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Biology of Human Tumors

Clinical Cancer Research

Large-Scale Characterization of DNA Methylation Changes in Human Gastric Carcinomas with and without Metastasis

Zhaojun Liu[1], Jun Zhang[1,2], Yanhong Gao[1], Lirong Pei[3], Jing Zhou[1], Liankun Gu[1], Lianhai Zhang[4], Budong Zhu[5], Naoko Hattori[6], Jiafu Ji[4], Yasuhito Yuasa[7], Wooho Kim[8], Toshikazu Ushijima[6], Huidong Shi[3], and Dajun Deng[1]

Abstract

Purpose: Metastasis is the leading cause of death for gastric carcinoma. An epigenetic biomarker panel for predicting gastric carcinoma metastasis could have significant clinical impact on the care of patients with gastric carcinoma. The main purpose of this study is to characterize the methylation differences between gastric carcinomas with and without metastasis.

Experimental Design: Genome-wide DNA methylation profiles between 4 metastatic and 4 nonmetastatic gastric carcinomas and their surgical margins (SM) were analyzed using methylated-CpG island amplification with microarray. The methylation states of 73 candidate genes were further analyzed in patients with gastric carcinoma in a discovery cohort ($n = 108$) using denatured high performance liquid chromatography, bisulfite-sequencing, and MethyLight. The predictive values of potential metastasis-methylation biomarkers were validated in cohorts of patients with gastric carcinoma in China ($n = 330$), Japan ($n = 129$), and Korea ($n = 153$).

Results: The gastric carcinoma genome showed significantly higher proportions of hypomethylation in the promoter and exon-1 regions, as well as increased hypermethylation of intragenic fragments when compared with SMs. Significant differential methylation was validated in the CpG islands of 15 genes ($P < 0.05$) and confirmed using bisulfite sequencing. These genes included *BMP3*, *BNIP3*, *CDKN2A*, *ECEL1*, *ELK1*, *GFRA1*, *HOXD10*, *KCNH1*, *PSMD10*, *PTPRT*, *SIGIRR*, *SRF*, *TBX5*, *TFPI2*, and *ZNF382*. Methylation changes of *GFRA1*, *SRF*, and *ZNF382* resulted in up- or down-regulation of their transcription. Most importantly, the prevalence of *GFRA1*, *SRF*, and *ZNF382* methylation alterations was consistently and coordinately associated with gastric carcinoma metastasis and the patients' overall survival throughout discovery and validation cohorts in China, Japan, and Korea.

Conclusion: Methylation changes of *GFRA1*, *SRF*, and *ZNF382* may be a potential biomarker set for prediction of gastric carcinoma metastasis. *Clin Cancer Res; 20(17); 4598–612. ©2014 AACR.*

Introduction

Gastric carcinoma is the second leading cause of cancer death throughout the world (1). Global statistics showed that in 2008 alone, nearly 989,000 people were diagnosed with gastric carcinoma, and approximately 464,000 people died from this disease (2). Currently, gastric carcinoma prognosis is primarily determined based on the clinical data and pathologic stages of patients at the time of diagnosis and treatment (3). However, successful management of patients with gastric carcinoma is still hampered by the lack of highly sensitive and specific biomarkers capable of predicting prognosis and likelihood of metastasis. Epigenetic alterations, including aberrant DNA methylation changes, may play an important role in gastric carcinogenesis as indicated by the increased hypermethylation of tumor suppressor genes in patients with gastric carcinoma (4–6). Given their important functions in cancer initiation and progression, methylation changes are being investigated as potential biomarkers for the early detection of cancers,

[1]Key Laboratory of Carcinogenesis and Translational Research (Ministry of Education), Division of Etiology, Peking University Cancer Hospital and Institute, Fu-Cheng-Lu, Beijing, China. [2]Shihezi University School of Medicine, Shihezi, China. [3]GRU Cancer Center, Georgia Regents University, Augusta, Georgia. [4]Department of Surgery, Peking University Cancer Hospital and Institute, Fu-Cheng-Lu, Beijing, China. [5]Department of Oncology, Peking University Cancer Hospital and Institute, Fu-Cheng-Lu, Beijing, China. [6]Division of Epigenetics, National Cancer Center Research Institute, Chuo-ku, Tokyo, Japan. [7]Department of Molecular Oncology, Tokyo Medical and Dental University, Bunkyo-ku, Tokyo, Japan. [8]Department of Pathology, Seoul National University College of Medicine, Jongno-gu, Seoul, Korea.

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Z. Liu, J. Zhang, and Y. Gao contributed equally to this article.

Corresponding Authors: Dajun Deng, Peking University Cancer Hospital and Institute, Fu-Cheng-Lu #52, Haidian District, Beijing 100142, China. Phone: 8610-88196752; Fax: 8610-88122437; E-mail: dengdajun@bjmu.edu.cn; and Huidong Shi, GRU Cancer Center, Georgia Regents University, Augusta, GA 30912; E-mail: hshi@gru.edu

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Translational Relevance

Gastric carcinoma is the second leading cause of cancer deaths in the world, with many occurring in East Asia. To identify DNA methylation biomarkers for prediction of gastric carcinoma metastasis, scientists and oncologists from China, USA, Japan, and Korea have carried out a 5-year collaborative study to profile differential methylation patterns in metastatic and nonmetastatic gastric carcinomas and perform an in-depth characterization of methylation changes in the CpG islands of 73 candidate genes. From this study, we established a methylation biomarker set composed of three genes, *GFRA1*, *SRF*, and *ZNF382*, that could be used to synergistically predict gastric carcinoma metastasis and patients' overall survival from multiple patient cohorts in China, Japan, and Korea. The established marker set will be a useful clinical tool for decision making on personalized postoperational therapy that is currently not available.

the prediction of cancer progression, and the prediction of chemotherapeutic sensitivity (7).

Recent advances in high-throughput technologies have significantly expanded our capability of interrogating genome-wide DNA methylation changes in cancer (6, 8, 9). Methylated CpG island amplification with microarray (MCAM) is one of the most powerful tools available for displaying differential methylation related to pathogenesis (10). A number of DNA methylome studies have been reported in a variety of primary cancers, including gastric carcinoma. However, few studies have been conducted to vigorously validate the methylation changes of the candidate genes at the single molecule level in numerous tumor samples (6, 11). Therefore, despite the long list of differentially methylated genes in patients with gastric carcinoma, a promising DNA methylation biomarker has not yet reached to the clinical utility.

In the present study, genome-wide DNA methylation analysis using the MCAM assay was performed in gastric carcinomas (10). A large number of differentially methylated regions were identified between gastric carcinomas and their corresponding surgical margin (SM). In addition, differential methylation profiles between metastatic and nonmetastatic gastric carcinomas were identified. Most importantly, the methylation status of promoter CpG islands (CGI) from 73 candidate genes was characterized using denatured high performance liquid chromatography (DHPLC) in 48 pairs of gastric samples from patients with gastric carcinoma and patients without cancer (12). The predictive values of three potential metastasis-related candidates were further validated in multiple cohorts from China, Japan, and Korea following the Reporting Prognostic Tumor Marker Study guidelines. We demonstrated that the methylation status of *GFRA1*, *SRF*, and *ZNF382* could be used as potential synergistic biomarkers for the prediction of gastric carcinoma metastasis.

Materials and Methods

Patient characteristics and sample collection

A total of 504 patients with gastric carcinoma from 3 academic medical centers in China, Japan, and Korea were included in this study. The study was approved by the local Institution Review Boards (IRB) at each institution, and all patients were given written informed consent unless the IRB permitted a waiver. The 2003 UICC-TNM (tumor–node–metastasis) system was used for the classification of gastric carcinomas (13). A total of 330 Chinese inpatients with gastric carcinoma that underwent surgical treatment at Peking University Cancer Hospital and Institute between 1999 and 2006 were enrolled in the discovery and validation cohorts based on the following criteria: (i) availability of frozen, fresh gastric carcinoma and SM samples; (ii) follow-up available for at least 5 years; (iii) falls into the proper pathologic TNM (pTNM) stages as described in the results section. In the validation cohort from Korea, 153 inpatients with gastric carcinoma that received surgical treatment were selected from Seoul National University Hospital during 2004 with a follow-up of at least 3 years. Paraffin-embedded samples were used in the Korea study. The validation cohort from Japan included 78 inpatients with gastric carcinoma that acquired surgical treatment between 1995 and 2002 with a follow-up of at least 5 years, as well as an additional 79 patients with gastric carcinoma between 2010 and 2011 who did not have survival data. The SM samples were not available for these Japanese patients. Gastric carcinomas were classified as cardiac or noncardiac in terms of location (14). Patients with preoperative chemotherapy were not included in the discovery or independent validation cohorts. Normal/gastritis biopsies (NorG) from 56 outpatients at Peking University Cancer Hospital were used as the cancer-free controls.

Study design

The discovery patient cohort from Peking University Cancer Hospital consisted of 54 randomly selected patients with nonmetastatic gastric carcinomas and 54 matched patients with distant metastatic gastric carcinomas. Among them, 8 paired gastric carcinoma and the corresponding SM samples from patients with or without distant and lymph metastasis were analyzed using MCAM on a customized Agilent promoter array. The clinical and histologic features of these 8 patients can be found in Supplementary Table S1. The remaining gastric carcinoma and SM samples from 100 patients were used for the characterization of 73 CGIs using DHPLC and bisulfite clone sequencing. The methylation states of the three most promising candidate CGIs were analyzed in three analogous-independent validation cohorts from China ($n = 222$), Japan ($n = 129$), and Korea ($n = 153$). The overall study design is outlined in Supplementary Fig. S1. Genomic DNA was isolated using phenol/chloroform extraction.

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Cell lines and culture

MKN74 cell line was kindly provided by Dr. Yasuhito Yuasa at Tokyo Medical and Dental University in 2010; RKO cell line, from Dr. Guoren Deng, at University California in San Francisco in 2001; AGS, by Dr. Chengchao Shou in 2009, HeLa and MGC803, by Dr. Yang Ke in 2004, at Peking University Cancer Hospital. All cells were grown in monolayer in appropriate medium supplemented with 10% FBS and maintained at 37°C in humidified air with 5% CO_2. These cell lines were tested and authenticated by Beijing JianLian Genes Technology Co., Ltd before they were used in this study. Short tandem repeat (STR) patterns were analyzed using Goldeneye20A STR Identifiler PCR Amplification Kit. Gene Mapper v3.2 software (ABI) was used to match the STR pattern with the online databases of National Platform of Experimental Cell Resources for Sci-Tech for MGC803 cell and the ATCC for other cells.

Genome-wide analysis of DNA methylation in gastric carcinoma tissues using MCAM

Genomic DNA (2 μg) from 8 pairs of fresh gastric carcinoma and SM samples was analyzed using the MCAM approach (10). Briefly, genomic DNA was digested consecutively with *SmaI* and *XmaI*, which cut unmethylated and methylated CCCGGG sites, respectively. The *XmaI* digestion produces sticky ends that can be ligated to linkers, whereas *SmaI* digestion results in blunt ends that are unable to be ligated to linkers. The ligation-mediated PCR products from gastric carcinoma and SM samples were purified and labeled with Alexafluor647 or 555, respectively, using the Bioprime Plus Array CGH Indirect Genomic Labeling Kit (Invitrogen) according to the manufacturers' instructions. The labeled DNA was cohybridized to a custom-designed Agilent oligonucleotide array, and the slides were washed and scanned as described previously (15). Data were extracted using the Feature Extraction Tool (Agilent Technologies) and exported for further analysis. The custom-designed Agilent oligonucleotide array was designed using Agilent eArray service (https://earray.chem.agilent.com/earray). The array consisted of approximately 99,028 probes (44–60 mers) that covered 29,879 *in silico SmaI*-digested DNA fragments (>60 bp and <2,000 bp) in the human genome. The probes were tiled within each fragment with 100-bp spacing. The methylation states of 6,177 genes were determined using this custom methylation array.

Microarray data normalization and probe/gene selection

The raw array data were processed and normalized by the Beijing CO-FLY Bioinformatic Company. Background model adjustment was carried out using the minimum normalization algorithm. Systematic differences between arrays were normalized using the quantile method as described (16, 17). The methylation array data, as well as the probe information, have been deposited into the Gene Expression Omnibus under accession number GSE47724.

The mean intensity of the normalized array hybridization (methylation) signal of each probe for sex-related chromosomes and autosomes in the SM samples from 4 males and 4 females (Supplementary Fig. S2A–S2C) was analyzed. As expected, the intensities of 784 of 2,390 X chromosome-probes (32.8%) were significantly higher in the female samples than the male samples (Student t test, $P < 0.05$); in contrast, 35 of 87 Y chromosome-probes (40.2%) were significantly higher in the male samples when compared with the female samples. These sex-specific differences were only observed in 1,250 of 96,550 (1.3%) probes in the 22 autosomes. These results confirmed that the quality of the normalized data is sufficient to differentiate sex-specific DNA methylation and suitable for studying gastric carcinoma– or metastasis-related methylation changes.

The methylation signal ratio ([gastric carcinoma]/[SM]) was calculated for each array probe. The Student paired t test ($P < 0.01$) was used to identify the differentially methylated probes between gastric carcinoma and SM samples from the 8 patients analyzed. The Mann–Whitney U test ($P < 0.029$) was used to identify the metastasis-specific differentially methylated probes between the 4 patients with metastatic gastric carcinoma and 4 patients with nonmetastatic gastric carcinoma. The methylation ratio data including the adjusted P values for each probe are included in Supplementary Data File S1.

The difference between gastric carcinoma–related hypermethylated and hypomethylated probes was calculated for each sliding window (sequence or region) using 51 probe-matched fragments, which included the target probe along with 25 probes both upstream and downstream of the target. Probes near the centromeres and telomeres of each chromosome were not included due to the absence of the 25 upstream or downstream probes. The numerical differences for 99K probes were charted to display the detailed regional methylation trend (or net methylation signal) for the corresponding chromosome arm.

Identification of differentially methylated candidate genes

To identify gastric carcinoma and metastasis-specific differentially methylated candidate genes for further evaluation, the promoter and exon-1 regions were focused on due to their known inverse correlation to epigenetic repression of gene transcription. The differentially methylated probes in these regions were defined as the top-100 probes and used in hierarchical clustering analysis and preparation of a heatmap, when their P values were less than 0.05 and their absolute mean difference values were within the top 100. Candidate genes were selected from these gastric carcinoma– or metastasis-related probes according to their function information in the public databases.

Hot-start PCR and DHPLC analysis

CpG-free universal primer sets and bisulfite-modified DNA (18) were used to amplify the genes of interest. The PCR reaction mixture (30 μL) included 20 ng DNA template, 0.15 mmol/L dNTP, 0.15 μmol/L of each primer, and 0.9 U of HotStart *Taq* DNA polymerase (Qiagen GmbH). The PCR products were then analyzed quantitatively by

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DHPLC using the WAVE DNA Fragment Analysis System (12, 19). PCR products of hypermethylated and hypomethylated genes were separated using a DNASep analytical column (Transgenomic) at the corresponding partial denaturing temperature as listed in the Supplementary Materials and Methods). *M.SssI*-methylated genomic DNA, obtained from blood samples, was used as a positive control. A sample containing a methylated PCR product peak was defined as methylation-positive and used to calculate methylation-positive rate (ratio of methylation-positive sample number to total sample number). The peak areas corresponding to the methylated and unmethylated PCR products were used to calculate the percentage of methylated copies (proportion of hypermethylated copies = methylation-peak area/total peak area) for each gene analyzed.

MethyLight

The methylation states of *GFRA1*, *SRF*, and *ZNF382* were determined using the MethyLight assays. Gene-specific probes labeled with 6FAM and TAMRA were used to quantify the relative copy number of methylated alleles compared with the *COL2A1* control (20). The sequences of the primer set and gene-specific probes can be found in the Supplementary Materials and Methods.

Statistical analysis

The SPSS 16.0 Trend test and Pearson χ^2 test were used to analyze the difference in methylation frequency between gastric carcinoma and SM samples and between metastatic and nonmetastatic gastric carcinoma samples. The Student paired *t* test, Kruskal–Wallis *H* test, and One-Way ANOVA were used to identify differentially methylated regions between the different groups of samples. The Mann–Whitney *U* test and Student *t* test were used to analyze the association between the percentage of methylated copies and the clinicopathological features. All statistical tests were two-sided, and $P < 0.05$ was considered statistically significant. The cutoff value was calculated according to the ROC curve using the percentage of methylated copies to predict gastric carcinoma metastasis. The log-rank test was used to compare survival time between groups. Cox proportional hazards models were used to identify independent predictors of survival (month) with adjustment for relevant clinical covariates. Functional annotation of the differentially methylated regions was performed using EpiExplorer (21).

Results

Genome-wide analysis of gastric carcinoma–related differential DNA methylation

To identify differentially methylated genes related to gastric carcinoma development and metastasis, genome-wide DNA methylation analysis was conducted in 8 pairs of gastric carcinoma and SM samples using the MCAM assay utilizing a 99K custom-designed Agilent oligonucleotide microarray as described above (10). Through this method, 9,860 probes in 4,047 genes were identified with significant methylation differences between the 8 gastric carcinoma and 8 SM samples (paired *t* test, $P < 0.01$). Of the differentially methylated probes, 4,177 showed hypermethylation (42%; [gastric carcinoma] > [SM]), whereas the remaining probes were hypomethylated (58%; [gastric carcinoma] < [SM]; Supplementary Data file S1). Nearly half of the hypomethylated probes (49%) were found to be within a $10^{2\sim3}$-bp region of the transcription start site (TSS), whereas 42% of the hypermethylated probes were within a $10^{3\sim4}$-bp region of the TSS (Supplementary Fig. S2D; $P < 0.0000001$). When compared with the hypermethylated probes, the hypomethylated probes showed a considerably higher gastric carcinoma content than the hypermethylated ones (median, 0.68 vs. 0.50), indicating the hypomethylation lies mainly in typical CGIs (Supplementary Fig. S2E). The promoter and exon-1 regions showed significantly higher proportions of hypomethylation to hypermethylation (26.8% vs. 23.4% for the promoter, $P = 1.2 \times 10^{-4}$; 13.8% vs. 3.0% for exon-1, $P = 5.5 \times 10^{-75}$) in gastric carcinomas compared with SMs. The opposite trend was seen in the intragenic regions, which showed a significantly lower proportion of hypomethylation to hypermethylation (32.1% vs. 41.5%, $P = 5.4 \times 10^{-22}$; Fig. 1A). A heatmap displaying the top-100 differentially methylated probes between gastric carcinomas and SMs in the promoter and exon-1 regions is provided in Fig. 1B.

Most gastric carcinoma–related differentially methylated probes were clustered in specific chromosomal regions, especially subtelomeric regions (Supplementary Data File S2). Although the presence of *SmaI*/*XmaI* restriction sites primarily determined the distribution patterns of probes with gastric carcinoma–related methylation changes, certain chromosomal locations showed increased hypomethylation with little to no overlapping hypermethylation. After being normalized with respect to the probe density, chromosomes 7, 8, and 20 were clearly shown to harbor multiple long-range hypermethylated domains. In contrast, most regions in chromosomes 3, 4, 14, 15, and 18 were found to be more favorable to long-range hypomethylation (Fig. 1C).

Genome-wide analysis of gastric carcinoma metastasis–related differential DNA methylation

Among the 8 pairs of gastric carcinoma and SM samples analyzed, half were metastatic gastric carcinomas and the other half were sex-, age-, location-, and differentiation-matched nonmetastatic control gastric carcinomas (Supplementary Table S1). The MCAM analysis identified 8,553 probes that were differentially methylated between the metastatic and nonmetastatic gastric carcinoma groups (Mann–Whitney *U* test, $P < 0.029$). Among these metastasis-related candidate probes, 623 probes corresponded to 480 genes that overlapped with the gastric carcinoma–related genes identified above. A heatmap displaying the top-100 metastasis-related, differentially methylated probes is provided in Fig. 1D.

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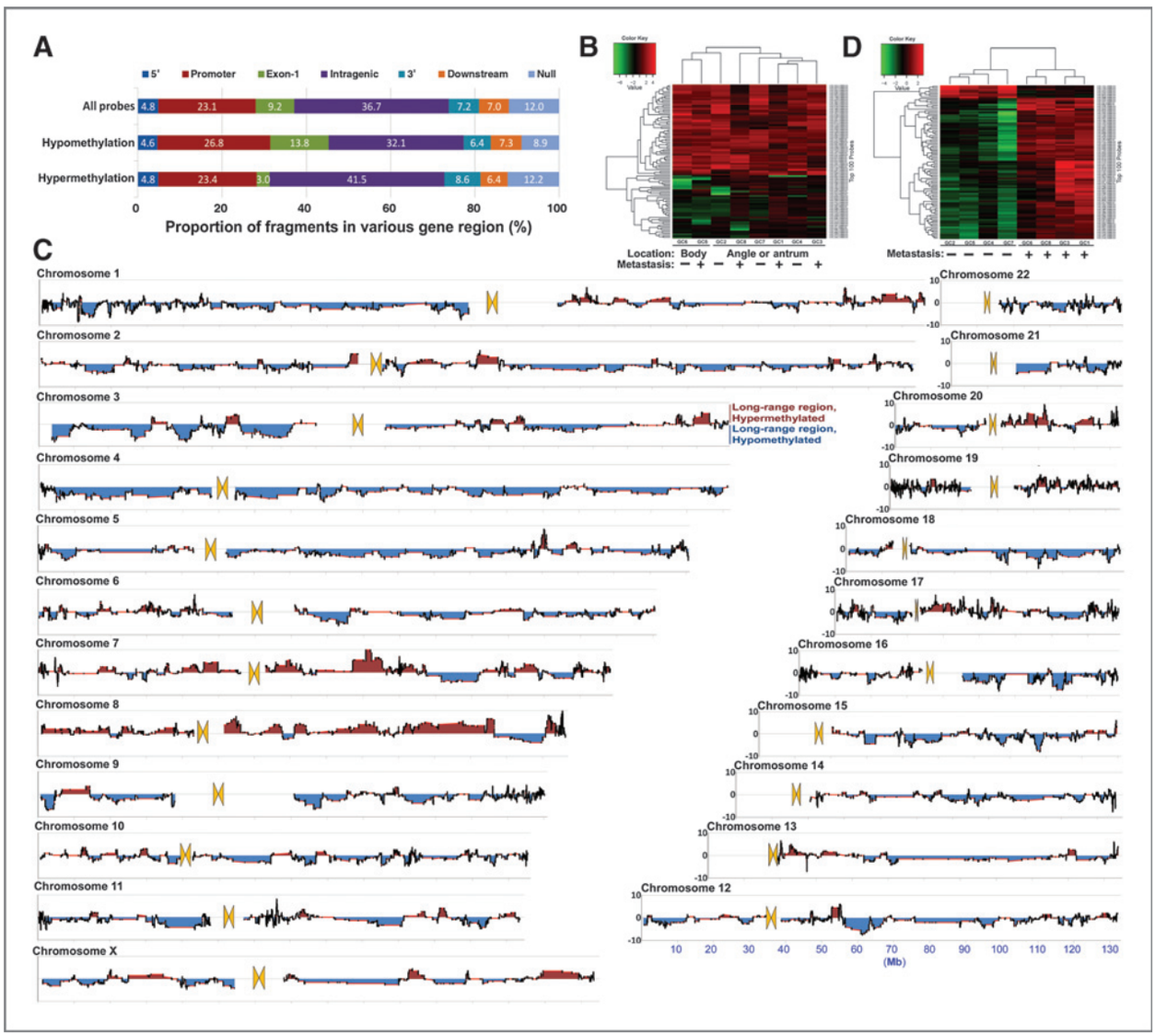

Figure 1. Distribution of probes with significant gastric carcinoma–related differential methylation changes in the human gastric carcinoma genome. A, more hypomethylation was observed in the promoter and exon-1 regions, whereas more hypermethylation was observed in the gene body region. B, heatmap of the top-100 probes with differential methylation changes between gastric carcinoma and SM samples in supervised analysis. C, patterns of detailed regional methylation trends for each chromosome arm in gastric carcinomas are displayed. The regional methylation value represents the average value of normalized methylation signal ratios between 8 gastric carcinomas and 8 paired SMs for each sliding window (sequence or region) covering 51 probe-matched fragments. The long-range hypermethylated and hypomethylated regions are indicated with deep-red and blue color, respectively. Double triangle, centromere. D, heatmap of the top-100 probes with differential methylation changes between metastatic samples (marked with "+") and nonmetastatic gastric carcinoma samples (marked with "−") in supervised analysis.

Identification in 15 gastric carcinoma–related aberrantly methylated genes

From the list of differentially methylated CGIs, 63 candidate genes were selected for further analysis based on their known functions and statistical significance of differential methylation signals between metastatic and nonmetastatic gastric carcinomas or between gastric carcinomas and SMs (Supplementary Table S2). Ten known tumor-related genes that were not included in the oligonucleotide array were also selected as complementary and control genes for the validation study. The CGIs of these 73 genes were amplified using CpG-free primer sets. The bisulfite-PCR products were then analyzed using DHPLC to quantify the methylation levels of these CGIs in the 8 paired gastric carcinoma and SM samples (Fig. 2A; Supplementary Data File S3). Differential methylation was observed in 37 CGIs between the 8 pairs of samples (Supplementary Table S2, underlined). The methylation levels of these 37 CGIs were further examined in additional 40 pairs of gastric carcinoma and SM samples, as well as 56 NorG samples. Significant differential methylation between paired gastric carcinoma/SM and NorG samples was observed in 15 CGIs ($P < 0.05$; Table 1). The

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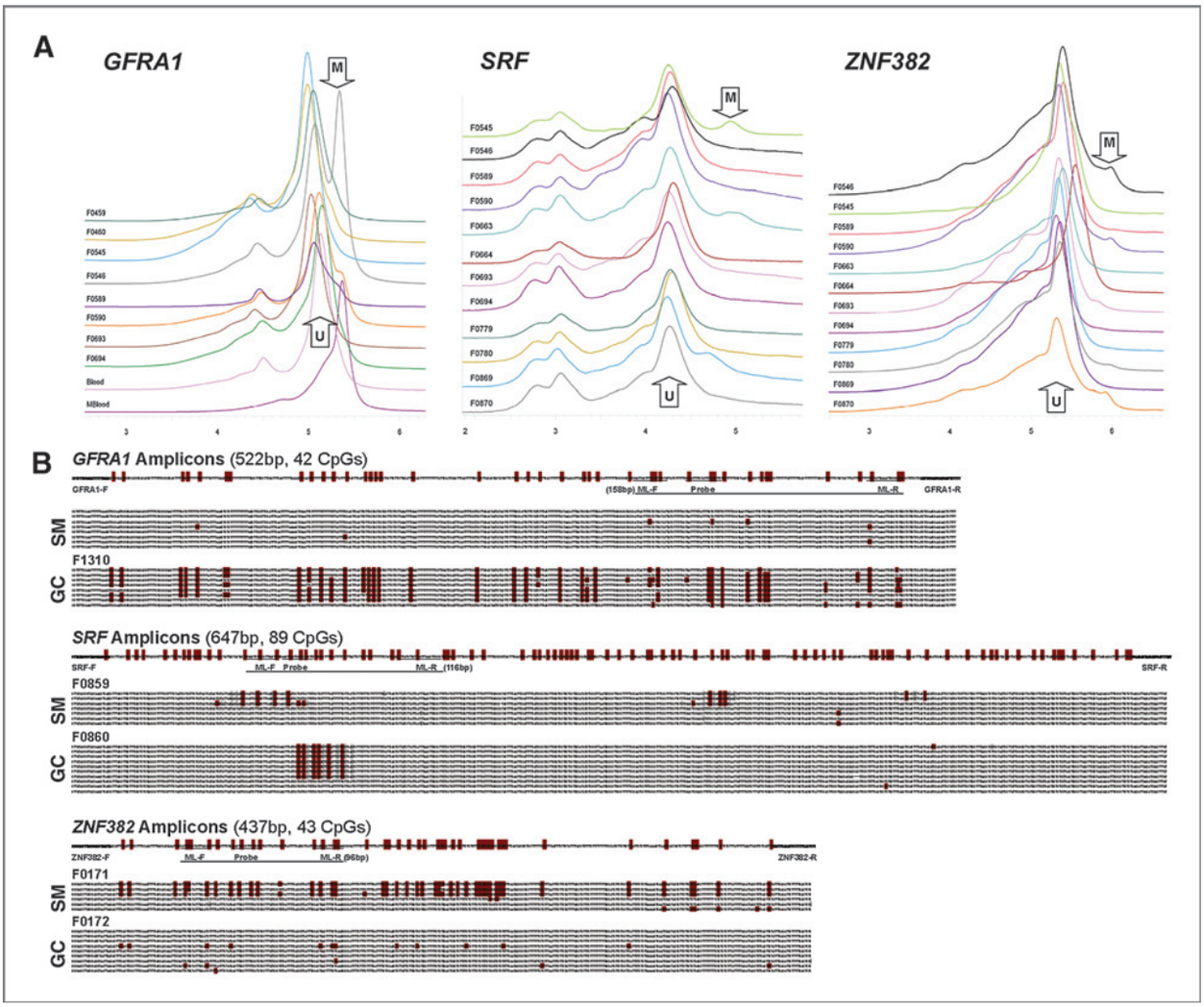

Figure 2. DNA methylation of *GFRA1*, *SRF*, and *ZNF382* in gastric carcinoma (GC) samples. A, representative DHPLC chromatograms of bisulfite PCR amplicons of *GFRA1*, *SRF*, and *ZNF382* CGIs, respectively. The hypermethylated (M) and hypomethylated (U) PCR products of each gene in the 8 pairs of gastric carcinoma and SM samples were separated with the DNASep analytical column at partial denaturing temperature as described in the Materials and Methods section. The peak areas corresponding to the methylated and unmethylated PCR products were used to calculate the percentage of methylated copies (proportion of hypermethylated copies = methylation-peak area/total peak area) for each gene analyzed. B, representative bisulfite clone sequencing results of *GFRA1*, *SRF*, and *ZNF382* in the representative gastric carcinoma and paired SM samples. The dark red dots, methylated CpG sites. Locations of the primer sets and probes used in the MethyLight assays are also illustrated.

number of samples with hypermethylated CGIs in the promoter and exon-1 of *BMP3*, *BNIP3*, *ECEL1*, *HOXD10*, *KCNH1*, *PSMD10*, *PTPRT*, *SRF*, *TBX5*, *TFPI2*, and *ZNF382* gradually increased from the NorG → SM → gastric carcinoma samples (Trend or χ^2 test, $P < 0.040$). These results suggest that hypermethylation of these 11 genes may play significant roles in gastric carcinoma development. Furthermore, the gastric carcinoma samples showed a significantly higher percentage of hypermethylated *CDKN2A* and *GFRA1* ($P < 0.050$) and significantly lower levels of methylation in *ELK1* and *SIGIRR* when compared with the SM samples.

The positive rate of methylation in *CDKN2A* and *PSMD10* was significantly higher in the gastric carcinoma and SM samples than was seen in the NorG samples. In contrast, the positive rate and proportion of methylated *ELK1* and *GFRA1* in the NorG samples were strikingly higher than in the gastric carcinoma and SM samples, indicating that hypomethylation of these genes occurs in gastric carcinogenesis as field effects. Furthermore, the positive rates of *BNIP3*, *KCNH1*, and *ZNF382* methylation in the gastric carcinoma samples were more than 3-times higher than the SM and NorG samples (29% vs. 7%–4%, 42% vs. 4%–14%, and 69% vs. 18%–23%, respectively). On the basis of this information, these genes are most likely involved in gastric carcinoma–specific methylation changes.

The methylation states of these CGIs were further confirmed using traditional bisulfite sequencing. The bisulfite

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Table 1. Prevalence of CGI methylation in gastric mucosa samples containing various pathologic changes from patients with gastric carcinoma and noncancerous control patients

		Methylation-positive rate[a]		Percentage of methylated copies in the methylation-positive samples	
CpG islands of genes	**Pathologic changes**	**Positive rate (%)**	***P***	**Median (25%–75%)**	***P***
BMP3	Gastric carcinoma	74/102 (72.5)	<0.001[b]	8.6 (4.0–29.3)	<0.001[c]
	SM	36/102 (35.3)	<0.001[d]	1.9 (1.0–5.4)	
	NorG	3/48 (6.3)		0.2 (0.1–0.9)	
BNIP3	Gastric carcinoma	17/58 (29.3)	<0.001[b]	7.3 (3.6–16.4)	
	SM	4/58 (6.9)		15.3 (6.0–27.8)	
	NorG	2/45 (4.4)		20.5	
CDKN2A	Gastric carcinoma	12/91 (13.2)	<0.001[e]	4.12 (0.3–13.7)	0.043[f]
	SM	15/91 (16.5)	<0.001[d]	0.45 (0.2–1.3)	
	NorG	1/46 (2.2)		0.63	
ECEL1	Gastric carcinoma	47/58 (81.0)	<0.001[b]	13.9 (2.3–26.7)	0.003[g]
	SM	26/58 (44.8)		0.0 (0.0–4.9)	
	NorG	15/42 (35.7)		0.0 (0.0–4.5)	
ELK1	Gastric carcinoma	43/48 (89.6)		56.0 (29.3–82.1)	0.001[c]
	SM	43/48 (89.6)		68.3 (47.9–100.0)	
	NorG	43/43 (100.0)		75.9 (67.7–100.0)	
GFRA1	Gastric carcinoma	59/98 (60.2)		5.4 (0.0–59.2)	0.002[g]
	SM	46/98 (46.9)	0.003[d]	0.0 (0.0–12.6)	0.000[h]
	NorG	35/48 (72.9)		44.4 (0.0–74.5)	0.017[i]
HOXD10	Gastric carcinoma	36/48 (75.0)	0.024[b]	16.1 (0.8–21.7)	0.012[g]
	SM	30/48 (62.5)		10.4 (0.0–15.0)	
	NorG	15/30 (50.0)		11.0 (0.0–61.8)	
KCNH1	Gastric carcinoma	20/48 (41.7)	<0.001[j]	1.0 (0.4–3.0)	0.005[g]
	SM	2/48 (4.2)		0.3	
	Nor	6/44 (13.6)		17.1 (13.7–29.0)	
PSMD10	Gastric carcinoma	19/48 (39.6)	0.011[e]	33.9 (18.5–45.3)	
	SM	17/48 (35.4)	0.023[d]	36.2 (11.6–45.8)	
	NorG	2/22 (9.1)		64.1	
PTPRT	Gastric carcinoma	42/58 (72.4)	<0.001[b]	10.6 (0.0–28.0)	0.009[g]
	SM	20/58 (34.5)		0.0 (0.0–11.0)	
	NorG	4/21 (19.0)		0.0 (0.0–0.0)	
SIGIRR	Gastric carcinoma	27/48 (56.3)	0.001[j]	18.9 (0.0–30.0)	0.023[g]
	SM	42/48 (87.5)	<0.004[d]	23.1 (18.1–30.0)	
	NorG	29/47 (61.7)		13.5 (0.0–24.6)	
SRF	Gastric carcinoma	30/102 (29.4)	0.030[b]	10.7 (2.8–18.4)	
	SM	20/102 (19.6)		13.5 (6.7–36.2)	
	NorG	4/31 (12.9)		2.1 (1.0–9.6)	
TBX5	Gastric carcinoma	45/58 (77.6)	0.032[b]	30.8 (20.0–48.1)	
	SM	36/58 (62.1)		25.7(14.1–38.1)	
	NorG	11/21 (52.4)		11.6 (7.9–37.2)	
TFPI2	Gastric carcinoma	38/58 (65.5)	<0.001[b]	25.7 (0.0–32.0)	<0.001[c]
	SM	16/58 (27.6)		0.0 (0.0–15.3)	
	NorG	4/47 (8.5)		0.0 (0.0–0.0)	
ZNF382	Gastric carcinoma	75/108 (69.4)	<0.001[b]	4.5 (2.0–11.8)	0.002[f]
	SM	25/108 (23.1)		1.9 (0.7–3.5)	
	NorG	10/56 (17.9)		3.9 (0.9–7.5)	

[a]The ratio between the number of methylation-positive sample and the number of total tested sample; [b]trend test; [c]gastric carcinoma versus SM versus NorG, Kruskal–Wallis test; [d/e]SM/gastric carcinoma versus NorG, χ^2 test; [f]gastric carcinoma versus SM, Mann–Whitney *U* test; [g]gastric carcinoma versus SM, paired *t* test; [h]SM versus NorG, Mann–Whitney *U* test; [i]NorG versus gastric carcinoma, Mann–Whitney *U* test; [j]gastric carcinoma versus SM, χ^2 test.

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Table 2. *SRF*, *ZNF382*, and *GFRA1* methylation prevalence comparison in SM and gastric carcinoma samples from Chinese patients in the discovery cohort with various clinicopathological characteristics

Clinicopathological features	*SRF* methylation-positive rate (%)		*ZNF382* methylation-positive rate (%)		*GFRA1* methylation-positive rate (%)		Percentage of methylated-*GFRA1* copies (%)[a]	
	SM	GC	SM	GC	SM	GC	SM	GC
Age								
<60	10/49 (20.4)	13/49 (26.5)	9/52 (17.3)	36/52 (69.2)	21/48 (43.8)	29/48 (60.4)	10.9 (7.5–54.3)	49.1 (5.4–62.4)
≥60	10/53 (18.9)	17/53 (32.6)	16/56 (28.6)	39/56 (69.6)	25/50 (50.0)	30/50 (60.0)	20.2 (6.5–44.5)	55.8 (17.3–85.7)
Sex								
Male	11/70 (15.7)	22/70 (31.4)	19/70 (27.1)	48/70 (68.6)	29/66 (43.9)	36/66 (54.5)	37.4 (7.1–63.7)	53.8 (18.7–84.3)
Female	9/32 (28.1)	8/32 (25.0)	6/38 (15.8)	28/38 (73.7)	17/32 (53.1)	23/32 (71.9)	8.8 (6.5–29.0)	41.4 (6.4–61.9)
Location								
Cardiac	4/19 (21.1)	8/29 (27.6)	4/20 (20.0)	12/20 (60.0)	11/17 (64.7)	10/17 (58.8)	34.8 (10.9–43.8)	52.5 (20.4–70.5)
Noncardiac	16/83 (19.3)	22/83 (26.5)	21/88 (23.9)	64/88 (72.7)	35/81 (43.2)	49/81 (60.5)	9.6 (6.9–47.3)	51.9 (8.6–70.5)
Differentiation								
Well/moderate	6/35 (17.1)	11/34 (32.4)	7/31 (22.6)	21/31 (67.7)	10/28 (35.7)	16/28 (57.1)	28.9 (5.5–71.4)	62.1 (49.8–96.8)
Poor	12/62 (19.4)	16/63 (25.4)	18/77 (23.4)	18/77 (23.4)[b]	35/64 (54.7)	40/64 (62.5)	12.5 (7.1–43.8)	42.4 (6.9–62.6)[c]
Vascular embolus								
No	16/50 (32.0)	17/50 (34.0)	14/53 (26.4)	41/53 (77.4)	21/44 (47.7)	25/44 (56.8)	12.9 (7.0–41.9)	61.0 (35.5–90.7)
Yes	3/50 (6.0)[d]	12/50 (24.0)	11/52 (21.2)	33/52 (63.5)	22/49 (44.9)	32/49 (65.3)	22.8 (6.7–45.7)	32.1 (5.3–61.1)[e]
pTNM stage								
I–II	14/45 (31.1)	18/45 (40.0)	13/47 (27.7)	37/47 (78.7)	23/42 (54.8)	24/42 (57.1)	12.9 (5.5–43.8)	61.4 (33.9–89.8)
III–IV	5/57 (8.8)[f]	15/57 (26.3)	11/61 (18.0)	39/61 (63.9)	23/56 (41.1)	35/56 (62.5)	20.2 (7.9–47.3)	41.4 (5.7–61.9)[g]
Local invasion								
T_{1-2}	7/19 (37.0)	7/19 (36.8)	4/19 (21.1)	13/19 (68.4)	11/20 (55.0)	10/20 (50.0)	16.2 (6.0–43.1)	59.9 (13.8–92.1)
T_3	11/60 (18.3)	20/61 (32.8)	16/64 (25.0)	47/64 (73.4)	25/55 (45.5)	45/55 (81.8)	12.5 (6.6–63.7)	43.4 (8.9–69.8)
T_4	2/23 (8.7)[h]	3/22 (13.6)[i]	5/25 (20.0)	16/25 (64.0)	10/23 (43.5)	14/23 (60.9)	17.9 (7.6–45.7)	50.9 (14.2–61.3)
Lymph metastasis								
N_0	16/55 (29.1)	19/56 (33.9)	18/58 (31.0)	45/58 (77.6)	30/55 (54.5)	32/55 (58.2)	11.0 (5.8–42.0)	60.6 (30.7–89.8)
N_{1-3}	4/47 (8.5)[j]	11/46 (23.9)	7/50 (14.0)[k]	31/50 (62.0)[l]	16/43 (37.2)[m]	27/43 (62.8)	39.1 (8.0–87.2)	22.8 (5.7–61.9)[n]
Distant metastasis								
M_0	16/51 (31.4)	17/51 (33.3)	17/54 (31.5)	43/54 (79.6)	26/49 (53.1)	29/49 (59.2)	12.7 (5.8–44.1)	60.3 (31.2–85.2)
M_1	4/51 (7.8)[o]	13/51 (25.5)	8/54 (14.8)[p]	33/54 (61.1)[q]	20/49 (40.8)	30/49 (61.2)	28.8 (8.4–66.9)	32.1 (6.2–66.2)
(Total)	20/102 (19.6)	30/102 (29.4)	25/108 (23.1)	76/108 (70.4)[r]	46/98 (46.9)	59/98 (60.2)	14.5 (7.0–45.7)	51.9 (10.4–69.8)[s]

NOTE: Numbers underlined: highlighted the values between them a statistically significant difference was observed.

Abbreviation: GC, gastric carcinoma.

[a]Median (25%–75% range) for methylation-positive samples; [b]χ^2 test, $P = 0.001$; [c]Mann–Whitney *U* test, $P = 0.026$; [d]Fisher test, $P = 0.002$; [e]Mann–Whitney *U* test, $P = 0.012$; [f]χ^2 test, $P = 0.004$; [g]Mann–Whitney *U* test, $P = 0.015$; [h]trend test, $P = 0.025$; [i]trend test, $P = 0.069$; [j]Fisher test, $P = 0.029$; [k]χ^2 test, $P = 0.036$; [l]χ^2 test, $P = 0.077$; [m]χ^2 test, $P = 0.028$; [n]Mann–Whitney *U* test, $P = 0.038$; [o]Fisher test, $P = 0.005$; [p]χ^2 test, $P = 0.040$; [q]χ^2 test, $P = 0.035$; [r]SM versus GC, $P < 0.001$; [s]paired *t* test, SM versus GC, $P = 0.002$.

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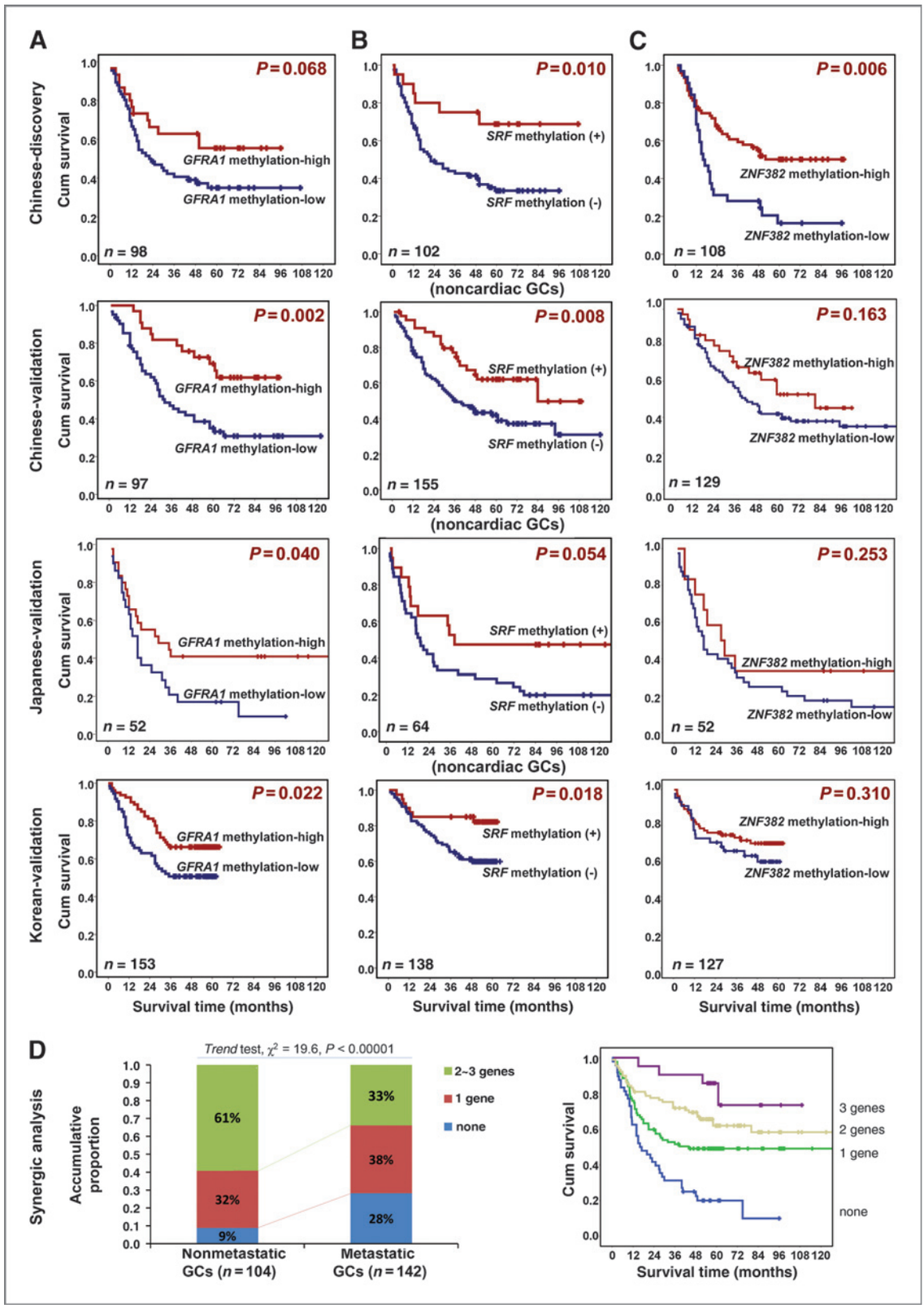

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sequencing results were consistently in agreement with the DHPLC analysis (Fig. 2B and Supplementary Fig. S3). In addition, quantitative MethyLight assays using fresh or formalin-fixed paraffin-embedded tissue samples further validated the DHPLC results (Supplementary Fig. S4A; Spearman test, $P < 0.020$). To understand if methylation changes in these CGIs affect gene expression, the mRNA levels of *SRF*, *ZNF382*, and *GFRA1* were analyzed in matched tissue samples using qRT-PCR. The qRT-PCR results showed that mRNA expression of all three genes was inversely correlated with the prevalence of methylation in their CGIs (Supplementary Fig. S4B; Spearman test, $P < 0.050$).

Confirmation of gastric carcinoma metastasis–related DNA methylation markers

Among the above 48 pairs of gastric carcinoma and SM samples, 24 pairs were from patients with lymphatic and distant metastasis, and 24 pairs were from sex-, age-, location-, and gastric carcinoma differentiation grade-matched patients without metastasis. Thus, the methylation states of these 15 CGIs were further analyzed to determine if they are associated with gastric carcinoma metastasis. DHPLC results showed that the methylation states of the *BMP3*, *GFRA1*, *SRF*, and *ZNF382* CGIs were significantly different between metastatic and nonmetastatic gastric carcinoma samples. The proportion of methylated *BMP3* and *GFRA1* was lower in the metastatic gastric carcinoma samples than the nonmetastatic gastric carcinoma samples (median, 1.8% vs. 5.9%; 8.6% vs. 38.6%; Mann-Whitney *U* test, $P < 0.040$). The positive rate of *SRF* and *ZNF382* methylation was also lower in the metastatic gastric carcinoma samples than the nonmetastatic gastric carcinoma samples (4% vs. 33%; 54% vs. 79%, $P = 0.020/0.066$). Therefore, the relationship between gastric carcinoma metastasis and methylation of these four CGIs was tested in additional gastric carcinoma and SM samples obtained from Chinese patients ($n = 50–60$). When these samples were taken together as a discovery cohort, the relationship between gastric carcinoma lymph/distant metastasis and the methylation changes in *GFRA1*, *SRF*, and *ZNF382* was statistically significant (Table 2); however, such an association was not observed for *BMP3* (data not shown).

To investigate whether the methylation status of the three potential biomarkers mentioned above had an impact on overall survival, Kaplan–Meier analysis was performed on each gene individually. Results showed that the overall survival of patients with gastric carcinoma with *GFRA1* or *ZNF382* methylation-high (cutoff value: percentage of methylated copies >26.4% for *GFRA1* or 1.3% for *ZNF382*) or *SRF* methylation-positive was elongated when compared with methylation-low or methylation-negative patients in the discovery cohort (log-rank test, $P = 0.068$, Fig. 3A; $P = 0.010$, Fig. 3B; $P = 0.001$, Fig. 3C, respectively). Substratification analysis revealed that *SRF* methylation was only correlated with overall survival in patients with noncardiac gastric carcinomas ($P < 0.033$) but not with cardiac gastric carcinomas ($P = 0.146$). Therefore, only patients with noncardiac gastric carcinoma were included in the survival analysis in the following *SRF* methylation validation cohorts.

The predictive value of these methylation markers for gastric carcinoma metastasis was further confirmed using three independent validation cohorts in China ($n = 222$), Japan ($n = 129$), and Korea ($n = 153$). Because the proportion of both methylated and unmethylated alleles of CGIs can be quantitatively and simultaneously determined using DHPLC, this method was consistently used to detect the methylation levels within these CGIs in freshly-frozen gastric samples from Chinese and Japanese patients. However, MethyLight was used to analyze the paraffin-embedded samples from the Korean patients, as fresh samples were not available. Results from these cohorts showed that the methylation-positive rates of *GFRA1*, *SRF*, and *ZNF382* were inversely and significantly correlated with pTNM stage and lymph metastasis in all three cohorts (Table 3). The Kaplan–Meier analysis also showed that the overall survival of patients with gastric carcinoma with higher methylation levels of *GFRA1* and *SRF* CGIs was consistently longer than those without methylation of these two genes across all three validation cohorts (Fig. 3A and B). However, correlation between *ZNF382* methylation and overall survival of patients with gastric carcinoma was not statistically significant in all three validation cohorts (Fig. 3C). These results indicate that *ZNF382* methylation may be a weak gastric carcinoma metastasis biomarker when compared with *GFRA1* and *SRF* methylation.

In addition, after adjustment for age, sex, differentiation, location, pTNM stage, and vascular embolus, *GFRA1* or *SRF* methylation was still an adequate prognostic indicator in multivariate analysis among all patients in these validation cohorts (HR, 0.543 or 0.395; 95% confidence interval, CI, 0.304–0.938 or 0.165–0.945; $n = 300$ or 452).

Substratification analysis showed that the overall survival of patients with stage I and II gastric carcinoma with methylated *SRF* was significantly elongated when compared with *SRF* methylation-negative patients in all four cohorts (HR, 0.357; 95% CI, 0.164–0.778; $n = 198$). Similar difference was also observed for *GFRA1* or *ZNF382* methylation-high, but not statistically significant (HR, 0.608 or 0.498; 95% CI, 0.336–1.099 or 0.243–1.023; $n = 173$ or

Figure 3. Kaplan–Meier survival curves of patients with gastric carcinoma (GC) with different *GFRA1*, *SRF*, and *ZNF382* methylation states. A–C, *GFRA1* and *ZNF382* methylation-high and *SRF* methylation-positive in gastric carcinoma or SM tissues were good survival factors with statistical significance for patients with gastric carcinoma in the Chinese-discovery cohorts, Chinese, Japanese, and Korean validation cohorts. D, synergistic analysis of three methylation markers. Distribution of the number of patients with methylation changes in one to three genes (*GFRA1*, *SRF*, and *ZNF382*) in metastatic and nonmetastatic gastric carcinoma groups. The number of patients with one or more differentially methylated genes in nonmetastatic gastric carcinomas was significantly higher than that in metastatic gastric carcinomas (left). The greater the number of genes associated with differential methylation, the longer the overall survival of patients with gastric carcinoma (right).

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167). Among patients with gastric carcinoma from Korea whose histologic types of gastric carcinomas were available, *GFRA1* methylation-high was significantly associated with low risk of metastasis of both intestinal- and diffuse-types of gastric carcinomas (positive rate: 82.4% and 76.5% for nonmetastatic gastric carcinomas; 43.2% and 43.1% for metastatic gastric carcinomas, $P < 0.05$). *GFRA1* methylation-high was also significantly correlated with longer overall survival of patients with diffuse-type gastric carcinoma (HR, 0.482; 95% CI, 0.247–0.938; $n = 67$). However, *ZNF382* methylation-high was significantly associated with low risk of metastasis of intestinal-type gastric carcinomas (93.8% vs. 62.5%, $P = 0.036$), but not diffuse-type gastric carcinomas.

Synergic analysis of three methylation markers

To investigate if a combination of the methylation markers (*GFRA1*, *SRF*, and *ZNF382*) has a synergistic effect on predicting gastric carcinoma metastasis, the merged data were reanalyzed in the above 4 patient cohorts. As expected, the number of patients with one or more methylated genes among the three-gene panel was significantly decreased in gastric carcinoma samples with lymph/distant metastasis (Fig. 3D left; linear-trend test, $P < 0.00001$; one gene vs. two genes, $P = 0.046$). The sensitivity and specificity of 2 to 3 positive methylation changes of 3 genes for detection of nonmetastatic gastric carcinomas were 60% and 67%, respectively. The positive and negative predictive values were 57% and 69%. In addition, multivariate analysis also showed that the number of combined methylation changes of *GFRA1*, *SRF*, and *ZNF382* was an independent predictor of overall survival for patients with gastric carcinoma ($n = 246$) after adjusting for the pTNM stage, gastric carcinoma location, differentiation, vascular embolus, age, and sex (HR, 0.734; 95% CI, 0.562–0.958; Fig. 3D, right). The pTNM stage and gastric carcinoma location were also independent survival factors (HR, 3.608; 95% CI, 2.648–4.917 and 2.723; 95% CI, 1.608–4.613, respectively). These results suggest that using a combination of this three-gene panel may function as a synergic biomarker set for predicting gastric carcinoma prognosis.

GFRA1, SRF, and ZNF382 expression changes in gastric carcinogenesis

The protein expression of the three genes in the paired gastric carcinoma and SM samples in both regular tissue sections and tissue microarray (TMA) were analyzed using the IHC assay as described in the Supplementary Materials and Methods (22). IHC analysis revealed that GFRA1 expression was predominantly observed in the cytoplasm of stromal cells, especially in the vessel cells in gastric carcinomas (Supplementary Fig. S5A). Among 38 pairs of IHC-informative cases, the proportion of gastric carcinomas with strong GFRA1 staining was significantly higher than SMs (24/38 vs. 12/38, $P < 0.01$). Among 28 pairs of informative cases, the proportion of gastric carcinomas with strong ZNF382 staining in epithelial cells was lower than SMs (4/28 vs. 11/28, $P < 0.07$; Supplementary Fig. S5B). Statistically significant association was not observed between GFRA1 (or ZNF382) staining and clinical parameters, such as invasion, lymph metastasis, embolus, differentiation, and overall survival. SRF staining was only observed in the nucleus of some stromal fibroblasts and smooth muscle cells in both regular gastric carcinoma and SM sections (Supplementary Fig. S5C). Therefore, SRF expression was not further examined using TMA.

Discussion

Over a 4-year period, a comprehensive epigenetic biomarker discovery and validation study involving over 500 patient samples from three large academic medical centers in China, Japan, and Korea had been conducted. The biomarker discovery effort started off with a genome-wide analysis of differentially methylated genes between metastatic and nonmetastatic gastric carcinomas in a small number of patient samples. The microarray-based methylation profiling identified a large number of gastric carcinoma–specific and metastasis-specific candidate genes that were differentially methylated. From the list of differentially methylated genes, a step-by-step elimination process identified a 15-gene panel associated with gastric carcinoma/metastasis-specific DNA methylation changes. The 15 genes were validated using multiple independent methods from a discovery cohort of gastric carcinoma patient samples. Finally, a methylation biomarker-set consisting of *GFRA1*, *SRF*, and *ZNF382* was validated for the prediction of gastric carcinoma metastasis and patients' overall survival in four cohorts from China, Japan, and Korea. This novel epigenetic biomarker set may be used in the decision-making process for personalized postoperational therapy. To our knowledge, this is the first such study which specifically focuses on the metastasis of gastric cancer.

A large number of genome-wide DNA methylation studies have been reported for many different tumor types in recent years (6, 10, 11). However, most of the studies failed to perform large-scale and in-depth follow-up studies to validate the candidate genes discovered through the genome-wide analyses. As a result, few methylation markers have been developed from the large number of DNA methylation studies published so far. The present study represents the most comprehensive and quantitative characterization of DNA methylation biomarkers in gastric carcinoma to date. Moreover, the three methylation biomarkers associated with gastric carcinoma metastasis and patients' survival were validated not only in multiple cohorts but also in freshly-frozen and paraffin-embedded samples using several independent methods such as DHPLC and MethyLight. The vigorous testing performed in this study ensures the high reliability and feasibility of these novel biomarkers in different clinical settings.

It has been previously reported that 2,540 of 17,800 tested genes are differentially expressed between 80 pairs of gastric carcinoma and SM samples. Furthermore, it was found that there are four times as many upregulated genes in gastric carcinomas than there are downregulated genes (1,983 vs. 557; GSE27342; ref. 23). Therefore, the frequent DNA hypomethylation in the promoter and exon-1 regions

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Table 3. Comparison of *SRF*, *ZNF382*, and *GFRA1* methylation-positive rates in patients with gastric carcinoma with various clinicopathological characteristics in the Chinese, Japanese, and Korean validation cohorts[a]

Clinicopathological features	Positive rate of *SRF* methylation (%)			Positive rate of *ZNF382* methylation-high (%)			Positive rate of *GFRA1* methylation-high (%)		
	Chinese	Japanese	Korean	Chinese	Japanese	Korean	Chinese	Japanese	Korean
Cutoff value[b]	None	None	None	>3.2	>31.4	>2.7	>39.5	>35.3	None
Age									
<60	31/101 (30.7)	11/47 (23.4)	24/77 (31.2)	16/62 (25.8)	8/41 (19.5)	40/65 (61.5)	12/44 (27.3)	19/41 (46.3)	34/74 (45.9)
≥60	34/121 (28.1)	12/31 (38.7)	21/75 (28.0)	20/67 (29.9)	27/88 (30.7)	43/62 (69.4)	21/53 (39.6)	40/88 (45.5)	33/79 (58.2)
Sex									
Male	47/164 (28.7)	12/48 (25.0)	30/112 (26.8)	30/102 (29.4)	27/89 (30.3)	57/91 (62.6)	23/67 (34.3)	40/89 (44.9)	56/106 (52.8)
Female	18/58 (31.0)	11/30 (36.7)	15/40 (37.5)	6/27 (22.2)	8/40 (20.0)	26/36 (72.2)	10/30 (33.3)	19/40 (47.5)	24/47 (51.1)
Location									
Cardiac	20/67 (29.9)	4/14 (28.6)	5/14 (35.7)	14/40 (35.0)	NA	16/21 (76.2)	12/32 (37.5)	NA	11/26 (42.3)
Noncardiac	45/155 (29.0)	19/64 (29.7)	40/138 (29.0)	22/88 (25.0)	NA	67/106 (63.2)	21/67 (31.3)	NA	69/127 (54.3)
Differentiation									
Well/moderate	19/54 (35.2)	4/15 (26.7)	20/67 (29.9)	11/30 (36.7)	10/31 (32.3)	35/48 (72.9)	14/29 (48.3)	17/31 (54.8)	49/96 (51.0)
Poor	42/157 (26.8)	19/63 (30.2)	25/84 (29.8)	25/94 (26.6)	22/92 (23.9)	46/77 (59.7)	19/65 (29.2)	39/92 (42.4)	31/57 (57.6)
Vascular embolus									
No	52/155 (33.5)	19/63 (30.2)	11/34 (32.4)	21/63 (33.3)	NA	21/31 (67.7)	0/3 (0.0)	NA	23/34 (67.6)
Yes	11/62 (17.7)[c]	4/15 (26.7)	34/118 (28.8)	14/60 (23.3)	NA	61/95 (64.2)	33/94 (35.1)	NA	57/119 (47.9)[d]
pTNM stage									
I–II	19/52 (36.5)	10/17 (58.8)	32/85 (37.6)	17/33 (51.5)	17/35 (48.6)	53/78 (67.9)	22/36 (61.1)	24/35 (68.6)	52/84 (61.9)
III–IV	44/167 (26.3)	13/61 (21.3)[e]	13/67 (19.4)[f]	19/96 (19.8)[g]	14/78 (17.9)[h]	30/49 (61.2)	10/59 (16.9)[g]	30/78 (38.5)[e]	28/69 (40.6)[g]
Local invasion									
T_{1-2}	16/35 (45.7)[i]	13/33 (39.4)	35/109 (32.1)	9/18 (50.0)[j]	22/62 (35.5)[k]	57/85 (67.1)	12/21 (57.1)	35/62 (56.5)[l]	58/100 (58.0)
T_3	33/133 (24.8)	10/36 (27.8)	9/38 (23.7)	18/77 (23.4)	11/59 (18.6)	22/35 (62.9)	16/58 (27.6)	21/59 (35.6)	21/44 (47.7)
T_4	15/52 (28.8)	0/9 (0.0)[m]	1/5 (20.0)	8/33 (24.2)	2/8 (25.0)	4/7 (57.1)	4/16 (25.0)[n]	3/8 (37.5)	1/9 (11.1)[o]
Lymph metastasis									
N_0	14/42 (33.3)	7/9 (77.8)	17/44 (38.6)	18/32 (56.3)	15/28 (53.6)	38/46 (82.6)	22/33 (66.7)	21/28 (75.0)	35/50 (70.0)
N_{1-3}	51/180 (28.3)	16/69 (23.2)[p]	28/108 (25.9)	18/97 (18.6)[g]	20/101 (19.8)[g]	45/81 (55.6)[p]	11/64 (17.2)[g]	38/101 (37.6)[e]	45/103 (43.7)[p]
Distant metastasis									
M_0	65/222 (29.3)	19/55 (34.5)	40/134 (29.9)	36/129 (28.4)	35/129 (27.1)	59/88 (67.0)	33/97 (34.0)	59/129 (42.4)	68/113 (60.2)
M_1	—	4/23 (17.4)	5/18 (27.8)	—	—	24/39 (61.5)	—	—	12/40 (30.0)[p]
(Total)	65/222 (29.3)	23/78 (29.5)	45/152 (29.6)	36/129 (28.4)	35/129 (27.1)	83/127 (65.4)	33/97 (34.0)	59/129 (45.7)	80/153 (52.3)

NOTE: Numbers underlined: highlighted the values between them a statistically significant difference was observed.

Abbreviations: GC, gastric carcinoma; NA, not available.

[a]The methylation states of three tested genes in frozen samples [from Chinese (SM) and Japanese (GC)] were analyzed by DHPLC; in fixed paraffin samples [from Korean (SM)], by MethyLight. In addition, 33, 20, and 13 patients with preoperative chemotherapy were included in the Chinese validation cohort for *SRF*, *GFRA1*, and *ZNF382* methylation, respectively. Significant differences in the methylation-positive rates were not observed between patients with and without preoperative chemotherapy.

[b]The cutoff value is calculated according to ROC curve (not shown) when more than half of samples are methylation-positive.

[c]χ^2 test, $P = 0.020$; [d]χ^2 test, $P = 0.042$; [e]χ^2 test, $P = 0.003$; [f]χ^2 test, $P = 0.014$; [g]χ^2 test, $P = 0.001$; [h]χ^2 test, $P = 0.040$; [i]χ^2 test, pT_{1-2} versus pT_{3-4}, $P = 0.018$; [j]χ^2 test, pT_{1-2} versus pT_{3-4}, $P = 0.042$; [k]χ^2 test, pT_{1-2} versus pT_{3-4}, $P = 0.040$; [l]χ^2 test, pT_{1-2} versus pT_{3-4}, $P = 0.019$; [m]trend test, $P = 0.028$; [n]trend test, $P = 0.028$; [o]trend test, $P = 0.011$; [p]χ^2 test, $P = 0.002$.

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of the gastric carcinoma methylome observed in this study may account for the prevalent increase in gene expression. In fact, an increasing number of studies have reported reactivation of proto-oncogenes by DNA hypomethylation in several cancers (24–26).

Long-range epigenetic silencing and large epigenetic structures have been reported in different cancers (27–29). Differential long-range hypermethylation and hypomethylation trends may be related to cancer/tissue-specific DNA methylation (11, 30). In the present study, it was found that chromosomes 7, 8, and 20 seemed more favorable for long-range hypermethylation (or amplification of methylated regions). In contrast, chromosomes 3, 4, 14, 15, and 18 had an affinity for long-range hypomethylation (or deletion of methylated regions). Further studies are warranted to determine which of these long-range hypermethylated and hypomethylated regions are gastric carcinoma–specific changes and which are changes across cancer-types.

Most of the 15 aberrantly methylated genes identified in gastric carcinomas are involved in cell proliferation, differentiation, apoptosis, adhesion, and embryonic development (Supplementary Table S2). Previous reports have demonstrated that silencing of *BMP3*, *BNIP3*, *CDKN2A*, *HOXD10*, *TFPI2*, and *ZNF382* via methylation correlates with both the development and progression of cancers (12, 19, 31–34). Similar associations were also observed in gastric carcinoma samples used in the present study. Methylation changes of *KCNH1*, *PSMD10*, and *SRF* in cancer tissues have not previously been reported. Furthermore, *BNIP3*, *KCNH1*, and *ZNF382* methylation levels were more than 3-times higher in the gastric carcinoma samples than in SM and NorG samples. It is needed to study whether methylation of these genes may affect their expression states in gastric carcinogenesis. In addition, though *TBX5* and *ELK1* methylation is not associated with gastric carcinoma metastasis, the overall survival of patients with gastric carcinoma with methylated *TBX5* or *ELK1* was longer than those without methylation ($P = 0.017$ or 0.003; data not shown). Because some methylation changes may occur in both gastric carcinoma and SM samples from patients with cancer, more gastric carcinoma–related methylation changes could potentially be identified if the NorG samples were used as the normal stomach reference.

Among three genes identified with gastric carcinoma development- and metastasis-related methylation changes, *GFRA1* is a cell surface GDNF (glial cell line-derived neurotrophic factor)/neurturin receptor and a tyrosine kinase that is normally expressed in the nervous system and kidney. However, this gene is overexpressed in gut neural crest stem cells and in many cancers (35–41). The present study provides the first evidence that hypomethylation of *GFRA1* CGIs may account for its overexpression in cancers. *SRF* is a master regulator of myogenesis and multiple cellular processes, including cell proliferation and migration. Furthermore, *SRF* is known to play important roles in the epithelial–mesenchymal transition and experimental invasion through cancer and stromal cells (42–48). The present study shows, for the first time, that methylation in the exon-1 region of its CGIs may epigenetically inactivate *SRF* transcription. Most importantly, we found that *SRF* methylation was correlated with overall survival in patients with noncardiac gastric carcinoma, but not in patients with cardiac gastric carcinoma. It is well known that *H. pylori* infection increases risk of noncardiac gastric carcinoma, but not cardiac gastric carcinoma (49). The incidence of cardiac gastric carcinoma is also gradually increased in Western countries coincided with a decrease in prevalence of *H. pylori* infection (50). Therefore, whether *H. pylori* infection contributes to *SRF* methylation and its biologic subsequence warrants future study.

GFRA1 and *SRF* are two crucial genes in the GDNF–GFRA–RET–RAS–MEK–ERK–ELK–SRF pathway involved in cell migration and cancer invasion (37, 41, 46–48). Therefore, epigenetic alterations of *GFRA1* and *SRF* may play important roles in gastric carcinoma metastasis through modulating this important pathway. *ZNF382* is a candidate tumor suppressor gene, and its methylation is associated with gastric carcinoma development (34). However, its link with cancer metastasis has not previously been reported. In the present study, it was found that the methylation status of *GFRA1*, *SRF*, or *ZNF382* was consistently and significantly associated with gastric carcinoma metastasis and patients' overall survival in multiple cohorts from different populations, suggesting that they may be used as potential biomarkers for predicting gastric carcinoma metastasis and prognosis. Most importantly, the combination of the three markers was not only identified as an independent survival factor but also as a strong synergistic biomarker set helping to distinguish metastatic gastric carcinomas from nonmetastatic gastric carcinomas. The TMA analysis of GFRA1 and ZNF382 from 40 patients with gastric carcinoma failed to demonstrate statistically significant association of their protein expression with clinicopathological parameters and overall survival of these patients; however, upregulation of GFRA1 protein and downregulation of ZNF382 were indeed observed in the gastric carcinomas compared with SMs, which is in agreement with hypo- and hypermethylation of *GFRA1* and *ZNF382* observed in gastric carcinomas. Our results suggest that DNA methylation analysis might be a more suitable diagnostic tool than IHC for these genes. To further prove the clinical utility of this marker panel on early prediction for gastric carcinoma metastasis, a prospective follow-up study among patients with nonmetastatic gastric carcinoma is being conducted.

In conclusion, through a comprehensive and collaborative epigenetic biomarker discovery effort, we have demonstrated that the DNA methylation changes of *GFRA1*, *SRF*, and *ZNF382* were coordinately associated with gastric carcinoma metastasis and overall patient survival, and this three-gene panel has potential to be used as a synergistic biomarker set capable of improving the prognosis and treatment for patients with gastric carcinoma.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Authors' Contributions

Conception and design: Y. Gao, H. Shi, D. Deng
Development of methodology: Z. Liu, J. Zhang, Y. Gao, L. Pei, B. Zhu, H. Shi, D. Deng
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Z. Liu, J. Zhang, Y. Gao, L. Pei, J. Zhou, L. Gu, B. Zhu, N. Hattori, J. Ji, W. Kim, T. Ushijima, H. Shi
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Z. Liu, J. Zhang, Y. Gao, B. Zhu, W. Kim, H. Shi, D. Deng
Writing, review, and/or revision of the manuscript: Z. Liu, Y. Gao, W. Kim, H. Shi, D. Deng
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J. Zhang, Y. Gao, L. Gu, L. Zhang, B. Zhu, J. Ji, W. Kim, H. Shi, D. Deng
Study supervision: Y. Gao, B. Zhu, Y. Yuasa, H. Shi, D. Deng

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Data and materials availability

The methylation array data have been deposited into the Gene Expression Omnibus under accession number GSE47724. Supplementary Data Files S1–S3 are available upon request.

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