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ORIGINAL ARTICLE

FHL1 on chromosome X is a single-hit gastrointestinal tumor-suppressor gene and contributes to the formation of an epigenetic field defect

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Tumor-suppressor genes on chromosome X can be inactivated by a single hit, any of the point mutations, chromosomal loss and aberrant DNA methylation. As aberrant DNA methylation can be induced frequently, we here aimed to identify a tumor-suppressor gene on chromosome X inactivated by promoter DNA methylation. Of 69 genes on chromosome X upregulated by treatment of a gastric cancer cell line with a DNA-demethylating agent, 5-aza-2′-deoxycytidine, 11 genes had low or no expression in the cell line and abundant expression in normal gastric mucosae. Among them, *FHL1* was frequently methylation-silenced in gastric and colon cancer cell lines, and methylated in primary gastric (21/80) and colon (5/50) cancers. Knockdown of the endogenous *FHL1* in two cell lines by two kinds of shRNAs significantly increased cell growth *in vitro* and sizes of xenografts in nude mice. Expression of exogenous *FHL1* in a non-expressing cell line significantly reduced its migration, invasion and growth. Notably, a somatic mutation (G642T; Lys214Asn) was identified in one of 144 colon cancer specimens, and the mutant *FHL1* was shown to lack its inhibitory effects on migration, invasion and growth. *FHL1* methylation was associated with *Helicobacter pylori* infection and accumulated in normal-appearing gastric mucosae of gastric cancer patients. These data showed that *FHL1* is a methylation-silenced tumor-suppressor gene on chromosome X in gastrointestinal cancers, and that its silencing contributes to the formation of an epigenetic field for cancerization.

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INTRODUCTION

Inactivation of tumor-suppressor genes is deeply involved in cancer development and progression.[1] The vast majority of tumor-suppressor genes are somatically inactivated by two hits of both alleles by genetic and/or epigenetic mechanisms, such as point mutations, chromosomal deletions and aberrant DNA methylation of promoter CpG islands (CGIs).[2,3] The two-hit theory makes tumor-suppressor genes on chromosome X unique because they can be inactivated by a single hit, and thus are 'risky' genes. So far, three examples have been identified, including *WTX* in Wilms tumors,[4] *FOXP3* in breast and prostate cancers[5,6] and *PHF6* in T-cell acute lymphoblastic leukemia (T-ALL),[7] all of which are inactivated by a point mutation or chromosomal loss.

Among the mechanisms of tumor-suppressor gene inactivation, aberrant DNA methylation can be present not only in tumor tissues but also in normal-appearing tissues, such as non-cancerous tissues of gastric,[8,9] colon,[10] liver,[11] esophageal,[12–14] breast[15] and renal cancer patients.[16] Levels of aberrant DNA methylation in non-cancerous tissues correlate with cancer risk clearly for gastric cancers[8,17] and other cancers, and accumulation of aberrant DNA methylation in a tissue is considered to form an epigenetic field for cancerization (epigenetic field defect).[18] Such association has been analyzed using methylation levels of marker genes, which are methylated in association with various tumor-suppressor genes and show much higher levels, and only a limited number of genes that functionally contribute to the field defect have been identified.

To identify risky genes that contribute to the formation of an epigenetic field defect, we here searched for genes on chromosome X from the 495 genes whose expression was upregulated fourfold or more after treatment with a DNA-demethylating agent, 5-aza-2′-deoxycytidine (5-aza-dC)[19] of a gastric cancer cell line (AGS), which is known to have very frequent methylation of CGIs.[20]

RESULTS

Screening of methylation-silenced genes on chromosome X

Among the 495 genes whose expression was upregulated fourfold or more by treatment of the AGS gastric cancer cell line with 5-aza-dC, 69 genes were located on chromosome X. Among the 69 genes, 11 genes had low expression (signal intensity <200) in non-treated AGS cells and had high expression (signal intensity >500) in a pool of gastric mucosae of three healthy volunteers.

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Genomic structures were analyzed for these 11 genes, and eight of them had CGIs in their promoter regions (Supplementary Table 1). Their mRNA expression levels were confirmed by quantitative reverse transcription–PCR (qRT–PCR) in non-treated AGS cells and gastric epithelial cells obtained by the gland isolation technique, and five (*MAOA, CXorf26, FHL1, SMARCA1* and *MAOB*) had consistent expression in gastric epithelial cells (Supplementary Table 1). Among the five genes, we focused on the *FHL1* gene, because it was reported to be able to inhibit growth, migration, invasion and metastasis of multiple types of cancer cells.[21–26] The other four genes were not reported to be involved in cancer development in the literature.

Promoter methylation and silencing of *FHL1* in gastrointestinal cancer cell lines

DNA methylation status of the *FHL1* promoter region was analyzed using two sets of methylation-specific PCR (MSP) primers designed to cover a region from the transcription start site to 220 bp upstream (Figure 1a). Among the 73 cancer cell lines analyzed (11 gastric, 7 colon, 12 lung, 12 skin, 7 pancreas, 4 esophageal, 4 prostate, 6 breast and 10 ovary cancer cell lines; Supplementary Table 2), *FHL1* was completely methylated (no unmethylated DNA molecules detected) in seven gastric, three colon (Figure 1b) and one lung cancer cell lines. In normal-appearing gastric and colonic mucosae, and peripheral leukocytes of healthy volunteers, *FHL1* was completely unmethylated in males, and partially methylated in females (Figure 1c). The partial methylation in females was considered to reflect methylation of the inactive chromosome X, which is shown later.

The role of the promoter methylation in downregulation of *FHL1* expression was analyzed. First, an association between the methylation and loss of expression was confirmed among the 11 gastric and 7 colon cancer cell lines. *FHL1* was consistently unexpressed in seven gastric and three colon cancer cell lines with its complete methylation (Figures 2a and b), but was expressed in most of the cancer cell lines without methylation, in normal colonic epithelial cells (CRL1790 and CRL1831) and in normal-appearing gastric and colonic mucosae. Second, when promoter methylation was removed by 5-aza-dC treatment of AGS and

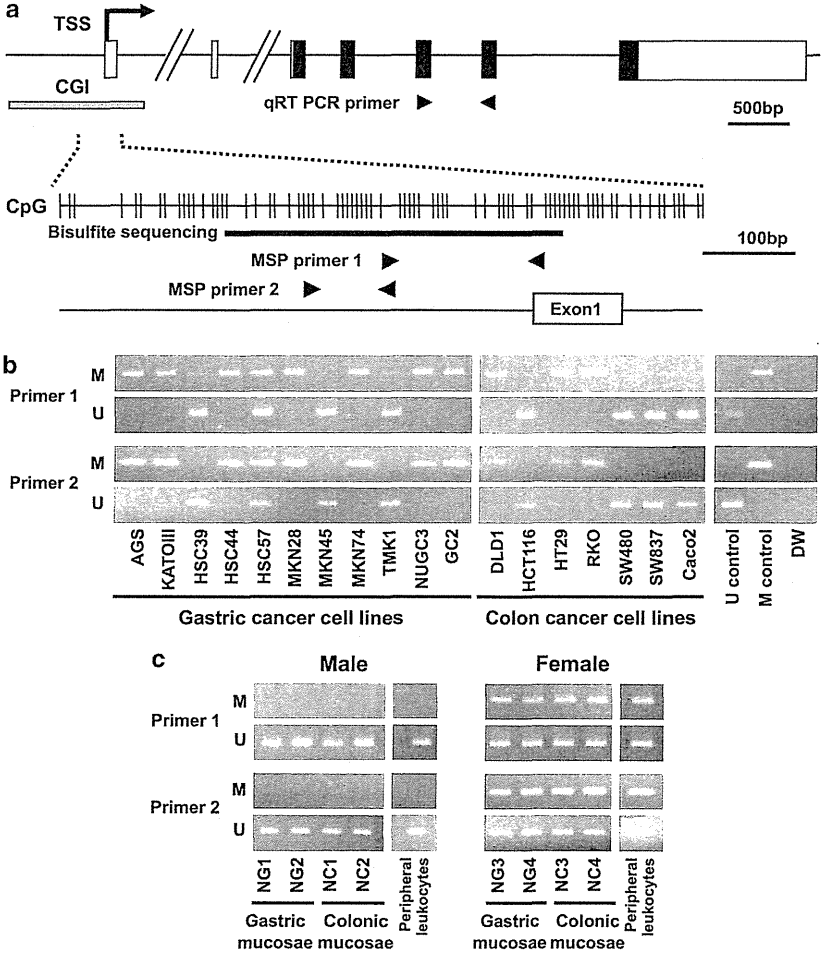

Figure 1. Genomic structure of *FHL1* and its methylation status in cancer cell lines, normal-appearing mucosae and peripheral leukocytes. (**a**) Genomic structure of *FHL1* and a CpG map of its promoter CGI. Open box, non-coding exon; closed box, coding exon; arrow, transcription start site (TSS); gray box, CGI region; vertical lines, individual CpG sites; arrowheads, primers for qRT–PCR and MSP; and bold line and number, the region and individual CpG sites analyzed by bisulfite sequencing. (**b**) Promoter methylation of *FHL1* in 11 gastric and seven colon cancer cell lines analyzed by MSP. M and U, primer sets specific to methylated and unmethylated DNA, respectively; U control, fully unmethylated genomic DNA; and M control, fully methylated genomic DNA. *FHL1* was frequently methylated in gastric and colon cancer cell lines. (**c**) Promoter methylation of *FHL1* in male and female normal-appearing gastric and colonic mucosae and peripheral leukocytes. *FHL1* was completely unmethylated in males and partially methylated in females.

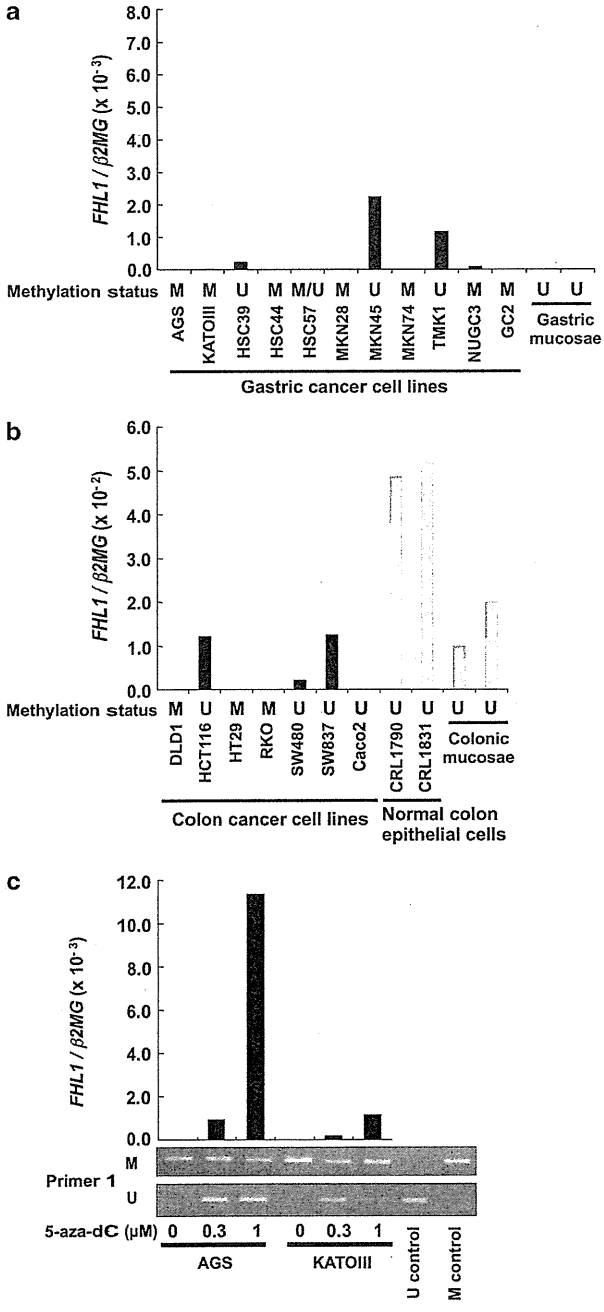

Figure 2. Methylation-silencing of *FHL1* in gastrointestinal cancer cell lines. (**a**) qRT–PCR of *FHL1* in gastric cancer cell lines and normal-appearing gastric mucosae. Results of MSP in Figure 1b are shown by M, M/U and U. M, only methylated DNA detected; M/U, both methylated and unmethylated DNA detected; and U, only unmethylated DNA detected. *FHL1* was not expressed in cell lines with complete methylation. (**b**) qRT–PCR of *FHL1* in colon cancer cell lines, normal colonic epithelial cells and normal-appearing colonic mucosae. *FHL1* was not expressed in cell lines with complete methylation. (**c**) Re-expression and demethylation of *FHL1* after 5-aza-dC treatment of AGS and KATOIII. *FHL1* expression was induced, along with its demethylation, after treatment with 5-aza-dC. U control, fully unmethylated genomic DNA; and M control, fully methylated genomic DNA.

KATOIII gastric cancer cell lines, *FHL1* expression was restored (Figure 2c). These data demonstrated that promoter methylation of *FHL1* caused its silencing.

Methylation of *FHL1* in surgical gastrointestinal cancer specimens

FHL1 methylation in surgical cancer specimens was analyzed by quantitative real-time MSP (qMSP) of 80 gastric and 50 colon cancers derived from male patients (Figure 3a). We adopted a cutoff value of 6%, which was previously determined based on the lowest methylation levels of tumor-suppressor genes in cancer samples,[9,27] and was also used in other researchers' report.[28] *FHL1* was methylated in 21 of the 80 (26%) gastric cancers and 5 of the 50 (10%) colon cancers. The presence of dense methylation of the promoter region was confirmed by bisulfite sequencing, and the fraction of densely methylated DNA molecules was in accordance with the methylation level obtained by qMSP (Figure 3b).

Association between promoter methylation and decreased expression was analyzed in 33 cancer specimens for which RNA was available. The mean *FHL1* expression level of 11 cancers with methylation was significantly lower than that of 22 cancers without methylation ($P=0.04$) (Figure 3c). Considering that surgical cancer specimens are contaminated with normal cells, the findings here supported that *FHL1* was methylation-silenced also in surgical cancer specimens.

Association between *FHL1* methylation and the CpG island methylator phenotype

Clinicopathological characteristics of cancers with *FHL1* methylation were analyzed in the 80 gastric cancers. *FHL1* methylation was not associated with tumor invasion, lymph node metastasis and histological type (Table 1). In contrast, *FHL1* methylation was associated with the presence of the CGI methylator phenotype (CIMP), 17 of 21 cancers with *FHL1* methylation (81%) and 13 of 59 without being CIMP-positive (22%; $P=2.9\times10^{-6}$). *FHL1* methylation was associated with the presence of Epstein–Barr virus (EBV) infection ($P=0.02$), but not with *hMLH1* methylation. This suggested that, between the two subtypes of CIMP-positive gastric cancers (those with EBV infection and those with *hMLH1* methylation),[29] *FLH1* methylation was associated with the former.

Growth-suppressive activity of *FHL1*

The effect of the *FHL1* expression loss on cell growth was analyzed by knocking down *FHL1* first *in vitro*. Two *FHL1*-specific shRNAs (sh1and sh2), along with a control shRNA (luciferase-specific shRNA; Luc-sh), were introduced into two cancer cell lines with *FHL1* expression (HCT116 and HSC39). *FHL1* expression was confirmed to be strongly suppressed by sh1 (11.7% of the control cells) and sh2 (14.8%) by qRT–PCR and also by western blot (Figure 4a). *FHL1* knockdown accelerated cell growth in HCT116 cells (sh1, 243% of control cells at 120 h, $P<0.001$, and sh2, 191%, $P<0.001$) and in HSC39 cells (sh1, 144% of control cells at 96 h, $P<0.01$, and sh2, 130%, $P<0.01$) (Supplementary Figure 1). Then, *in vivo* growth assay using a nude mouse xenograft model showed that HCT116 cells with *FHL1* knockdown formed 2.7-fold larger tumors than control cells (Luc-sh) ($P<0.001$) (Figure 4b), and that their mean weight was 2.8-fold heavier than that of control cells (Figure 4c). The maintenance of *FHL1* decrease by shRNA was confirmed (Supplementary Figure 2).

The growth-suppressive activity was further analyzed by expressing exogenous *FHL1* in two non-expressing cell lines (AGS and MKN28). By qRT–PCR and western blot, expression levels of the exogenous *FHL1* in AGS and MKN28 were shown to be ~10-and 40-fold, respectively, of those in non-cancerous gastric mucosae (Figures 4d and 5a, and Supplementary Figure 3a). *FHL1* expression reduced the cell growth in AGS (72.2% of control

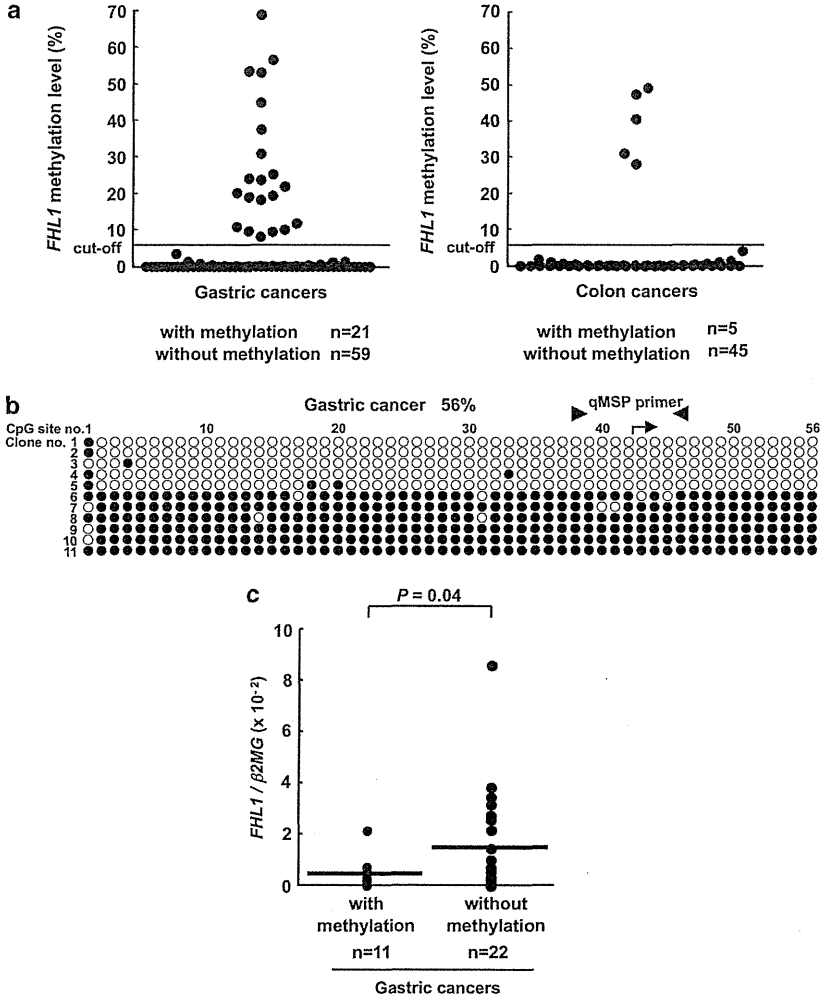

Figure 3. Methylation of *FHL1* in surgical gastrointestinal cancer specimens and its effect on expression. (**a**) Methylation levels in gastric (left) and colon (right) cancers derived from male patients. A horizontal line shows a cutoff value of 6%. *FHL1* was methylated in 21 of 80 primary gastric cancers and 5 of 50 colon cancers, respectively. (**b**) Confirmation of *FHL1* methylation by bisulfite sequencing. Fifty-six CpG sites were analyzed in a gastric cancer with a methylation level of 56%, and six of 11 DNA molecules were densely methylated. Closed circle, methylated CpG site; open circle, unmethylated CpG site; arrowheads, primers for qMSP; and arrow, transcription start site. (**c**) Decreased expression of *FHL1* in gastric cancers with methylation analyzed by qRT–PCR. A horizontal line represents the mean expression level in each group.

cells at 120 h, $P<0.05$; Figures 4d and 5b) but not in MKN28 (Supplementary Figure 3b).

Inhibitory effects of *FHL1* on migration and invasion

To clarify the mechanisms of how *FHL1* works as a tumor-suppressor gene, inhibitory effects of *FHL1* on cell migration and invasion were analyzed in two cell lines (AGS and MKN28). *FHL1* inhibited cell migration both in AGS (26.6% of control cells, $P<0.01$, Figure 5c) and in MKN28 (33.1% of control cells, $P<0.01$, Supplementary Figure 3c). In addition, *FHL1* inhibited cell invasion both in AGS ($P<0.05$, Figure 5d) and in MKN28 ($P<0.05$, Supplementary Figure 3d). In contrast, no induction of apoptosis was observed in AGS by terminal deoxynucleotidyl transferase dUTP nick end labeling assay (Supplementary Figure 4).

An *FHL1* mutation and its loss of function

FHL1 mutations were analyzed by sequencing its seven exons in 58 gastric and 144 colon cancer specimens derived from male patients. A somatic mutation (G642T; Lys214Asn) in exon 6 was identified in a colon cancer (Figure 5e). Also, a synonymous polymorphism (C450T) was observed in two gastric cancers. In the cancer with the G642T mutation, *FHL1* methylation was absent (data not shown), suggesting that either this mutation or promoter methylation was sufficient to inactivate *FHL1*. Further, the effects of the G642T mutation were analyzed by exogenously expressing the mutant and wild-type *FHL1* at similar levels (Figure 5a and Supplementary Figure 3a) in non-expressing AGS and MKN28 cells. The mutant *FHL1* lacked the inhibitory effects on migration and invasion both in AGS (Figures 5c and d) and in MKN28 (Supplementary Figures 3c and d). The mutant *FHL1* also lacked its inhibitory effect on cell growth in AGS (Figure 5b), whereas such effect could not be analyzed in MKN28, whose growth was not suppressed even by wild-type *FHL1*. These data indicated that the mutation was a loss-of-function mutation.

FHL1 methylation levels in non-cancerous gastric and colonic mucosae

To analyze the association between *FHL1* methylation and *Helicobacter pylori* (*H. pylori*) infection, and the contribution of

Table 1. Association between clinicopathological characteristics of patients and *FHL1* promoter methylation

Characteristics	*FLH1 methylation*		P
	Positive (N = 21)	*Negative (N = 59)*	
Tumor invasion			
⩽T2	13	33	0.80
>T2	8	26	
Lymph node metastasis			
Positive	15	50	0.20
Negative	6	9	
Histological type			
Intestinal	8	27	0.61
Diffuse	13	32	
CIMP			
Positive	17	13	2.9×10^{-6}
Negative	4	46	
EBV infection			
Positive	4	1	0.02
Negative	17	58	
hMLH1 *methylation*			
Positive	4	5	0.23
Negative	17	54	

Abbreviations: CIMP, CGI methylator phenotype; EBV, Epstein–Barr virus.

FHL1 methylation to the formation of an epigenetic field defect, *FHL1* methylation levels were quantified in gastric mucosae of male healthy volunteers (with and without *H. pylori* infection; 16 each) and non-cancerous mucosae of male gastric cancer patients (with and without *H. pylori* infection; 26 each) (Figure 6a). Among the healthy volunteers, *FHL1* methylation was elevated only in *H. pylori*-positive individuals (10 of 16, 62.5%; $P = 0.01$, *t*-test). As potent methylation induction by *H. pylori* can mask a difference in *H. pylori*-positive individuals,[8] *FHL1* methylation levels were compared between healthy volunteers and gastric cancer patients among the *H. pylori*-negative individuals. *FHL1* methylation level was shown to be elevated only in gastric cancer patients (5 of 26, 19.2%; $P = 0.09$, *t*-test). In the case of the colon, *FHL1* methylation was elevated in colonic mucosae of only 2 of 50 colon cancer patients (4%) (Supplementary Figure 5).

FHL1 methylation levels in female specimens

FHL1 methylation levels were analyzed in female specimens, including gastric mucosae of healthy volunteers (18 with *H. pylori* infection and 10 without), those of gastric cancer patients (7 with *H. pylori* infection and 11 without) and one specimen of peripheral leukocytes (Figure 6b). As in male specimens, among the healthy volunteers, *FHL1* methylation levels were significantly elevated in *H. pylori*-positive individuals ($P = 0.01$, *t*-test). Among the *H. pylori*-negative individuals, they tended to be higher in cancer patients than those in healthy volunteers ($P = 0.06$, *t*-test). *FHL1* methylation levels in *H. pylori*-negative female specimens were expected to be 50% because *FHL1* is located on chromosome X, but its actual distribution was between 20 and 40%. Bisulfite sequencing of the *FHL1* promoter region showed that female specimens contained DNA molecules with sparse methylation of CpG sites (Figure 6c), which was in contrast with the dense methylation in cancer specimens (Figure 3b). It was considered that the inactive chromosome X had sparse methylation of the *FHL1* promoter region not detected by qMSP.

DISCUSSION

The *FHL1* gene on chromosome X was shown to be a tumor-suppressor gene in gastrointestinal cancers by the presence of its methylation-silencing, its inhibitory effects on migration, invasion and growth, and the presence of a loss-of-function mutation. Notably, a loss-of-function mutation was identified for the first time in any type of cancers. This added *FHL1* as a new member of 'risky' tumor-suppressor genes on chromosome X, and the first tumor-suppressor gene on chromosome X that can be inactivated by methylation-silencing. *FHL1* methylation was associated with *H. pylori* infection and strongly accumulated in gastric mucosae of gastric cancer patients. Together with the fact that *FHL1* is a tumor-suppressor gene, the accumulation of *FHL1* methylation was considered to contribute to the formation of a field for cancerization as a driver.

Downregulation of *FHL1* in surgical specimens has been reported in breast, renal, prostate,[23] gastric,[25] liver,[21] and lung cancers.[22] The downregulation was associated with short patient survival and deep invasion in gastric cancers,[25] and with poor differentiation in lung cancers.[22] As a mechanism for the downregulation, methylation silencing was described in bladder cancers.[24] Functionally, *FHL1* has been reported to suppress growth of lung, liver and breast cancer cells and transformed fibroblasts,[21,22,26,30] and migration and invasion of bladder cancer cells and transformed fibroblasts.[24,26] The data obtained here were in line with previous reports, and demonstrated that *FHL1* inhibits migration and invasion in gastrointestinal cancer cells.[22]

Mechanistically, FHL1 is characterized by the presence of four and a half highly conserved LIM domains, which are involved in a wide range of protein–protein interactions, including actin cytoskeleton, cellular signaling proteins and transcriptional machinery.[31] In hepatocellular carcinomas, FHL1 was shown to interact with Smad2 and activate TGF-β pathway independently of TGF-β.[21] In breast cancers, FHL1 was shown to interact with estrogen receptor-α and estrogen receptor-β, and repress estrogen-responsive gene transcription.[30] Proteins that interact with FHL1 in gastric and colonic epithelial cells have not been clarified yet. However, inactivation of the TGF-β pathway is known to be involved in these cancers,[32] and is a strong candidate mechanism of how *FHL1* inactivation is involved in these gastrointestinal cancers.

FHL1 methylation was present not only in cancer tissues, but also in non-cancerous gastric mucosae of gastric cancer patients (5 of 26) and in non-cancerous colonic mucosae of colon cancer patients (2 of 50). This showed, for the first time in any types of cancers, that *FHL1* methylation silencing is involved in the formation of the epigenetic field defect as a driver. So far, only a limited number of driver genes, including *CDKN2A*, *CDH1* and *LOX*, are known to be involved in the formation of an epigenetic field defect.[18] For those genes on autosomes, it is difficult to estimate what fraction of cells has biallelic methylation. In contrast, in the case of *FHL1*, its methylation level linearly correlates with the fraction of cells with its inactivation, and, even if its methylation level is low, the presence of its methylation is expected to bring a significant impact. *H. pylori* infection is known to induce aberrant methylation that consists of temporary and permanent components,[8,33] and the high methylation levels in individuals with current *H. pylori* infection were in accordance with this previous finding.

In females, approximately half of the DNA molecules were methylated, densely or sparsely, in gastric mucosae and peripheral leukocytes of healthy volunteers without *H. pylori* infection by bisulfite sequencing. As no methylated DNA molecules were detected in a male specimen, both the densely and sparsely methylated DNA molecules in female specimens were considered to be derived from the inactive X allele.[34] However, we were not able to demonstrate it because a polymorphism that can

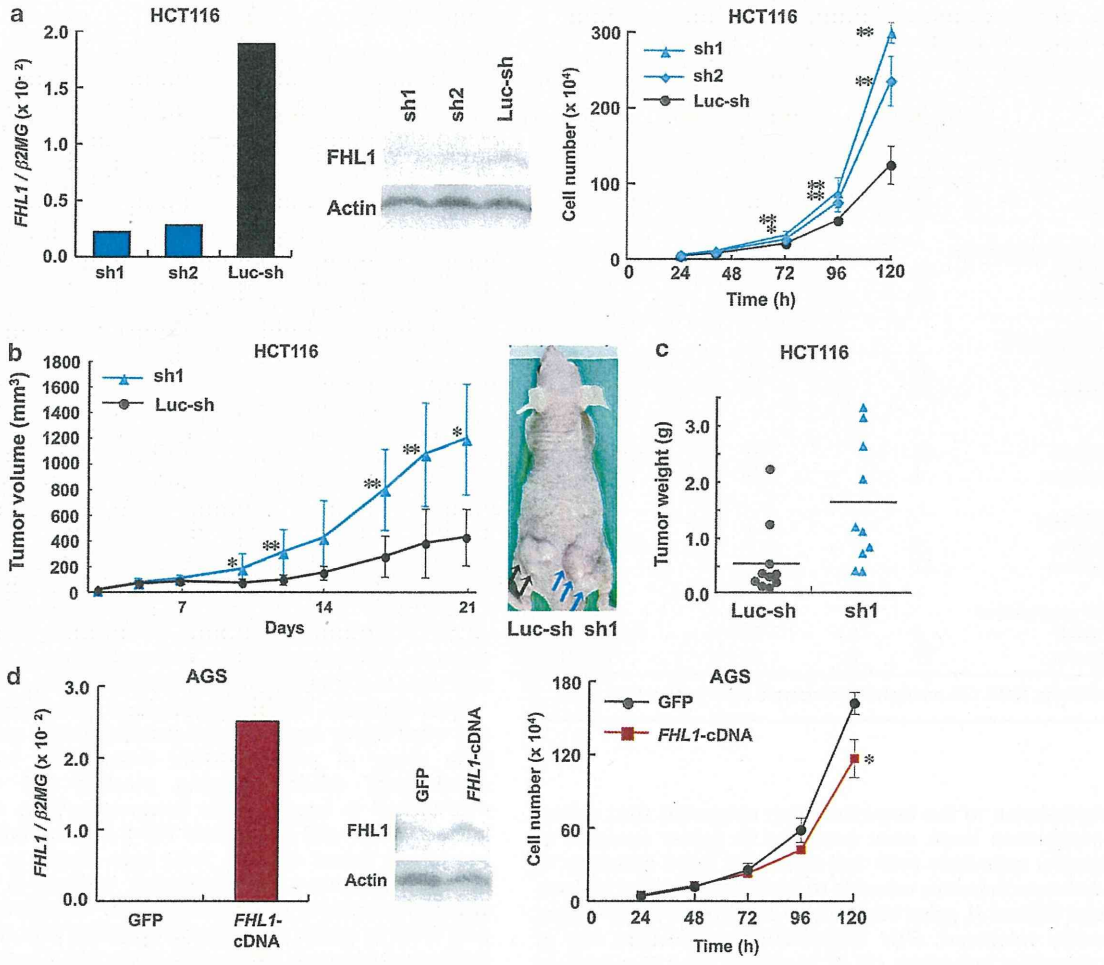

Figure 4. Growth-suppressive activity of *FHL1 in vitro* and *in vivo*. (**a**) *FHL1* knockdown and the resultant increased growth of HCT116 cells. Decreased expression of *FHL1* by its knockdown was confirmed by qRT–PCR (left) and western blot (middle). Growth rates of cells with *FHL1* knockdown were shown to be increased (*$P<0.01$, **$P<0.001$) (right). Data are shown as the mean of three independents ± s.d. (**b**) Increased *in vivo* growth of HCT116 cells with *FHL1* knockdown. Cells with *FHL1* knockdown (sh1) showed a 2.7-fold larger tumor volume compared with the control cells (Luc-sh) (*$P<0.01$, **$P<0.001$). Data are shown as the mean ± s.d. Arrows, tumors produced. (**c**) Increased tumor weight of cells with *FHL1* knockdown (sh1). Mean tumor weight of cells with knockdown (sh1) ($n=10$) was 2.8-fold heavier than that of controls (Luc-sh) ($n=10$). (**d**) Exogenous *FHL1* expression and the resultant decreased growth of AGS cells. Increased levels of *FHL1* expression were confirmed by qRT–PCR (left) and western blot (middle). Growth rates of cells with exogenous *FHL1* were shown to be significantly decreased (*$P<0.01$) (right).

distinguish the allelic origin of mRNA was not present. As qMSP detects only molecules that have dense methylation at primer sites, it was considered that it detected only densely methylated molecules, and methylation levels between 20 and 40% were observed in females.

In conclusion, we showed that *FHL1* on chromosome X is a methylation-silenced tumor-suppressor gene in gastrointestinal cancers, and its methylation in non-cancerous gastric mucosae contributes to the formation of an epigenetic field for cancerization.

MATERIALS AND METHODS

Cell lines and treatment with 5-aza-dC

Sixty-eight cancer cell lines (6 gastric, 7 colon, 12 lung, 12 skin, 7 pancreas, 4 esophageal, 4 prostate, 6 breast and 10 ovary cancer cell lines) and two normal colonic epithelial cells (CRL1790 and CRL1831) were obtained from the American Type Culture Collection (Manassas, VA, USA), Japanese Collection of Research Bioresources (Tokyo, Japan), RIKEN Cell Bank (Tsukuba, Japan) and Tohoku University Cell Resource Center for Biomedical Research (Sendai, Japan)(Supplementary Table 2). HSC39, HSC44 and HSC57 were gifted by Dr K Yanagihara; TMK1 was gifted by Dr W Yasui at Hiroshima University; and GC2 was established by MT For 5-aza-dC treatment. AGS and KATOIII cells were seeded on day 0; media containing freshly prepared 0.3 μM 5-aza-dC were added on days 1 and 3, and cells were harvested on day 5.[35]

Tissue specimens and analysis of *H. pylori* infection status

Cancer specimens were obtained from 80 male gastric cancer patients (average age = 60.4, range = 29–88) and 144 male colon cancer patients (average age = 70, range = 39–98) who underwent gastric and colon resection, respectively, with informed consent. All cancers were histologically diagnosed, and histological types of gastric cancers were classified according to the Lauren classification system (35 intestinal and 45 diffuse type).[36] EBV positivity was determined by *in situ* hybridization targeting *EBER1* using formalin-fixed and paraffin-embedded specimens.[37] The proportion of EBV-positive specimens (5 of 80, 6.3%) was close to EBV prevalence in a previous report (11 of 172, 6.4%).[38]

Normal-appearing gastric mucosae were obtained by endoscopic biopsy of the antral region from 60 healthy volunteers (32 male and 28 female; average age = 52, range = 25–91) and 70 gastric cancer patients

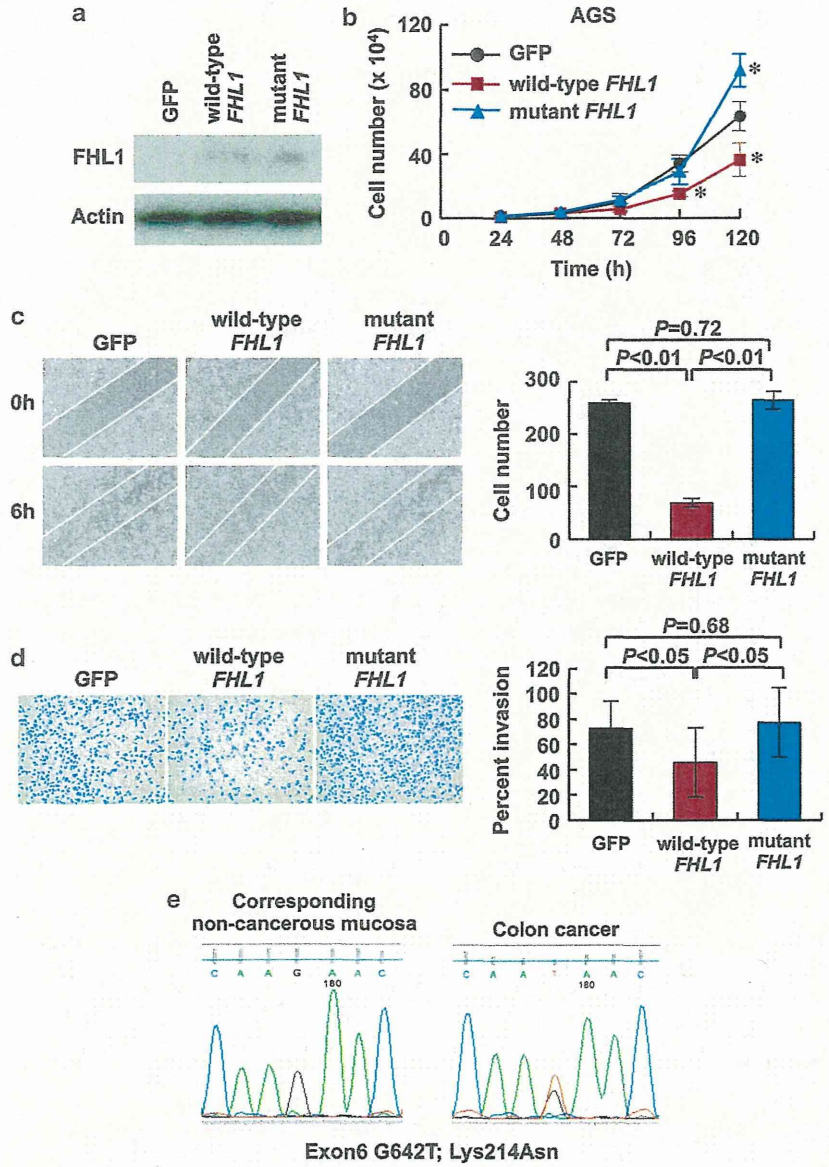

Figure 5. Inhibitory effects of *FHL1* on migration and invasion, and the lack of such functions in *FHL1* with the G642T mutation in AGS. (**a**) Expression levels of exogenous wild-type and mutant *FHL1* detected by western blot. (**b**) The growth-suppressive effect of the wild-type *FHL1*, and the lack of the effect in mutant *FHL1*. Whereas wild-type *FHL1* suppressed cell growth, mutant *FHL1* did not (*$P<0.01$). (**c**) Migration inhibition by wild-type *FHL1*, and the lack of the effect in the mutant *FHL1*. Whereas wild-type *FHL1* inhibited cell migration to 26.6% of the control cells, mutant *FHL1* did not. Photographs were taken at 0 and 6 h after scratching (left), and the number of cells that migrated into the scratched area was counted (mean ± s.d.; right). (**d**) Invasion inhibition by wild-type *FHL1*, and the lack of the effect in the mutant *FHL1*. Whereas wild-type *FHL1* inhibited cell invasion, mutant *FHL1* did not. Representative fields with invading cells on Matrigel-precoated membrane (left). Percent invasion is shown as the mean ± s.d. (right). (**e**) Sequence analysis of colon cancer specimens and corresponding non-cancerous colonic mucosae showed a somatic mutation (G642T; Lys214Asn) in exon 6 of *FHL1*.

(52 male and 18 female; average age = 65, range = 38–85). *H. pylori* infection status was analyzed by a serum anti-*H. pylori* IgG antibody test (SRL, Tokyo, Japan), rapid urease test (Otsuka, Tokushima, Japan) or culture test (Eiken, Tokyo, Japan). Gastric epithelial cells for qRT–PCR analysis were isolated by the gland isolation technique.[39] Normal-appearing colonic mucosae were obtained from a mucosal area distant from colon cancers of surgically resected specimens. Leukocytes were collected from one male (age = 47) and one female (age = 32) volunteer. Specimens were kept frozen at − 80 °C until DNA/RNA extraction. All the analyses using human-derived specimens were approved by the Institutional Review Boards.

Data processing of expression microarray analysis

Expression microarray analysis data in our previous report[19] were used. Signal intensities were scaled so that average signal intensity of all the 18 602 genes would become 500.

Sodium bisulfite modification, MSP, qMSP and bisulfite sequencing

Bisulfite modification was performed using 1 μg of *Bam*HI-digested genomic DNA as previously described.[40] MSP was performed with

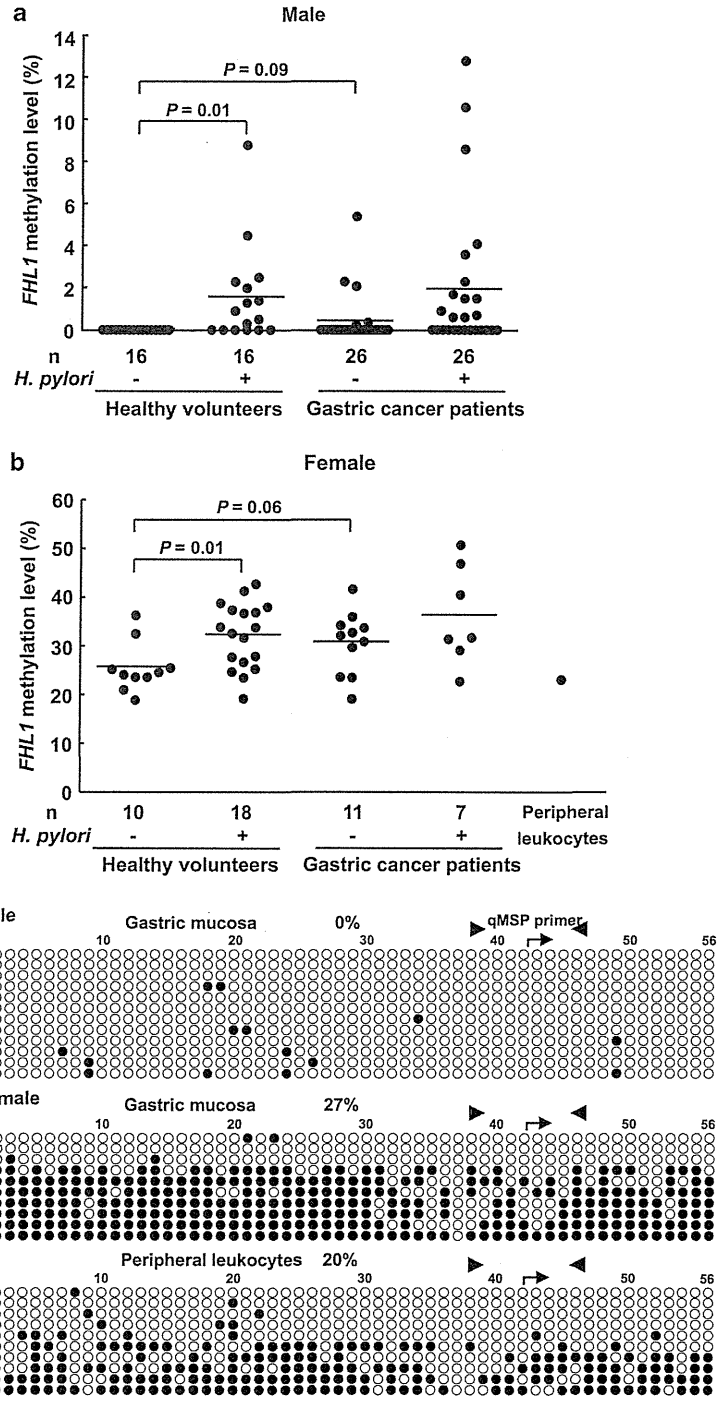

Figure 6. *FHL1* methylation levels in male and female gastric mucosae. (**a**) Methylation levels in male gastric mucosae of healthy volunteers and non-cancerous mucosae of gastric cancer patients. A horizontal line represents the mean methylation level for each group. Among healthy volunteers, *FHL1* methylation was present only in *H. pylori*-positive individuals ($P = 0.01$). Among individuals without *H. pylori* infection, *FHL1* methylation was present only in gastric cancer patients. (**b**) Methylation levels in female gastric mucosae and peripheral leukocytes. *FHL1* methylation levels distributed between 20 and 40%. Methylation levels were higher in *H. pylori*-positive healthy volunteers and gastric cancer patients also in female. (**c**) Bisulfite sequencing of male gastric mucosae, female gastric mucosae and female peripheral leukocytes. Female specimens contained both densely methylated and sparsely methylated DNA molecules, and it was considered that the inactive chromosome X can be densely and sparsely methylated. Closed circle, methylated CpG site; open circle, unmethylated CpG site; arrowheads, primers for qMSP; and arrow, transcription start site.

primer sets specific to methylated and unmethylated sequences (Supplementary Table 3). As controls, fully methylated and unmethylated DNA were prepared by methylating genomic DNA with *SssI* methylase (New England Biolabs, Beverly, MA, USA) and by amplifying genomic DNA with the GenomiPhi amplification system (GE Healthcare, Buckinghamshire, UK), respectively.

Quantitative real-time MSP was performed by real-time PCR using SYBR Green I (BioWhittaker Molecular Applications, Rockland, ME, USA) and an iCycler Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA). Although a primer set for MSP was also used for qMSP, a specific annealing temperature in the presence of SYBR Green I was determined (Supplementary Table 3). The number of molecules in a specimen was determined by comparing its amplification with those of standard DNA that contained known numbers of molecules (10^1–10^6 molecules). Based on the numbers of methylated (M) and unmethylated (U) molecules, a methylation level was calculated as the fraction of M molecules in the total number of DNA molecules (no. of M molecules + no. of U molecules). Standard DNA was prepared by cloning PCR products of methylated and unmethylated sequences into a vector (pGEM-T Easy, Promega, Madison, WI, USA). The CIMP status in a gastric cancer was determined as described previously.[27]

Bisulfite sequencing was conducted with primers common to methylated and unmethylated DNA sequences (Supplementary Table 4). The PCR product was cloned into pGEM-T Easy, and 10–12 clones were cycle-sequenced for each specimen.

qRT–PCR

cDNA was synthesized from 1 μg of total RNA using a Superscript III (Invitrogen, Carlsbad, CA, USA). qRT–PCR was performed by real-time PCR using SYBR Green I and an iCycler Thermal Cycler. Standard DNA was prepared by serial dilution of PCR products quantified by the QIAxcel system (QIAGEN, Valencia, CA, USA) after purification using Zymo-Spin I Columns (Zymo Research, Orange, CA, USA).[41] The measured number of cDNA molecules was normalized to that of b2-microglobulin (*b2MG*). The primers and PCR conditions are shown in Supplementary Table 5.

Knockdown and cDNA introduction assays

For a knockdown assay, two pairs and one pair of oligonucleotides were designed against *FHL1* and *Luciferase* (control), respectively (Supplementary Table 6). After annealing of sense and antisense oligonucleotides, the fragment was cloned into a pGreenPuro lentiviral vector (System Biosciences, Mountain View, CA, USA). For cDNA cloning, the entire coding region of human *FHL1* was amplified by RT–PCR (Supplementary Table 7), and cloned into a pCDH-CMV-MCS-EF1-Puro lentiviral vector (System Biosciences). As a control, *copGFP* was cloned into the vector in the same manner. The mutant cDNA was synthesized using the site-directed mutagenesis technique.[42] Using complementary primers carrying mutated sequence (mutation site forward and reverse primers; Supplementary Table 7) and primers for each end of the entire coding region (entire region reverse and forward primers), RT–PCR was performed to generate two DNA fragments that had overlapping ends. These two PCR products were combined by a subsequent PCR with primers for each end of the entire coding region to obtain the mutant cDNA. The mutant cDNA was cloned into a pCDH-CMV-MCS-EF1-Puro lentiviral vector.

The viral vectors and packaging vectors (pPACKH1 HIV Lentivector Packaging Kit, System Biosciences) were cotransfected into 293TN packaging cells, and culture media-containing pseudoviral particles were retrieved. Infection of cancer cell lines with pseudoviral particles was performed according to the manufacturer's protocol (System Biosciences), and stably expressing cells were selected by puromycin without cloning.

Cell growth, migration, invasion and apoptosis analysis

Cell growth was analyzed by seeding cells in triplicate in a six-well plate (3×10^4 cells, AGS; 1×10^5 cells, HSC39) and in a 12-well plate (5×10^3 cells, HCT116). Their numbers were counted at 24, 48, 72, 96 and 120 h. Three independent cultures were performed for one experiment.

Cell migration was analyzed by a wound-healing assay.[43] Cells were seeded in triplicate in a 6-cm dish coated with type I collagen (1×10^6 cells, AGS; 4×10^6 cells, MKN28), and cultured in RPMI-1640 medium containing 1% fetal calf serum to form a monolayer. The cell monolayer was scraped in a straight line with a pipette tip. After incubation for 6 and 12 h, the migrating cells were observed under bright-field microscopy. Three independent cultures were performed for one experiment.

Cell invasion was analyzed by a Matrigel invasion assay, using a Boyden chamber with the Matrigel-precoated membrane or Matrigel-free membrane in the top chamber (BD Biosciences, Bedford, MA, USA). Cells were seeded in top chambers in serum-free RPMI1640 (5×10^4 cells, AGS; 1×10^5 cells, MKN28), and the bottom chambers were filled with RPMI1640 containing 10% fetal calf serum. After incubation for 24 and 48 h (AGS and MKN28, respectively), the area of cells invading through the top chambers was measured by ImageJ software (version 1.38, National Institutes of Health, Bethesda, MD, USA). Percent invasion was calculated as the area of cells invading through the Matrigel-precoated membrane relative to those through Matrigel-free membrane. Three independent cultures were performed for one experiment and the experiment was repeated three times.

The apoptosis of the cells was analyzed by terminal deoxynucleotidyl transferase dUTP nick end labeling assay, using an *in situ* cell death detection kit, TMRred (Roche, Basel, Switzerland).

Tumor formation assay in nude mice

Cells (8×10^6 cells, HCT116) were inoculated subcutaneously on both flanks of 7-week-old male athymic nude mice (BALB/cAJc1-nu/nu; CLEA, Tokyo, Japan). Tumor sizes were measured with calipers every 3 days and the volume was calculated as (length × width^2) × 0.5, and tumor weights were measured at their killing on day 22. All the animal experiments were approved by the Animal Experiment Ethical Committee at the National Cancer Center.

Mutation analysis

All seven exons of *FHL1* were amplified using 100 ng of genomic DNA with primers located in introns, except for one primer on exon 7 (Supplementary Table 8). The PCR products were directly cycle-sequenced with a BigDye Terminator kit (PE Biosystems, Foster City, CA, USA) and an ABI PRISM 310 automated DNA sequencer (PE Biosystems).

Statistical analysis

Differences in mean methylation levels, expression levels, cell numbers and tumor sizes were analyzed by the Welch *t*-test. Association between *FHL1* methylation and clinicopathological factors was analyzed by the χ^2 test. All the analyses were performed using SPSS (SPSS, Inc., Chicago, IL, USA), and the results were considered significant when a P value < 0.05 was obtained by two-sided tests.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Comprehensive DNA methylation and extensive mutation analyses reveal an association between the CpG island methylator phenotype and oncogenic mutations in gastric cancers

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ABSTRACT

Recent development of personal sequencers for extensive mutation analysis and bead array technology for comprehensive DNA methylation analysis have made it possible to obtain integrated pictures of genetic and epigenetic alterations on the same set of cancer samples. Here, we aimed to establish such pictures of gastric cancers (GCs). Comprehensive methylation analysis of 30 GCs revealed that the number of aberrantly methylated genes was highly variable among individual GCs. Extensive mutation analysis of 55 known cancer-related genes revealed that 19 of the 30 GCs had 24 somatic mutations of eight different genes (*CDH1*, *CTNNB1*, *ERBB2*, *KRAS*, *MLH1*, *PIK3CA*, *SMARCB1*, and *TP53*). Integration of information on the genetic and epigenetic alterations revealed that the GCs with the CpG island methylator phenotype (CIMP) tended to have mutations of oncogenes, *CTNNB1*, *ERBB2*, *KRAS*, and *PIK3CA*. This is one of the first studies in which both genetic and epigenetic alterations were extensively analyzed in the same set of samples. It was also demonstrated for the first time in GCs that the CIMP was associated with oncogene mutations.

1. Introduction

Both genetic and epigenetic alterations are important for human carcinogenesis [1,2]. Genetic alterations are responsible for activation of oncogenes and inactivation of tumor-suppressor genes [2]. In human gastric cancers (GCs), oncogenes activated by mutations include *CTNNB1* (β-catenin), *ERBB2*, and *PIK3CA* [3–10], and tumor-suppressor genes inactivated by mutations include *CDH1* (E-cadherin), *CDKN2A* (*p16*), *TP53*, and *ARID1A* [11,12]. Even by whole exome sequencing of GCs, the vast majority of driver genes identified were known cancer-related genes, and novel genes identified, such as *ARID1A* and *FAT4*, had only low incidences of mutations [11,12]. This indicates that extensive mutation analysis of a large number of known cancer-related genes can provide an overall picture of a cancer sample, and this is now possible with high speed and low cost by using next-generation personal sequencers [13,14].

Epigenetic alterations, namely aberrant DNA methylation of promoter CpG islands (CGIs), are also responsible for inactivation of various tumor-suppressor genes [1]. DNA methylation statuses of the entire genome can be now comprehensively analyzed using microarray technologies, and bead array technology is especially useful for its quantitative measurement [15]. In GCs, tumor-suppressor genes inactivated by promoter methylation include *CDH1*, *CDKN2A*, *FHL1*, *LOX*, *MLH1*, and *SFRP* family genes (*SFRP1*, *SFRP2*, and *SFRP5*) [16–21]. These tumor-suppressor genes are more frequently inactivated by aberrant methylation than by genetic alterations in GCs [22]. In addition, aberrant methylation is induced in gastric mucosae by *Helicobacter pylori* (*H. pylori*)

Abbreviations: GC, gastric cancer; CGI, CpG island; *H. pylori*, *Helicobacter pylori*; CIMP, CpG island methylator phenotype; EB virus, Epstein–Barr virus; TSS, transcription start site; COSMIC, Catalogue Of Somatic Mutations In Cancer.

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infection [23,24], a well-established major inducer of human GCs [25]. The frequent inactivation of tumor-suppressor genes by aberrant methylation and the deep involvement of *H. pylori* infection in its induction indicate the importance of epigenetic alterations in GCs.

Not only in GCs but also in other types of cancers, a subgroup of cancers is known to have frequent aberrant DNA methylation of CGIs, referred to as the CpG island methylator phenotype (CIMP). The CIMP was first described in colorectal cancers [26], and is associated with unique clinicopathological features. For example, the CIMP is associated with poor prognosis in colorectal cancers, lung cancers, and neuroblastomas [27–29]. In contrast, depending on the number and set of genes used for the determination of the CIMP status, the CIMP can be associated with either poor or good prognosis in GCs [30–33]. The CIMP in specific cancers is associated with genetic alterations, such as mutations of *BRAF*, *KRAS*, and *PIK3CA* in colorectal cancers [34–37], and amplification of *ERBB2* in breast cancers [38]. In contrast, little is known on a specific association between the CIMP and genetic alterations in GCs.

In this study, we aimed to establish integrated pictures of genetic and epigenetic alterations of GCs. To this end, we conducted comprehensive analysis of DNA methylation statuses using bead array technology, and extensive analysis of mutations of 55 known cancer-related genes using a next-generation personal sequencer.

2. Materials and methods

2.1. Samples

Thirty GC samples were obtained from patients who underwent gastrectomy with informed consents. Three normal gastric mucosae samples were obtained endoscopically from healthy volunteers without *H. pylori* infection with informed consents. The study was approved by the Institutional Review Boards. The samples were stored in RNAlater (Life Technologies, Carlsbad, CA) at −80 °C until the extraction of genomic DNA (GC samples and normal gastric mucosae samples) and RNA (normal gastric mucosae samples). Clinical information of the 30 GCs is shown in Supplementary Table 1. The status of Epstein–Barr (EB) virus infection was evaluated by PCR using primers specific to genomic DNA of EB virus (forward, CGGTATTATGTTTTTGGTATGTGTA; reverse, ATAACAACAACGTCATAAAAACCAC), and no infection was present in the 30 GCs.

Genomic DNA was extracted from GC and normal gastric mucosae samples by the phenol/chloroform method, and was quantified by using a Quant-iT PicoGreen dsDNA Assay Kit (Life Technologies). Total RNA was isolated using ISOGEN (Nippon Gene, Tokyo, Japan).

2.2. Analysis of DNA methylation

Analysis of DNA methylation was performed using an Infinium HumanMethylation450 BeadChip array, which covered 482,421 CpG sites (Illumina, San Diego, CA) as described previously [39]. CpG sites with low signals (signal <500, 0.19–2.19% of total CpG sites) were excluded from further analyses. The methylation level of each CpG site was represented by β values which ranged from 0 (unmethylated) to 1 (fully methylated).

A total of 193,531 genomic "segments" were defined by their location against a transcription start site (TSS) [TSS1500 (regions between 200 bp upstream and 1500 bp upstream from TSS), TSS200 (200 bp upstream region from TSS), 5′-UTR, 1st exon, gene body, 3′-UTR, and intergenic regions] and their relative location against a CGI (N Shelf, N Shore, CGI, S Shore, S Shelf, and non-CGI). A genomic segment >500 bp was further divided into genomic "blocks". A genomic block was defined as a 500-bp region from an initial CpG site (probe), and the next genomic block started from the next CpG site (Supplementary Fig. 1). A genomic segment ⩽500 bp was counted as one genomic block. A total of 282,805 genomic blocks were produced, and 276,456 genomic blocks on autosomes were analyzed to enable comparison between males and females. A DNA methylation level of a genomic block was evaluated using the average of β value of the CpG sites within the block. A genomic block was considered as methylated when its β value was 0.4 or more, and as unmethylated when its β value was 0.2 or less.

2.3. Analysis of sequence variations

A library DNA containing 226 amplicons of 55 cancer-related genes was prepared from a sample by multiplex PCR using 50 ng of genomic DNA and an Ion *AmpliSeq* Cancer Panel Kit (Life Technologies) with 36 customized primers (Supplementary Table 2). The 226 amplicons covered the vast majority of samples with mutations reported (91.9% or more) for 15 oncogenes and the *TP53* tumor-suppressor gene (83.1%), and variable fractions of samples with mutations reported (3.3–88.5%) for 39 genes (Supplementary Table 3). Then, the entire library DNA was uniquely barcoded by using an Ion Xpress Barcode Adaptors 1-16 Kit (Life Technologies). The barcoded libraries from five to six samples were pooled, and mixed with Ion Spheres for emulsion PCR using the Ion OneTouch System (Life Technologies) with an Ion OneTouch Template Kit (Life Technologies). From the product of emulsion PCR, the complexes of Ion Spheres with amplified DNA were enriched by using Ion OneTouch ES (Life Technologies) and were loaded onto an Ion 316 chip (Life Technologies). Sequencing was performed by using Ion PGM Sequencer (Life Technologies) with an Ion Sequencing Kit (Life Technologies). Obtained sequences were mapped onto the human reference genome hg19, and sequence variations with frequencies of 10% or more were identified by using CLC Genomics Workbench 5.1 (CLC bio, Aarhus, Denmark). Common SNPs were excluded from further analysis. Reading depths of individual regions analyzed are shown in Supplementary Table 4.

2.4. Dideoxy sequencing

A region containing a sequence variation identified was amplified using 20 ng of genomic DNA with primers listed in Supplementary Table 5. The PCR product was purified by a DNA Clean and Concentrator-5 Kit (Zymo Research, Irvine, CA), and directly cycle-sequenced by using a DYEnamic ET Terminator Cycle Sequencing kit (GE Healthcare, Buckinghamshire, UK) and an ABI PRISM 310 automated DNA sequencer (PE Biosystems).

2.5. Analysis of gene expression by GeneChip oligonucleotide microarray

Gene expression levels in normal gastric mucosae were analyzed by using the GeneChip Human Genome U133 Plus 2.0 microarray (Affymetrix, Santa Clara, CA) as described [40]. Genes with signal intensities of 250 or more were defined as expressed genes.

2.6. Cluster analysis

Unsupervised hierarchical clustering analysis was performed by using R 2.15 [R Core Team (2012) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org/] with the Heatplus package [Alexander Ploner (2011) Heatplus: Heatmaps with row and/or column covariates and colored clusters, R package version 2.2.0.] from Bioconductor [41]. The Euclidean distance was used as distance function both for samples and genes. Due to the limitation in the calculation algorithm for the hierarchical clustering, 25,000 elements or less were analyzed.

2.7. Survival curve

Survival curves were analyzed using the Kaplan-Meier method, and the Kaplan-Meier curve was drawn by using SPSS 13.0J (SPSS, Chicago, IL, USA).

2.8. Statistical analysis

The association between the CIMP and oncogene mutations, and that between genes aberrantly methylated in GCs and target genes of polycomb repressive complex (PRC) 2 in human embryonic stem (ES) cells were tested by the chi-square test. The differences in the survival rates among groups were evaluated using the Mantel-Cox test.

3. Results

3.1. Comprehensive analysis of DNA methylation profiles

DNA methylation levels were compared between GCs and normal gastric mucosae. First, using all the 276,456 genomic blocks, some GCs, such as S24TP, S33TP, and S37TP, had a larger fraction of aberrantly methylated blocks than other GCs, such as S2TP, S4TP, and S15TP (Fig. 1 and Supplementary Fig. 2). Second, the analysis was conducted using 6877 TSS200 CGIs unmethylated in normal gastric mucosae (genes unmethylated in normal gastric mucosae) because a TSS200 CGI is known to play a critical role in methylation-silencing [42]. The number of aberrantly methylated genes ranged from three to 1211. Third, we focused on TSS200 CGIs of genes with positive expression in normal cells but aberrantly methylated in cancer cells because this group of genes is known to frequently contain driver genes in carcinogenesis [43]. Using 263 TSS200 CGIs whose downstream genes were expressed in

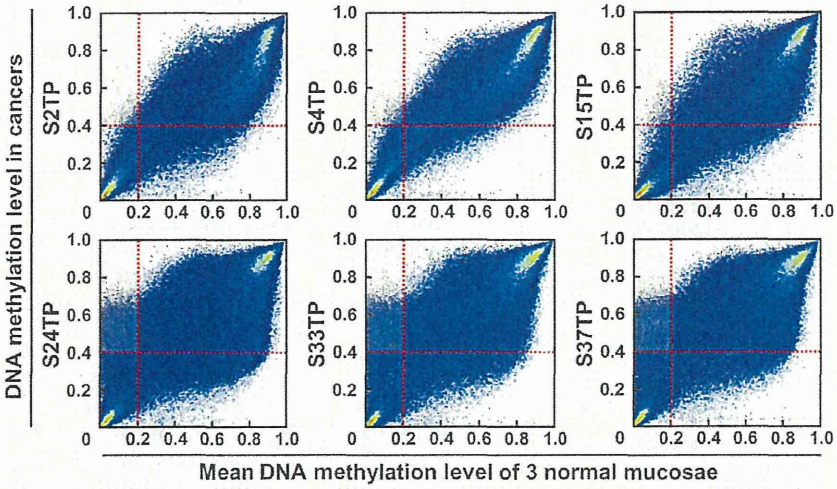

Fig. 1. Comprehensive analysis of DNA methylation profiles in GCs. DNA methylation levels were compared between GCs and normal gastric mucosae for the 276,456 genomic blocks. S24TP, S33TP, and S37TP (lower three panels) had a larger fraction of aberrantly methylated genes (yellow-colored areas) than S2TP, S4TP, and S15TP (upper three panels). The vertical and horizontal axes indicate the methylation levels in GCs and the mean methylation levels of three normal mucosae, respectively.

normal gastric mucosae and aberrantly methylated in one or more GCs (methylation-silenced genes), the number ranged from 0 to 166. These results showed that the number of aberrantly methylated genes was highly variable among individual GCs.

3.2. Extensive mutation analysis of the 55 cancer-related genes

Mutations were analyzed for the 55 cancer-related genes. Among the 30 GCs, 22 GCs had 30 sequence variations of at least one gene (Table 1 and Supplementary Table 6), and all the 30 sequence variations were confirmed by dideoxy sequencing (Supplementary Fig. 3). The confirmed sequence variations were analyzed whether or not they were somatic mutations using corresponding non-cancerous tissues. The 24 of the 30 sequence variations were shown to be somatic mutations (Fig. 2 and Table 1), and were present in 19 GCs. Among the 24 mutations, 22 were missense mutations, and two were nonsense mutations. Three GCs (S5TP, S13TP, and S33TP) had two or more mutations of different genes. Four oncogenes, *CTNNB1*, *ERBB2*, *KRAS*, and *PIK3CA*, and four tumor-suppressor genes, *CDH1*, *MLH1*, *SMARCB1*, and *TP53*, were mutated. *TP53* was most frequently mutated (43%, 13 of the 30 GCs), and *CTNNB1*, *ERBB2*, *KRAS*, and *PIK3CA* were mutated in two GCs. These results showed that 63% of GCs (19 out of the 30 GCs) had at least one somatic mutation of known cancer-related genes.

3.3. The association between the CIMP and mutations of oncogenes

Unsupervised hierarchical clustering analysis was conducted first using DNA methylation profiles of 25,000 genomic blocks randomly selected from all the 276,456 genomic blocks. However, the numbers of aberrantly methylated genes in GCs of different clusters did not appear to be different (Supplementary Fig. 4). Then, we again conducted unsupervised hierarchical clustering using DNA methylation profiles of CGIs, namely 25,000 genomic blocks randomly selected from 59,992 blocks with CGIs (Fig. 3A). This time, clusters I ($n = 3$) and IIb ($n = 13$) contained GCs with a larger number of aberrantly methylated genes than GCs in cluster IIa ($n = 14$). Among the 16 GCs in clusters I and IIb, seven GCs were shown to have mutations of oncogenes, *CTNNB1*, *ERBB2*, *KRAS*, and *PIK3CA*.

Thirdly, using DNA methylation profiles of 6877 genes unmethylated in normal gastric mucosae, two major clusters were observed (Fig. 3B). Cluster III ($n = 11$) contained GCs with a relatively large number of aberrantly methylated genes, and seven of the 11 GCs of this cluster were shown to have mutations of oncogenes, *CTNNB1*, *ERBB2*, *KRAS*, and *PIK3CA*. In contrast, cluster IV ($n = 19$) contained GCs with a relatively small number of aberrantly methylated genes, and none of the 19 GCs in this cluster had mutations of oncogenes. The difference was markedly statistically significant ($P = 7.15 \times 10^{-5}$), and GCs in cluster III and IV were considered to be the CIMP-positive [CIMP(+)] and the CIMP-negative [CIMP(−)], respectively.

Fourth, using DNA methylation profiles of the 263 methylation-silenced genes, three major clusters were produced (Fig. 3C). Cluster V ($n = 3$) contained GCs with the largest number of aberrantly methylated genes, and two of the three GCs were shown to have mutations of *PIK3CA*. Cluster VIa ($n = 8$) contained GCs with a relatively larger number of aberrantly methylated genes than GCs in cluster VIb ($n = 19$). Five of the eight GCs in this cluster were shown to have mutations of oncogenes, *CTNNB1*, *ERBB2*, *KRAS*. Clusters VIb contained the same sets of GCs as cluster IV, the previous clustering, except for one. These results showed that the CIMP(+) GCs were associated with mutations of oncogenes, such as *CTNNB1*, *ERBB2*, *KRAS* and *PIK3CA*, in GCs.

3.4. Possible association between the CIMP and good prognosis

To analyze an association between the CIMP status and prognosis of patients, Kaplan-Meier curves were drawn using overall survival (OS). Using the CIMP status based on the DNA methylation of the 6877 genes unmethylated in normal gastric mucosae, it was revealed that the prognosis of the CIMP(+) patients (Cluster III in Fig. 3B) tended to be better than that of the CIMP(−) patients (Cluster IV in Fig. 3B) ($P = 0.285$; Fig. 4). Also, using the CIMP status based on the methylation of the 263 methylation-silenced genes, the prognosis of the CIMP(+) patients (Cluster V and VIa in Fig. 3C) tended to be better than that of the CIMP(−) patients (Cluster VIb in Fig. 3C) ($P = 0.285$; Supplementary Fig. 5). These results suggested that the CIMP(+) status is possibly associated with good prognosis in GCs.

Table 1
List of somatic mutations identified in the 30 GCs.

Sample #	Sample name	Gene	Coverage	Variant frequencies	Nucleotide change	Amino acid change
1	S1TP	*CDH1*	339	10.3	c.1198G > A	p.Asp400Asn
2	S2TP	*TP53*	496	34.1	c.581T > G	p.Leu194Arg
3	S4TP	*TP53*	438	74.2	c.581T > G	p.Leu194Arg
4	S5TP	*KRAS*	1626	54.4	c.38G > A	p.Gly13Asp
		SMARCB1	50	56	c.1130G > A	p.Arg377His
5	S6TP	*TP53*	2077	24.7	c.820G > C	p.Val274Leu
6	S9TP			No mutation		
7	S11TP	*TP53*	10,211	53.4	c.844C > T	p.Arg282Trp
8	S12TP	*ERBB2*	24,516	63.8	c.2264T > C	p.Leu755Ser
9	S13TP	*TP53*	70	15.7	c.478A > G	p.Met160Val
		ERBB2	482	23.9	c.2264T > C	p.Leu755Ser
10	S14TP			No mutation		
11	S15TP	*TP53*	534	40.3	c.743G > A	p.Arg248Gln
12	S16TP	*TP53*	453	36.2	c.660T > G	p.Tyr220Ter
13	S17TP			No mutation		
14	S18TP	*TP53*	1946	26.5	c.844C > T	p.Arg282Trp
15	S19TP			No mutation		
16	S20TP			No mutation		
17	S22TP			No mutation		
18	S23TP	*TP53*	565	67.8	c.537T > A	p.His179Gln
19	S24TP			No mutation		
20	S32TP			No mutation		
21	S33TP	*MLH1*	4092	45.4	c.1744C > G	p.Leu582Val
		CTNNB1	11,994	20.5	c.101G > A	p.Gly34Glu
		PIK3CA	276	49.3	c.1633G > A	p.Glu545Lys
		TP53	1142	34.9	c.524G > A	p.Arg175His
22	S34TP	*TP53*	551	28.3	c.641A > G	p.His214Arg
23	S35TP	*KRAS*	770	41.3	c.35G > T	p.Gly12Val
24	S36TP	*TP53*	1142	34.9	c.524G > A	p.Arg175His
25	S37TP	*PIK3CA*	59	15.3	c.1624G > A	p.Glu542Lys
26	S40TP			No mutation		
27	S42TP			No mutation		
28	S43TP	*TP53*	239	74.9	c.1024C > T	p.Arg342Ter
29	S45TP			No mutation		
30	S47TP	*CTNNB1*	4591	33.7	c.121A > G	p.Thr41Ala

3.5. Association between the genes aberrantly methylated in GCs and genes targeted by PRC2 in ES cells

The fraction of genes targeted by PRC2 in ES cells was analyzed in the genes aberrantly methylated in GCs and those unmethylated in GCs because genes methylated in GCs were reported to be associated with PRC2 target genes [33]. Using the information on the PRC2 target genes in human ES cells [44,45], it was shown that the genes aberrantly methylated in GCs consisted of a larger fraction of PRC2 target genes than those unmethylated in GCs ($P = 6.64 \times 10^{-79}$) (Supplementary Fig. 6). These results confirmed that genes aberrantly methylated in GCs were associated with genes targeted by PRC2 in ES cells.

4. Discussion

In this study, we conducted comprehensive DNA methylation analysis and extensive mutation analysis of 30 GCs, and showed (1) that the number of aberrantly methylated genes was highly variable among the 30 GCs, (2) that 19 of the 30 GCs had 24 somatic mutations of 8 different genes (*CDH1*, *CTNNB1*, *ERBB2*, *KRAS*, *MLH1*, *PIK3CA*, *SMARCB1*, and *TP53*), and (3) that the CIMP was associated with mutations of oncogenes, including *ERBB2*, *CTNNB1*, *KRAS*, and *PIK3CA*, in GCs. This is one of the first studies in which both genetic and epigenetic alterations were extensively analyzed in the same set of samples, and the association between the CIMP and mutations of oncogenes in GCs was revealed here for the first time.

A similar association has been known also in colorectal cancers, but the mechanisms for this association are still unclear. As a possible mechanism, it has been proposed (1) that cancers with the CIMP can escape senescence caused by *BRAF* mutation owing to silencing of regulators of senescence by *BRAF* mutation, such as *IGFBP7* [46,47], and (2) that overexpression of the *BRAF* mutant can induce aberrant methylation at various genes, such as *MLH1* [48]. Similar possibilities can be hypothesized in GCs. As a mechanism for methylation induction by oncogenic mutation, if this applies to GCs, there is a possibility that oncogenic mutations displace factors involved in the susceptibility to methylation induction, such as RNA polymerase II [40,49–53].

Somatic mutations of four tumor-suppressor genes, *CDH1*, *MLH1*, *SMARCB1*, and *TP53*, and four oncogenes, *CTNNB1*, *ERBB2*, *KRAS*, and *PIK3CA*, were identified. Among these mutated genes, *TP53* (32%), *CDH1* (20%), *PIK3CA* (10%), *CTNNB1* (9%), *KRAS* (7%), and *ERBB2* (2%) are listed in the top 15 mutated genes in GCs in the Catalogue Of Somatic Mutations In Cancer (COSMIC) database. In contrast, mutations of *SMARCB1* have not been identified in GCs, even by whole exome sequencing [11,12], but were identified for the first time in this study, showing the usefulness of extensive mutation analysis of known cancer-related genes. *SMARCB1* encodes a component of chromatin remodeling complex, SWI/SNF, and is mutated in malignant rhabdoid tumors [54]. In GCs, the defects of components of SWI/SNF, such as mutation of *ARID1A* [11,12] and loss of BRM expression, are known [55]. Therefore, it is considered that the dysfunction of chromatin remodeling activity plays an important role in gastric carcinogenesis.

The selection of genomic blocks heavily influenced the results of unsupervised hierarchical clustering analysis. The association between the CIMP and mutations of oncogenes was clearly observed using DNA methylation profiles of the selected 6877 and 263 blocks, and some association was observed using the methylation profiles of the 25,000 blocks with CGIs. In contrast, no association was observed using the 25,000 blocks randomly selected from all the blocks. Therefore, it is considered that the selection of biologically important probes (or genes) is required to extract meaningful information from the huge amount of data obtained by comprehensive DNA methylation analysis.

We previously found that the CIMP statuses in GCs were not associated with DNA methylation statuses in background non-cancerous mucosae, contrary to expectations [30]. The presence of the CIMP(+) GCs suggested that CGIs methylated in GCs are composed of those methylated as a result of the CIMP and those methylated in background non-cancerous mucosae.

The genes aberrantly methylated in GCs here were associated with genes targeted by PRC2 in ES cells, confirming previous reports. It has been known that genes methylated in other types of cancers are associated with genes targeted by PRC2 in ES cells [49,50,53] or normal cells [40,50–52]. A recent comprehensive analysis in GCs also revealed that genes methylated in GCs were associated with genes targeted by PRC2 in ES cells [33]. EZH2, a component of PRC2, and CBX7, a component of PRC1, are known to interact with DNA methyltransferases [56,57], and these interactions seem to be a possible mechanism of the high frequency of DNA methylation of the genes targeted by PRC2.

The prognosis of the CIMP(+) patients tended to be better than that of the CIMP(−) patients. The association between the CIMP and prognosis is highly dependent upon cancer types. For example, the CIMP is associated with poor prognosis in colorectal cancers [28], lung cancers [29], and neuroblastoma [27]. In GCs, some studies showed association with good prognosis [30,31], and others showed that with poor prognosis [32,33]. The reason why the CIMP in GCs was associated with good prognosis in some studies is unknown, but it might be possible that genes involved

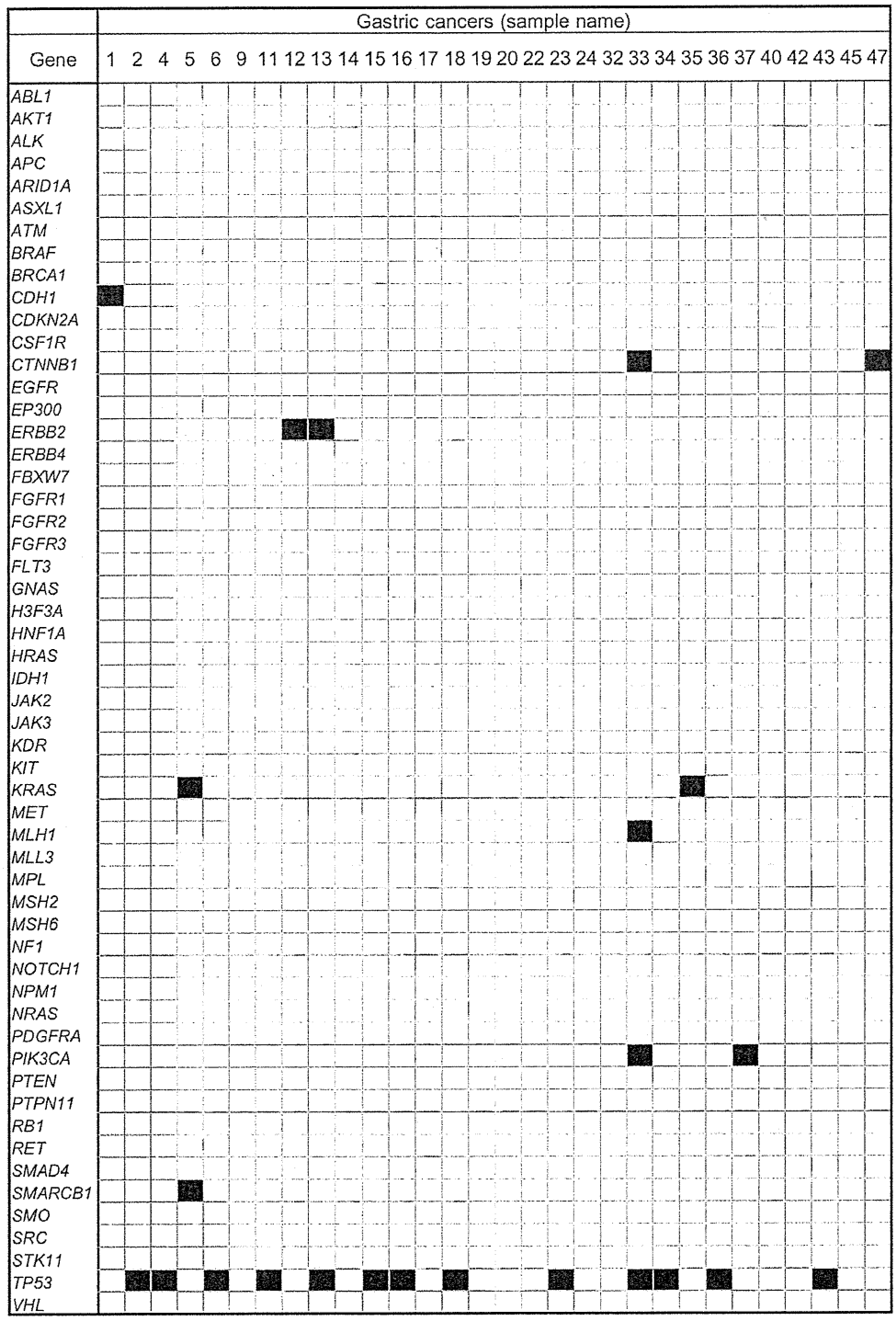

Fig. 2. Results of extensive mutation analysis of the 30 GCs. Mutations of the 55 known cancer-related genes were analyzed by Ion Torrent PGM sequencer. Among the 30 GCs, 19 had 24 somatic mutations of 8 different genes. *TP53* was mutated in 13 GCs (43%, 13 of the 30 GCs), and *CTNNB1*, *ERBB2*, *KRAS*, and *PIK3CA* were mutated in two GCs, respectively. The presence of a somatic mutation is shown by a filled square.

in tumor progression are silenced by aberrant DNA methylation in GCs with the CIMP.

In conclusion, integrated analysis of genetic and epigenetic alterations revealed that the CIMP was associated with mutations of oncogenes, including *ERBB2*, *CTNNB1*, *KRAS* and *PIK3CA*, in GCs.

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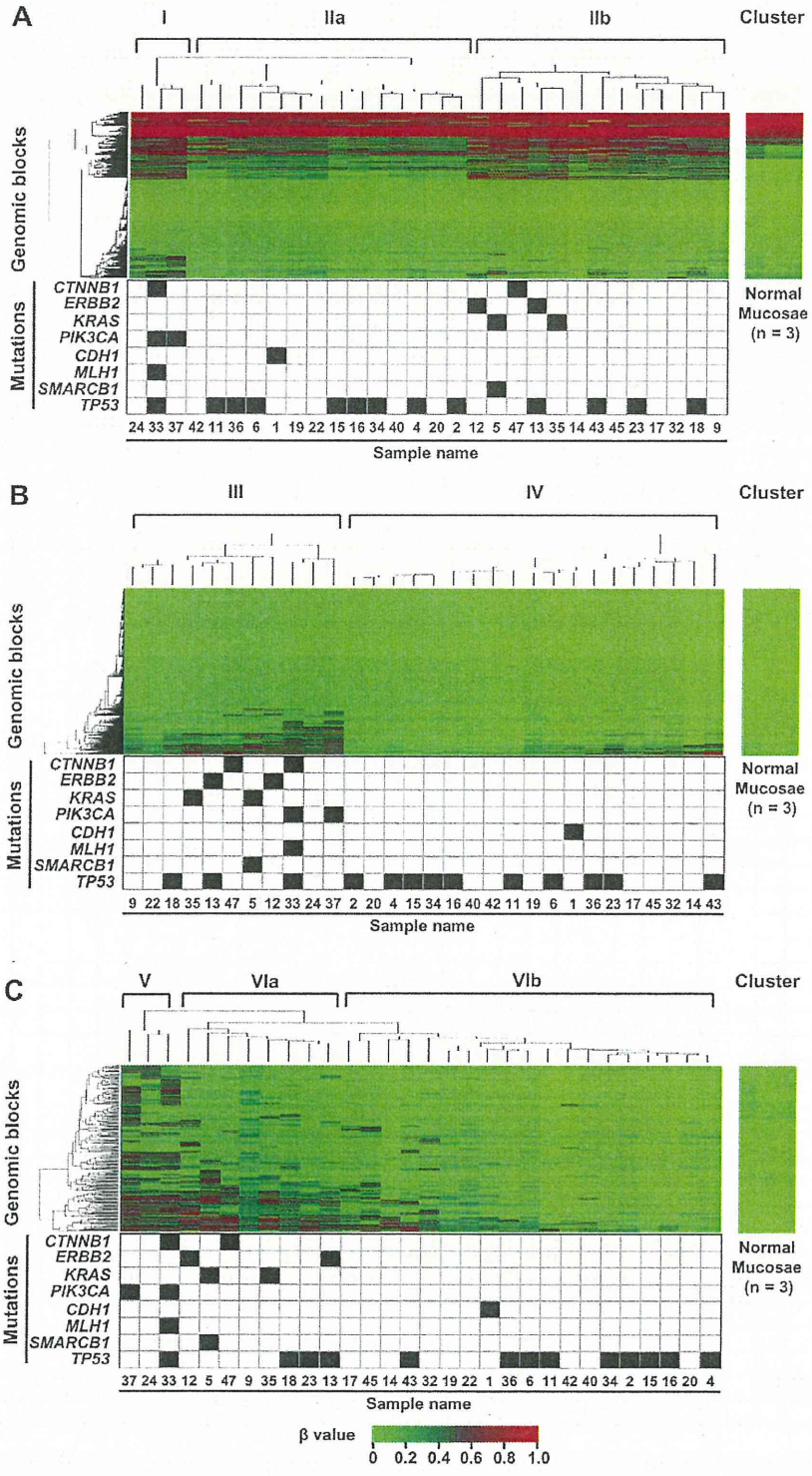

Fig. 3. The association between the DNA methylation profile and gene mutations. (A) Unsupervised hierarchical clustering analysis using DNA methylation profiles of 25,000 genomic blocks with CGIs. Clusters I ($n = 3$) and IIb ($n = 13$) contained GCs with a relatively large number of aberrantly methylated genes, and seven of the 16 GCs were shown to have mutations of oncogenes. (B) Unsupervised hierarchical clustering analysis using DNA methylation profiles of the 6877 blocks (genes) unmethylated in normal gastric mucosae. Cluster III ($n = 11$) contained GCs with a relatively large number of aberrantly methylated genes, and seven of the 11 GCs were shown to have mutations of oncogenes. (C) Unsupervised hierarchical clustering analysis using DNA methylation profiles of the 263 methylation-silenced genes. Cluster V ($n = 3$) contained GCs with the largest number of aberrantly methylated genes, and two of the three were shown to have *PIK3CA* mutations.

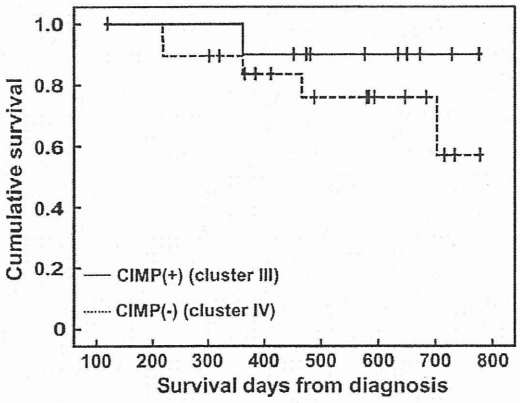

Fig. 4. The possible association between the CIMP and good prognosis. Kaplan-Meier curves were drawn using overall survival (OS). The CIMP status was determined based on the DNA methylation profile of the 6877 genes unmethylated in normal gastric mucosae. The prognosis of the CIMP(+) patients ($n = 11$) tended to be better than that of the CIMP(−) patients ($n = 19$) ($P = 0.285$).

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.canlet.2012.11.022.

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Research Article
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Prevention of *Helicobacter pylori*–Induced Gastric Cancers in Gerbils by a DNA Demethylating Agent

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Abstract

Suppression of aberrant DNA methylation is a novel approach to cancer prevention, but, so far, the efficacy of the strategy has not been evaluated in cancers associated with chronic inflammation. Gastric cancers induced by *Helicobacter pylori* infection are known to involve aberrant DNA methylation and associated with severe chronic inflammation in their early stages. Here, we aimed to clarify whether suppression of aberrant DNA methylation can prevent *H. pylori*–induced gastric cancers using a Mongolian gerbil model. Administration of a DNA demethylating agent, 5-aza-2′-deoxycytidine (5-aza-dC), to gerbils (0.125 mg/kg for 50–55 weeks) decreased the incidence of gastric cancers induced by *H. pylori* infection and *N*-methyl-*N*-nitrosourea (MNU) treatment from 55.2% to 23.3% ($P < 0.05$). In gastric epithelial cells, DNA methylation levels of six CpG islands (HE6, HG2, SB1, SB5, SF12, and SH6) decreased to 46% to 68% ($P < 0.05$) of gerbils without 5-aza-dC treatment. Also, the global DNA methylation level decreased from 83.0% ± 4.5% to 80.3% ± 4.4% (mean ± SD) by 5-aza-dC treatment ($P < 0.05$). By 5-aza-dC treatment, *Il1b* and *Nos2* were downregulated (42% and 58% of gerbils without, respectively) but *Tnf* was upregulated (187%), suggesting that 5-aza-dC treatment induced dysregulation of inflammatory responses. No obvious adverse effect of 5-aza-dC treatment was observed, besides testicular atrophy. These results showed that 5-aza-dC treatment can prevent *H. pylori*–induced gastric cancers and suggested that removal of induced DNA methylation and/or suppression of DNA methylation induction can become a target for prevention of chronic inflammation–associated cancers. *Cancer Prev Res; 6(4); 263–70. ©2013 AACR.*

Introduction

DNA methylation is an epigenetic mechanism for gene regulation. Methylation of promoter CpG islands (CGIs) consistently suppresses expression of their downstream genes (1), and physiologic methylation of retrotransposons is involved in their transcriptional repression (2). In cancers, tumor suppressor genes are frequently inactivated by aberrant methylation of their promoter CGIs (3, 4). Such aberrant methylation is present not only in cancers but also in noncancerous tissues exposed to chronic inflammation, such as colonic mucosae with ulcerative colitis, liver tissues exposed to hepatitis, and gastric mucosae exposed to chronic gastritis (5–10). In the case of the stomach, *Helicobacter pylori* infection is known to induce severe chronic inflammation (11–13) and aberrant methylation in gastric epithelial cells (GEC; ref. 14). Accumulation levels of aberrant methylation in gastric mucosae correlate with risk of gastric cancers (8–10).

Suppression of aberrant methylation is considered as one of the novel targets for cancer chemoprevention (15, 16). Traditionally, chemoprevention has used substances based on 2 strategies: the anti-initiation and anti-promotion/progression strategies (17–19). In the former strategy, blockage of activity of carcinogens that induce genetic or epigenetic alterations and enhancement of repair systems have been targeted. In the latter strategy, suppression of proliferation of initiated cells and induction of their apoptosis have been targeted. However, neither of these strategies targeted removal of genetic or epigenetic alterations accumulated in the cells, which can be achieved by DNA demethylating agents, such as 5-aza-2′-deoxycytidine (5-aza-dC; refs. 20, 21).

The usefulness of DNA demethylating agents in cancer chemoprevention has been shown in several animal models, including intestinal tumors in $Apc^{min/+}$ mice (22, 23), prostate tumors in transgenic mice harboring probasin promoter-driven SV40 antigen (24), 4-(methyl-nitrosamino)-1-(3-pyridyl)-1-butanone–induced mouse lung tumors (25), and 4-nitroquinoline 1-oxide–induced mouse

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oral tumors (26). Genetic suppression of a maintenance DNA methyltransferase (*Dnmt1*) also suppressed tumor development in some of these models (22, 27, 28). However, so far, the efficacy of suppression of aberrant DNA methylation was not evaluated in chronic inflammation–associated cancers, in which aberrant DNA methylation is heavily involved (29, 30). From this aspect, gastric cancers induced by *H. pylori* infection of Mongolian gerbils (*Meriones unguiculatus*) have several advantages. In gerbils, *H. pylori* infection induces severe chronic inflammation, as in humans, and promotes gastric cancers initiated by *N*-methyl-*N*-nitrosourea (MNU; refs. 31, 32). Also, 10 CGIs have already been established as markers that can be methylated by *H. pylori* infection, and a critical role of inflammation triggered by *H. pylori* infection, not a direct effect of *H. pylori*, in methylation induction has been shown (14). In contrast, few markers for methylation induction have been isolated in *H. pylori*- or *Helicobacter felis*–infected mice, except *Tff2* promoter (33).

In this study, using the gerbil model, we aimed to clarify whether 5-aza-dC treatment can prevent chronic inflammation–induced gastric cancers and evaluate its effects on methylation induction and inflammation triggered by *H. pylori* infection.

Materials and Methods

Animals and sample preparation

Male Mongolian gerbils (MGS/Sea) were purchased from Kyudo and divided into 10 groups (G1–6 in Fig. 1A and G7–10 in Fig. 2A). Gerbils were inoculated with *H. pylori* [~4×10^8 colony-forming units (CFU)/gerbil, ATCC 43504; American Type Culture Collection] at 5 weeks of age (34). In a carcinogenicity experiment, 10 ppm of MNU (Sigma-Aldrich) was given in drinking water to gerbils. 5-Aza-dC (125 μg/kg body weight in sterilized PBS; Sigma-Aldrich) was administered to gerbils intraperitoneally twice per week. The dose was selected from the 3 doses (125, 250, and 500 μg/kg) tested in a preliminary experiment for lack of toxicity. Timing and duration of the treatments are shown in Figs. 1A and 2A.

The stomach was resected and cut along the greater curvature. In a carcinogenicity experiment (G1–6), the antral region was fixed in formalin for histologic analysis. From the body region, GECs were isolated by the gland isolation technique (35) and stored in 100% ethanol at −80°C until DNA extraction. The testes, small intestine, liver, and kidneys were resected, and half parts were fixed in formalin. The other halves were snap-frozen for DNA extraction. In an experiment to induce *H. pylori*–triggered gastritis (G7–10), the antral region was cut into 2 pieces—one was snap-frozen for RNA extraction and the other half was fixed in formalin.

In both experiments, samples (tissues or GECs) were digested with proteinase K, and gDNA was extracted by the standard phenol/chloroform method. RNA of gastric tissue was isolated using ISOGEN (Nippon Gene). Whole blood was obtained from the inferior vena cava, and gDNA was extracted by a QuickGene DNA Whole Blood Kit (Fujifilm). All the animal experiments were approved by the Committee for Ethics in Animal Experimentation at the National Cancer Center.

Histological analysis

Formalin-fixed tissues were sliced along the longitudinal axis into strips of 5 mm width and embedded in paraffin. Sections of 3 μm thickness were prepared and stained with hematoxylin and eosin. Neoplastic lesions in the stomach were diagnosed as previously described (36). The size of a gastric cancer was determined as the maximum diameter of the gastric cancer in the neighboring sections. The degree of infiltration of mononuclear and polymorphonuclear cells was graded on a 4-point scale (0–3; 0, no or faint; 1, mild; 2, moderate; 3, marked) as described (37).

Luminometric methylation assay

gDNA from whole blood was amplified by an illustra GenomiPhi V2 DNA Amplification Kit (GE Healthcare) and used as fully unmethylated DNA. The unmethylated DNA was methylated by *Sss*I methylase (New England Biolabs) and used as fully methylated DNA. A series of standard DNA was prepared by serial mixing of the unmethylated DNA and the methylated DNA.

Luminometric methylation assay (LUMA) was conducted as described (38) with slight modifications. Briefly, 3 μg of DNA was digested with 2 pairs of restriction enzymes (*Hpa*II and *Eco*RI or *Msp*I and *Eco*RI) in independent tubes (all restriction enzymes were purchased from Toyobo). The DNA was purified with a DNA Clean & Concentrator Kit (Zymo Research) and eluted in 40 μL of an annealing buffer (2 mmol/L magnesium acetate and 20 mmol/L Tris-acetate, pH7.6). Using the PSQ 96 Pyrosequencing System (Qiagen), 5′-CG overhang produced by *Hpa*II (or *Msp*I) and 5′-AATT overhang produced by *Eco*RI was sequenced, and an *Hpa*II/*Eco*RI (or *Msp*I/*Eco*RI) signal ratio was determined. An *Hpa*II/*Msp*I value was obtained as (*Hpa*II/EcoRI)/(*Msp*I/*Eco*RI) in each sample. The *Hpa*II/*Msp*I value was compared with those of the standard DNA series, and the global methylation level (GML), which is equivalent to the percentage of methylated DNA in the standard DNA, was determined.

Quantitative methylation-specific PCR

DNA digested with *Bam*HI was treated with sodium bisulfite as described (39) and used as a template for real-time PCR. With primer sets specific to methylated CGIs (HE6, HG2, SA9, SB1, SB5, SC3, SD2, SE3, SF12, and SH6; Supplementary Fig. S1) and a B2 repeat sequence, real-time PCR was carried out as described (14). On the basis of the copy number of sequences measured by real-time PCR, the methylation level was calculated as a percentage of the methylated reference (PMR), which was obtained as [(number of methylated fragments of a target CGI in sample)/(number of the B2 repeat in sample)]/[(number of