* may play a tumor suppressor role that is itself suppressed in gastric cancer.

Discussion

Identification of individuals at high risk of developing gastric cancer is essential for the prevention and early detection of gastric cancer. Esophagogastroduodenoscopy (EGD) is the most useful method for detecting gastric cancers, although population-based screening for gastric cancers using only EGD is generally considered ineffective for reducing mortality (21, 22). Severe atrophic gastritis is strongly associated with an increased risk of intestinal gastric cancer, and screening for high-risk individuals based on serum pepsinogen levels followed by careful observation using EGD is an effective strategy for surveillance of this disease (12, 23). In contrast, diffuse type gastric cancers lack those characteristic features because they do not progress through atrophic gastritis. Consequently, identification of sensitive and reliable biomarkers for diffuse type gastric cancer would be highly desirable. A number of studies have shown aberrant DNA methylation in precancerous lesions, including chronic gastritis and intestinal metaplasia, and detection of such an epigenetic field defect would provide useful information for identifying individuals at high risk for developing gastric cancer (7, 24, 25). The majority of those studies focused on well-studied genes, such as *CDH1* and *p16* (26, 27), but recent advances in microarray technology have enabled us to conduct a genome-wide analysis of CpG island methylation status. For instance, a recent study reported by Nanjo and colleagues identified a series of 7 methylation markers that can predict gastric cancer risk in individuals with past *H. pylori* infection (28).

In this study, we carried out high-throughput methylation analysis using a set of gastric mucosa specimens from healthy individuals and patients with intestinal or diffuse type gastric cancer. Our MCAM analysis identified a number of methylated genes in noncancerous gastric mucosae from the patients with gastric cancer. A larger number of methylated genes were identified in gastric mucosae from patients with intestinal type gastric cancer than with diffuse type gastric cancer, which is consistent with the earlier observation that aberrant DNA methylation is not induced by the presence of *H. pylori*, itself, but by inflammatory processes triggered by the infection (29). Our list of genes methylated in the background mucosa in intestinal type gastric cancer includes a number of methylation-prone genes (e.g., *SFRP2* and *IRF4*) that confirms the reliability of our screening method (13, 30). The methylation status in

Table 2. ROC analysis of the ability of *RASGRF1* methylation to discriminate between patients with gastric cancer and healthy individuals

	AUC			Sensitivity		Specificity	
	Estimate	95% confidence interval (CI)	Cut-off	Estimate	95% CI	Estimate	95% CI
Total ($n = 161$)	0.821	(0.775%–0.887%)	7.71%	78.02%	(68.12%–86.03%)	75.36%	(63.51%–84.94%)
			11.68%	74.73%	(64.53%–83.25%)	84.06%	(73.26%–91.76%)
			12.76%	70.33%	(59.84%–79.45%)	86.96%	(86.68%–93.86%)
PG-positive ($n = 35$)	0.763	(0.601%–0.926%)	6.79%	80.00%	(56.34%–94.27%)	60.00%	(32.29%–83.66%)
			13.67%	70.00%	(45.72%–88.11%)	86.67%	(59.54%–98.34%)
			22.29%	55.00%	(31.53%–76.94%)	93.33%	(68.05%–99.83%)
PG-negative ($n = 41$)	0.844	(0.719%–0.969%)	7.13%	77.78%	(52.36%–93.59%)	73.91%	(51.60%–89.77%)
			10.64%	72.22%	(46.52%–90.31%)	86.96%	(66.41%–97.22%)
			13.39%	6.67%	(40.99%–86.66%)	91.30%	(71.96%–98.93%)

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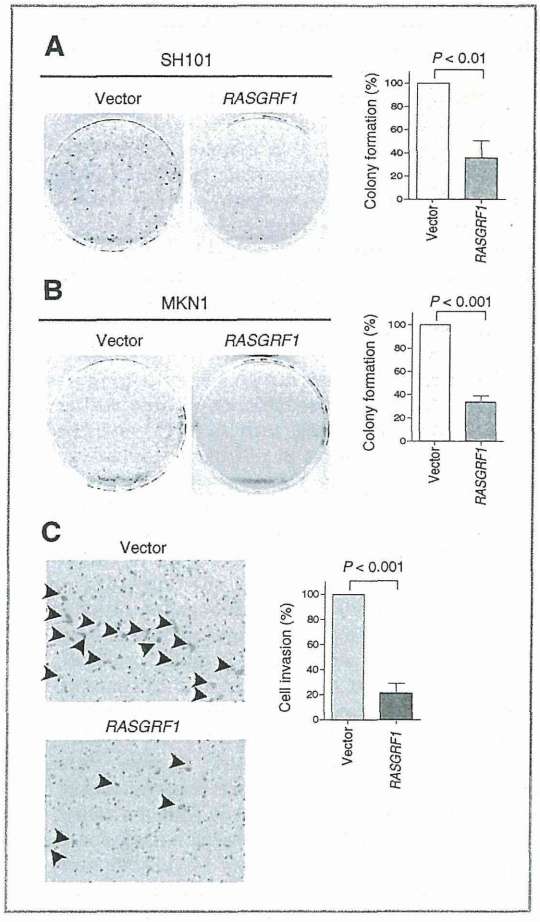

Figure 4. Functional analysis of *RASGRF1*. A and B, colony formation assays using the indicated gastric cancer cells transfected with a *RASGRF1* expression vector or a control vector. Representative results are on the left, and relative colony formation efficiencies are on the right. Shown are means of 3 replications; error bars represent SDs. C, Matrigel invasion assay using SNU638 cells transfected with a *RASGRF1* expression vector or a control vector. Invading cells are indicated by arrows. Shown on the right are the means of 3 random microscopic fields per membrane; error bars represent the SDs.

the background mucosa of diffuse type gastric cancer had remained largely unknown and, to our knowledge, this study is the first to examine the genome-wide CpG island methylation status in the gastric mucosa from diffuse type patients with gastric cancer. It is noteworthy that we found that approximately half of the genes methylated in diffuse type gastric cancer were also methylated in intestinal type gastric cancer. It is generally believed that intestinal and diffuse type gastric cancers develop through distinctly different molecular pathways; however, our data may be indicative of a pathogenic mechanism common to both types. Furthermore, our results suggest that methylation of these genes could be an ideal molecular marker for assessing the risk for both gastric cancer types.

Among the genes identified, we selected *RASGRF1*, *GALNT14*, and *SOX5* for further analysis and found that the elevation of their methylation levels was specific to patients with gastric cancer. *SOX5* is a member of the high-mobility group superfamily and is reportedly overexpressed in several malignancies, including nasopharyngeal carcinoma and prostate cancer, which suggests it has oncogenic properties (31, 32). On the other hand, one recent study showed that *SOX5* suppresses platelet-derived growth factor B–induced gliomas (33). *GALNT14* belongs to a large subfamily of glycosyltransferases, and its expression in cancer cells is associated with cellular sensitivity to the proapoptotic ligand Apo2L/TRAIL (34). Up to now, however, methylation of *GALNT14* and *SOX5* has not been reported in human cancer, and further study will be needed to clarify their functional significance.

RASGRF1 and *RASGRF2* constitute a gene family encoding guanine nucleotide exchange factors (GEF), which activate Ras GTPase by promoting the release of bound GDP, enabling activating GTP to take its place (35). RASGRF proteins are predominantly expressed in adult neurons in the central nervous system, and are involved in a wide range of neuronal functions. In mice, *Rasgrf1* is an imprinted gene. The imprinted *Rasgrf1* locus is methylated on the paternal allele at a differentially methylated region (DMR) located 30 kb upstream of the promoter, and it is expressed only from the paternal allele (36). Interestingly, a recent study showed that Piwi-interacting RNAs (piRNAs), a subset of noncoding small RNAs, play a pivotal role in the establishment of methylation at the *Rasgrf1* DMR (37). In contrast, we found the promoter CpG island of *RASGRF1* to be hypermethylated in gastric cancer, and that this methylation is unlikely to be associated with gene imprinting. Levels of *RASGRF1* methylation are also significantly elevated in the noncancerous background gastric mucosa in both the intestinal and diffuse types of gastric cancer and are highly discriminative between gastric mucosa from cancer-free individuals and patients with gastric cancer. This suggests *RASGRF1* methylation may be a gastric cancer risk factor that is independent of gastric mucosal atrophy, and that *RASGRF1* methylation could be a predictive marker of gastric cancer risk that would overcome the disadvantages of other screening methods, such as the serum pepsinogen test and EGD.

The function of *RASGRF1* in normal stomach and during gastric tumorigenesis is largely unknown, but it may exert oncogenic effects through activation of Ras proteins (35). On the other hand, one recent study showed that RASGRF proteins bind directly to Cdc42, another Ras-related GTP-binding protein, and suppress Cdc42-mediated cellular processes, including tumor cell invasion and transformation (38). In this study, we found that ectopic expression of *RASGRF1* suppressed proliferation and invasion by gastric cancer cells, which is suggestive of its tumor suppressor role, although we carried out only overexpression experiments. Alteration of *RASGRF1* gene in human cancer has not yet been reported; in fact, this is the first report of its epigenetic