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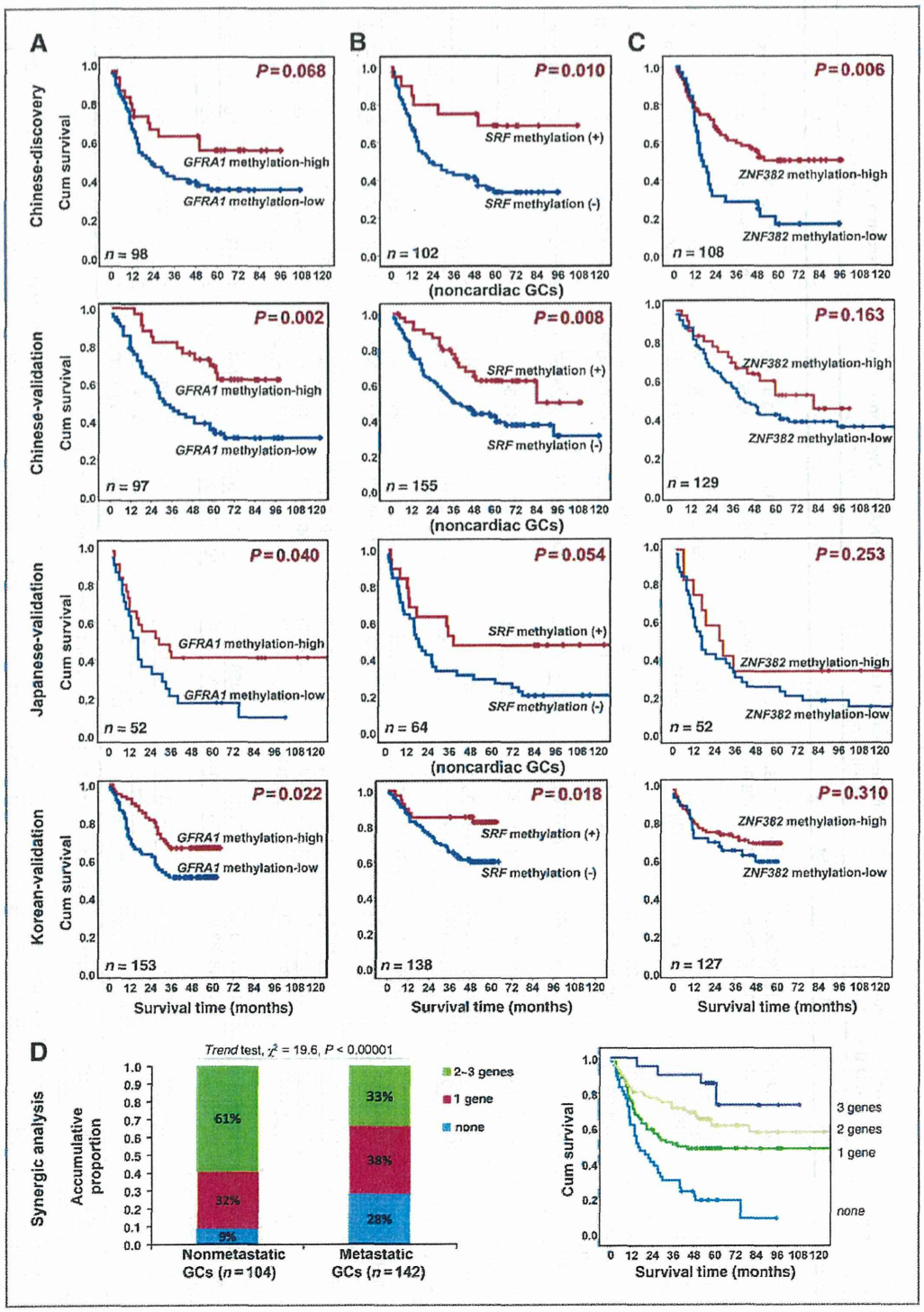

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sequencing results were consistently in agreement with the DHPLC analysis (Fig. 2B and Supplementary Fig. S3). In addition, quantitative MethyLight assays using fresh or formalin-fixed paraffin-embedded tissue samples further validated the DHPLC results (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

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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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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CASE REPORT

Hereditary diffuse gastric cancer in a Japanese family with a large deletion involving *CDH1*

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Abstract Hereditary diffuse gastric cancer (HDGC), characterized by susceptibility to gastric signet ring cell carcinomas (SRCCs) and caused by *CDH1* germline mutations, is rare in the Japanese. We present here a Japanese family with HDGC identified by comparative genomic hybridization (CGH) analysis. A 55-year-old woman was treated with completion gastrectomy for multiple SRCCs, and pathological examination revealed approximately 200 foci of SRCC with loss of E-cadherin expression. Her 30-year-old son had surveillance endoscopy and was found to have multiple SRCCs. He underwent total gastrectomy, and 32 foci of SRCC with loss of E-cadherin expression were histologically found. Although no point mutations were detected in *CDH1* by sequencing, CGH revealed a 275-kb deletion involving exons 7–16 of *CDH1* in both patients. While only a few HDGCs have been reported in East Asia, patients with multiple SRCC may need to be offered appropriate genetic counseling and testing in this area.

Keywords Hereditary diffuse gastric cancer · Gastric cancer · E-cadherin · Comparative genomic hybridization

Introduction

Hereditary diffuse gastric cancer (HDGC) is an autosomal dominant disease associated with multiple signet ring cell carcinomas (SRCCs) and is caused by a germline mutation in the E-cadherin gene (*CDH1*). Guilford et al. [1] first reported that HDGC is caused by truncating mutations of *CDH1* in New Zealand Maori families with early-onset, multiple SRCCs. In Western countries, inherited gastric cancers (GCs) are thought to account for 1–3 % of all GCs [2]. Clinically, approximately 25 % of families fulfilling the criteria for the diagnosis of HDGC have inactivating *CDH1* germline mutations [2, 3]. In contrast, in East Asian countries, including Japan, Korea, and China, with high incidences of GCs [4], HDGC has rarely been reported [5, 6]. Recently, Yamada et al. [7] reported two germline alterations in the *CDH1* gene in two Japanese familial GCs. However, HDGC is still rarely diagnosed in East Asian countries, and it is still unknown whether HDGC is really rare or overlooked because of the high incidence of coincidental familial GC. We report here clinical characteristics and genomic analysis of a Japanese HDGC family with a *CDH1* germline mutation.

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Case reports

A 55-year-old female patient was referred to our hospital for treatment of multiple SRCCs detected by endoscopic examination during an annual health check. She had a past history of intramucosal SRCC at the age of 34 and had undergone distal gastrectomy with *Billroth I* reconstruction in another hospital. She had no past history of other malignancies, including lobular breast cancer. Family history of GC was noted, affecting the patient's father and paternal grandfather (Fig. 1). Esophagogastroduodenoscopy (EGD) in our hospital detected 10–12 small pale mucosal patches, mainly in the greater curvature of the remnant stomach (Fig. 2a), but no atrophic gastritis indicative of *Helicobacter pylori* infection. The size of each focus was endoscopically estimated as less than 10 mm. In one of the largest lesions, narrow band imaging with magnification showed a wavy-shaped irregular microvessel pattern suggesting undifferentiated adenocarcinoma (Fig. 2b). All the biopsy specimens from six lesions demonstrated SRCC. Completion gastrectomy was performed, and the entire gastric mucosa was histologically examined.

Histopathological examination revealed approximately 200 SRCC foci, and their maximum size was 10 mm in diameter (Fig. 2f). None of the lesions showed submucosal invasion, and tumor cells were mostly confined to the upper mucosal layer (Fig. 2c). Periodic acid-Schiff (PAS) staining highlighted intracytoplasmic mucin in SRCC (Fig. 2d).

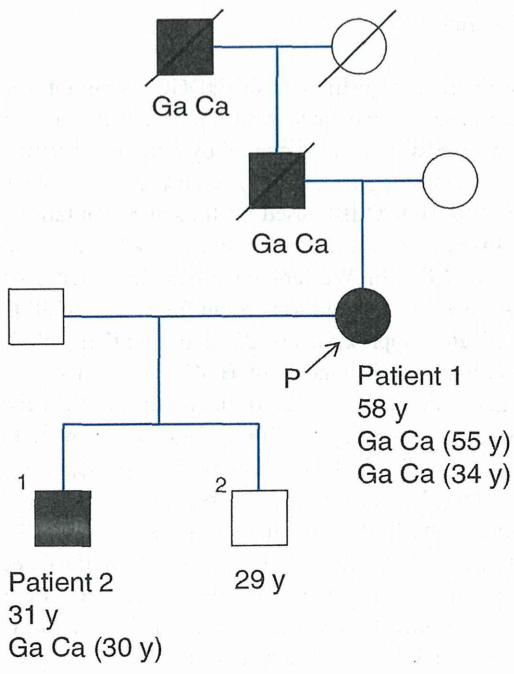

Fig. 1 Pedigree chart. The presence of the deletion mutation (Fig. 4) was confirmed in patients 1 and 2 in two generations

Immunohistochemistry was performed using anti-E-cadherin antibody (NCH-38; 1:100 dilution; DAKO, Glostrup, Denmark) as previously described [8]. The results showed loss of E-cadherin expression in SRCCs. (Fig. 2e). These histopathological findings were exactly identical to the findings in the HDGC cases reported by Guilford et al. [1]. No lymph node metastasis was observed.

Based on the pathological findings of the proband (patient 1), we suspected that she might have HDGC. Therefore, we performed surveillance endoscopy of two sons of the proband. Neither son had a history of malignancy. EGD for the 30-year-old elder son (Patient 2) detected three tiny pale areas at the body-antrum junction of the stomach (Fig. 3a, b). Apart from these tiny pale patches, no other endoscopic findings were suggestive of SRCC, but biopsy specimens obtained from each of the three pale areas revealed SRCC. Based on these findings, we clinically diagnosed HDGC in both patients, and total gastrectomy was performed on patient 2. Histopathological assessment of the entire gastric mucosa revealed a total of 32 SRCCs (Fig. 3f) along with loss of E-cadherin expression in SRCCs (Fig. 3c, d, e). No apparent findings of recurrence or distant metastasis have been noted on the follow-up thus far. EGD in the younger son identified no significant endoscopic findings.

The presence of a 275-kb deletion involving *CDH1*

In order to perform genetic analysis, genomic DNA was extracted from peripheral leukocytes of patient 1, patient 2, and a healthy volunteer. Genomic DNA was also extracted from biopsy specimens obtained from the cancer site(s) in the stomachs of both patients. All 16 exons of *CDH1* were sequenced by PCR-direct sequencing from both directions using previously reported primers [9]. Promoter methylation was analyzed by bisulfite modification and quantitative real-time methylation specific PCR (MSP) as previously described [10]. Primer sequences for the methylated DNA were 5′-TCG TTT TGG GGA GGG GTT C-3′ (forward) and 5′-CAA ATA AAC CCC GAA AAC ACC G-3′ (reverse), and those for the unmethylated DNA were 5′-GGA GGT ATT GTT TTT TGT ATT-3′ (forward) and 5′-AAC AAA CCA TCA ACT CCA-3′ (reverse). However, no *CDH1* mutation or aberrant methylation was detected in peripheral lymphocytes or biopsy specimens of patient 1.

Array-comparative genomic hybridization (CGH) analysis was performed according to the manufacturer's protocol using genomic DNA of peripheral leukocytes and human reference DNA (Caucasian, male #5190-4370, female #5190-4371, Agilent Technologies, Santa Clara, CA). DNA was digested with *Alu*I and *Rsa*I, labeled with Cy5 and Cy3, respectively, using a SureTag DNA

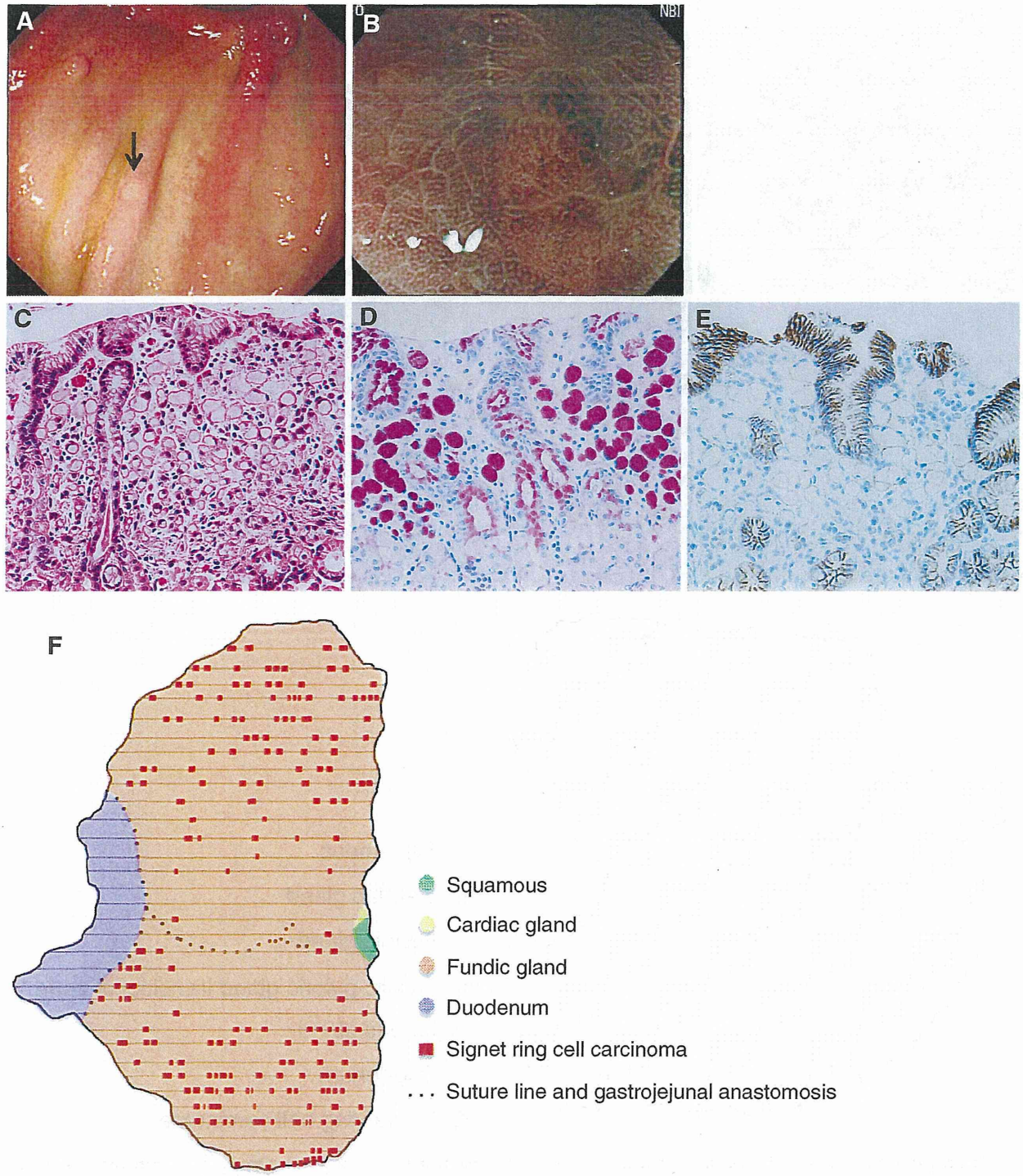

Fig. 2 Clinicopathological findings in patient 1. **a** Conventional endoscopy revealed pale lesions in the greater curvature of the remnant stomach (*arrow*). **b** Magnifying endoscopy with narrow-band imaging showed irregular microvessels. **c** Histology of the tumor showing SRCC proliferating in the upper layer of the fundic gland mucosa. **d** Periodic acid-Schiff (PAS) staining highlighted intracytoplasmic mucin in SRCC. **e** Immunohistochemistry for E-cadherin. Although normal gastric epithelial cells showed clear membrane staining, SRCCs showed loss of immunoreactivity. **f** Gastrectomy mapping study. Approximately 200 SRCC foci were observed in the resected specimen, predominantly near the greater curvature