Table 1. Prognostic power of methylation of individual genes and CpG island methylator phenotype (CIMP)

Marker	Japanese ($n = 140$)				German ($n = 152$)			
	No. of NBLs with methylation or amplification	HR	95% CI for HR	*P* value	No. of NBLs with methylation or amplification	HR	95% CI for HR	*P* value
CASP8	26	3.1	1.5–6.4	0.002	30	4.8	2.1–11	0.0002
EMP3	4	1.7	0.4–6.8	0.49	2	NA	–	0.70
HOXA9	27	0.79	0.48–1.3	0.36	2	14	3.1–62	0.0006
NR1I2	15	1.3	0.62–2.7	0.49	13	4.2	1.6–11	0.003
CD44	3	0.23	0.055–0.94	0.04	2	NA	–	0.66
CIMP	67	22	5.3–93	1.5×10^{-5}	50	9.5	3.2–28	4.7×10^{-5}
MYCN amplification	38	9.5	4.4–21	4.0×10^{-9}	23	12	4.9–29	4.8×10^{-8}

NBL, neuroblastoma; HR, hazard ratio; CI, confidence interval; NA, not applicable.

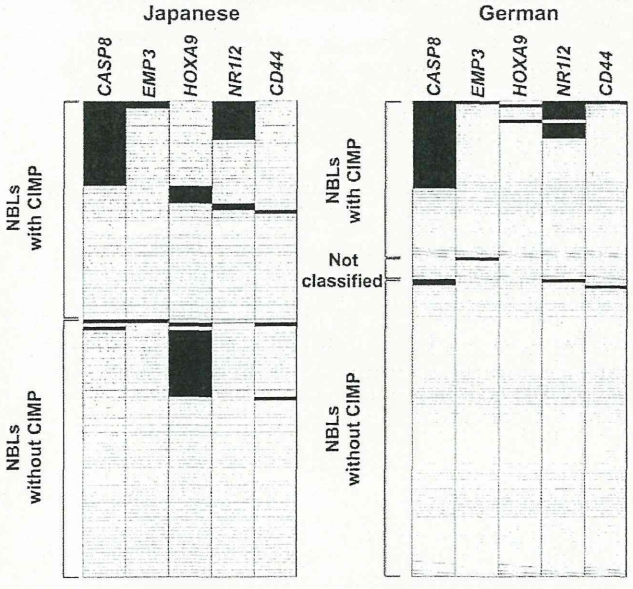

Figure 3. *Methylation profiles of the five individual genes in NBLs with and without CIMP.* Left panel, 140 Japanese NBLs; and right panel, 152 German NBLs. NBLs were classified by CIMP status determined as in our previous studies (2,3), and then aligned by methylation statuses of the five genes. In German NBLs, seven cases were not classified as NBLs with CIMP or without CIMP (3). The NBLs with CIMP tended to show methylation of multiple promoter CGIs. Closed box, methylated DNA detected; open box, only unmethylated DNA detected; and box with a slash; neither methylated nor unmethylated DNA detected, possibly due to low DNA quality.

Regarding the assessment of CIMP, besides the use of the *PCDHB* gene family, a combination of silenced genes has been proposed. Yang et al. (17) analyzed methylation of eight genes (*HIC-1*, *RASSF1A*, *BLU*, *DCR2*, *CASP8*, *TIG-1*, *HIN-1*, *TMS-1*), and identified that methylation of two and three genes had no effects on survival ($P = 0.719$ and 0.214, respectively), but methylation of ≥4 genes had a trend toward decreased survival ($P = 0.055$). Also, Lau et al. (18) identified that methylation of at least one of three genes (*FOLH1*, *MYOD1* and *THBS1*) was associated with event-free survival (HR = 2.2; 95% CI = 1.1–4.2; $P = 0.022$), and the association was stronger in methylation of all the three genes (HR = 4.5; 95% CI = 1.6–13; $P = 0.006$). These data support the model that CIMP leads to methylation of promoter CGIs of tumor-related genes with low incidences, which leads to poor survival.

Among the individual genes, *CASP8* and *RASSF1A* methylation have been repeatedly shown to be associated with poor survival (9,17,19–23). *CASP8* methylation was consistently associated with poor survival in the present study. By the analysis of methylation and survival data in our previous study (2), *RASSF1A* methylation was also revealed to be associated with poor survival in Japanese NBLs (HR = 4.2; 95% CI = 1.9–9.3; $P = 0.0005$). However, HRs of these genes were smaller than that of CIMP. These data indicated that these two genes play critical roles in a fraction of NBLs but not in the other NBLs. Indeed, a recent genome-wide methylation study revealed that methylation of numerous genes was associated with poor survival in NBLs (24).

In conclusion, the stronger prognostic power of CIMP than of methylation of individual genes was shown, and methylation silencing of various tumor-suppressor genes with low incidences was suggested to be involved in poor survival.

Supplementary data

Supplementary data are available at http://www.jjco.oxfordjournals.org.

Funding

This study was supported by Grants-in-Aid for the Third-Term Cancer Control Strategy Program from the Ministry of Health, Labour and Welfare, Japan and by the National Cancer Center Research and Development Fund, Japan.

Conflict of interest statement

None declared.

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Oncogene (2013) 32, 2140–2149

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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

K Asada[1,7], T Ando[1,2,7], T Niwa[1], S Nanjo[1], N Watanabe[1], E Okochi-Takada[1], T Yoshida[1], K Miyamoto[3], S Enomoto[4], M Ichinose[4], T Tsukamoto[5], S Ito[6], M Tatematsu[5], T Sugiyama[2] and T Ushijima[1]

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.

Oncogene (2013) **32,** 2140–2149; doi:10.1038/onc.2012.228; published online 11 June 2012

Keywords: field for cancerization; chromosome X; DNA methylation; gastrointestinal cancer; *Helicobacter pylori*

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.

[1]Division of Epigenomics, National Cancer Center Research Institute, Tokyo, Japan; [2]Third Department of Internal Medicine, University of Toyama, Toyama, Japan; [3]Institute for Clinical Research and Department of Surgery, National Hospital Organization Kure Medical Center/Chugoku Cancer Center, Hiroshima, Japan; [4]Second Department of Internal Medicine, Wakayama Medical University, Wakayama, Japan; [5]Division of Oncological Pathology, Aichi Cancer Center Research Institute, Nagoya, Japan and [6]Department of Gastroenterological Surgery, Aichi Cancer Center Hospital, Nagoya, Japan. Correspondence: Dr T Ushijima, Division of Epigenomics, National Cancer Center Research Institute, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan.
E-mail: tushijim@ncc.go.jp
[7]These authors contributed equally to this work.
Received 20 September 2011; revised 25 April 2012; accepted 4 May 2012; published online 11 June 2012

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

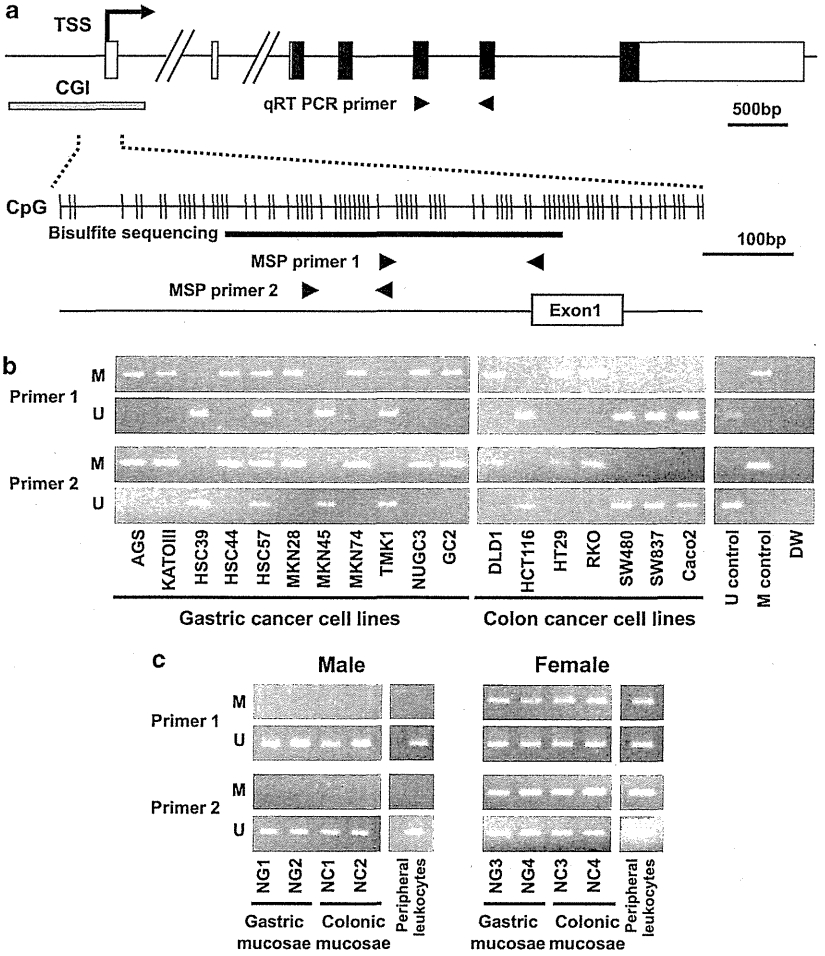

Figure 1. Genomic structure of *FHL1* and its methylation status in cancer cell lines, normal-appearing mucosae and peripheral leukocytes. (**a**) Genomic structure of *FHL1* and a CpG map of its promoter CGI. Open box, non-coding exon; closed box, coding exon; arrow, transcription start site (TSS); 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.

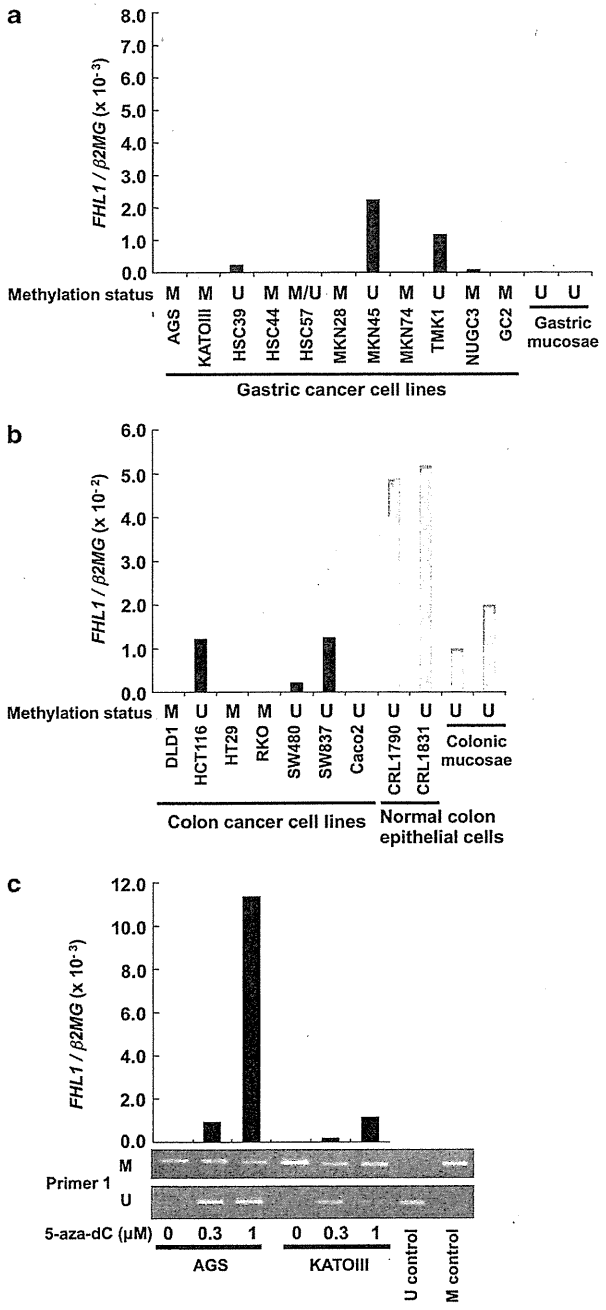

Figure 2. Methylation-silencing of *FHL1* in gastrointestinal cancer cell lines. (**a**) qRT–PCR of *FHL1* in gastric cancer cell lines and normal-appearing gastric mucosae. Results of MSP in Figure 1b are shown by M, M/U and U. M, only methylated DNA detected; M/U, both methylated and unmethylated DNA 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

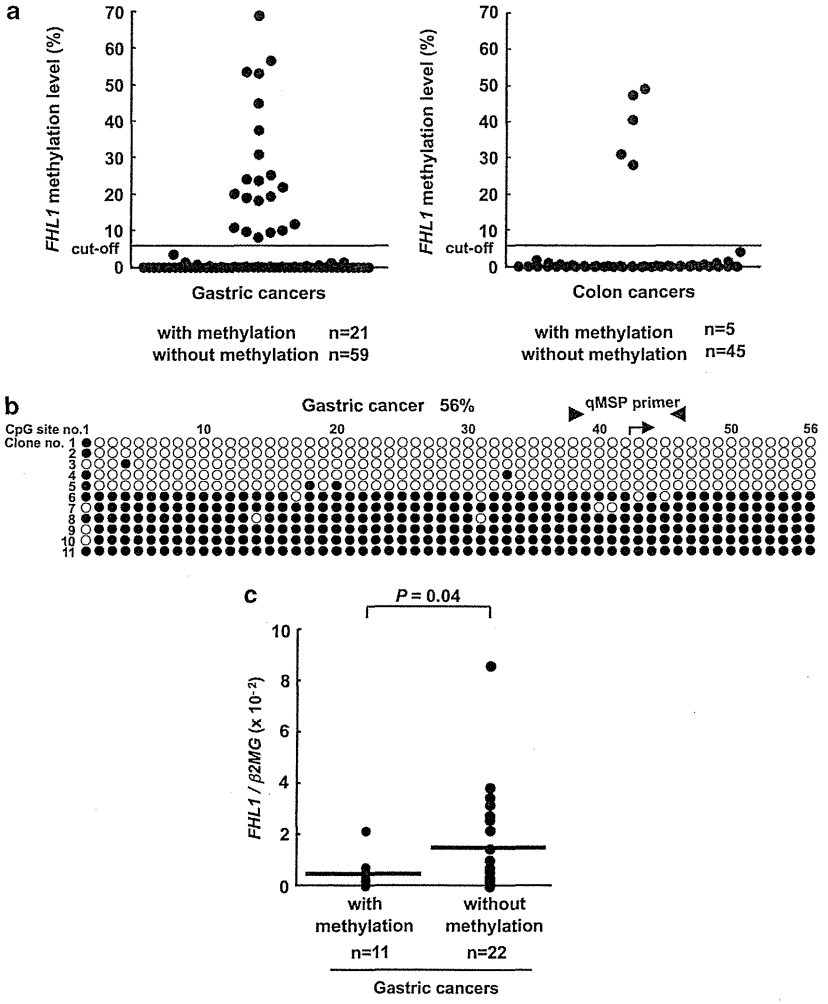

Figure 3. Methylation of *FHL1* in surgical gastrointestinal cancer specimens and its effect on expression. (**a**) Methylation levels in gastric (left) and colon (right) cancers derived from male patients. A horizontal line shows a cutoff value of 6%. *FHL1* was methylated in 21 of 80 primary gastric 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

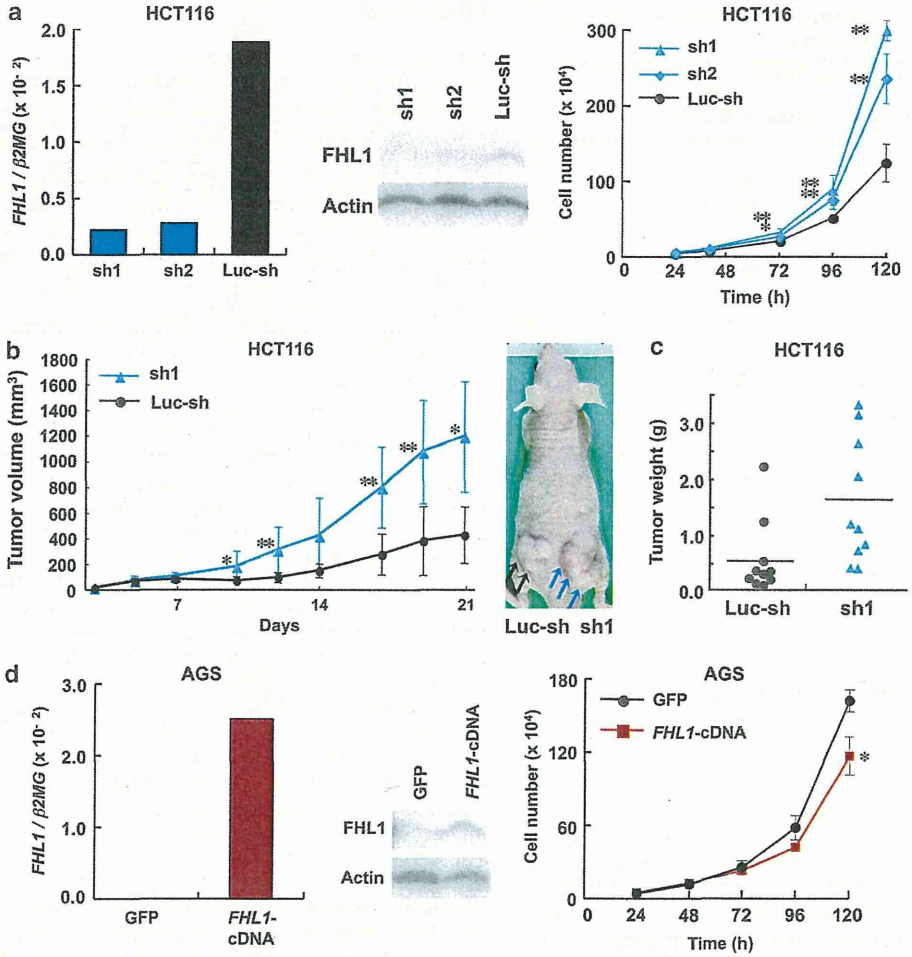

Figure 4. Growth-suppressive activity of *FHL1 in vitro* and *in vivo*. (**a**) *FHL1* knockdown and the resultant increased growth of HCT116 cells. Decreased expression of *FHL1* by its knockdown was confirmed by qRT–PCR (left) and western blot (middle). Growth rates of cells with *FHL1* knockdown were 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

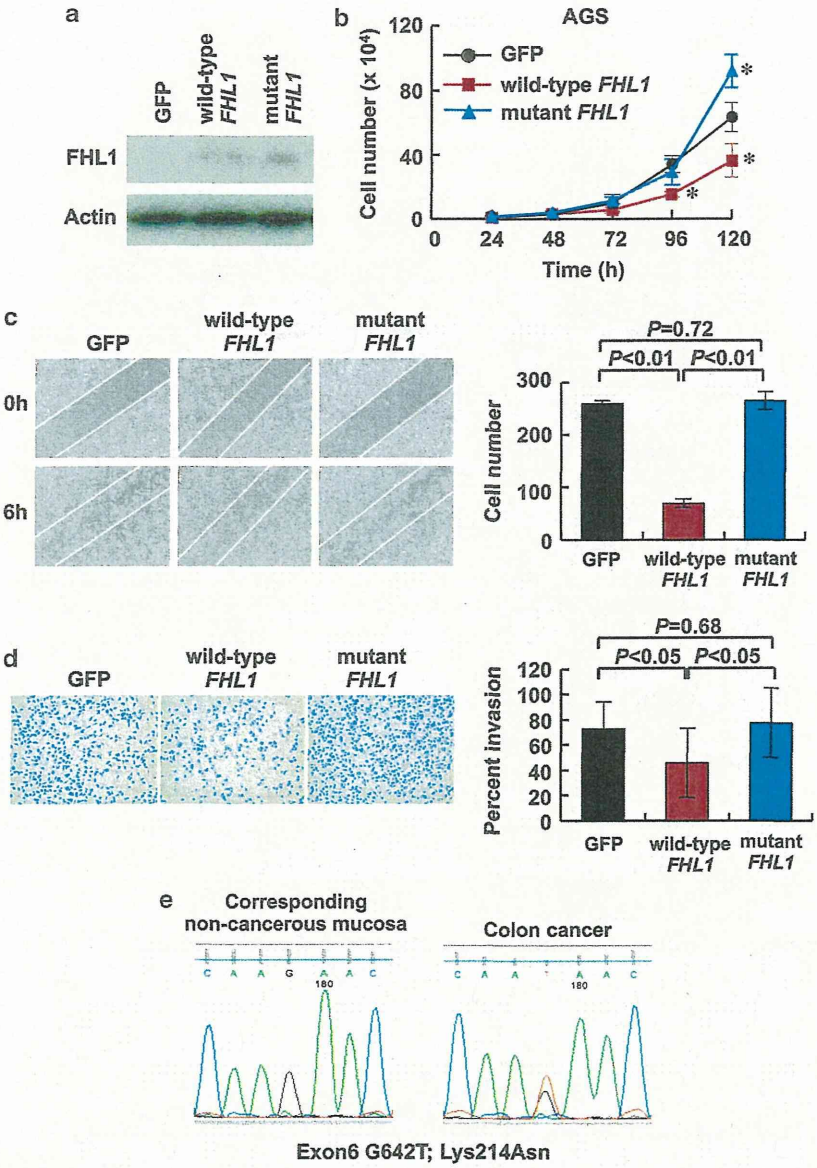

Figure 5. Inhibitory effects of *FHL1* on migration and invasion, and the lack of such functions in *FHL1* with the G642T mutation in AGS. (**a**) Expression levels of exogenous wild-type and mutant *FHL1* detected by western blot. (**b**) The growth-suppressive effect of the wild-type *FHL1*, and the 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

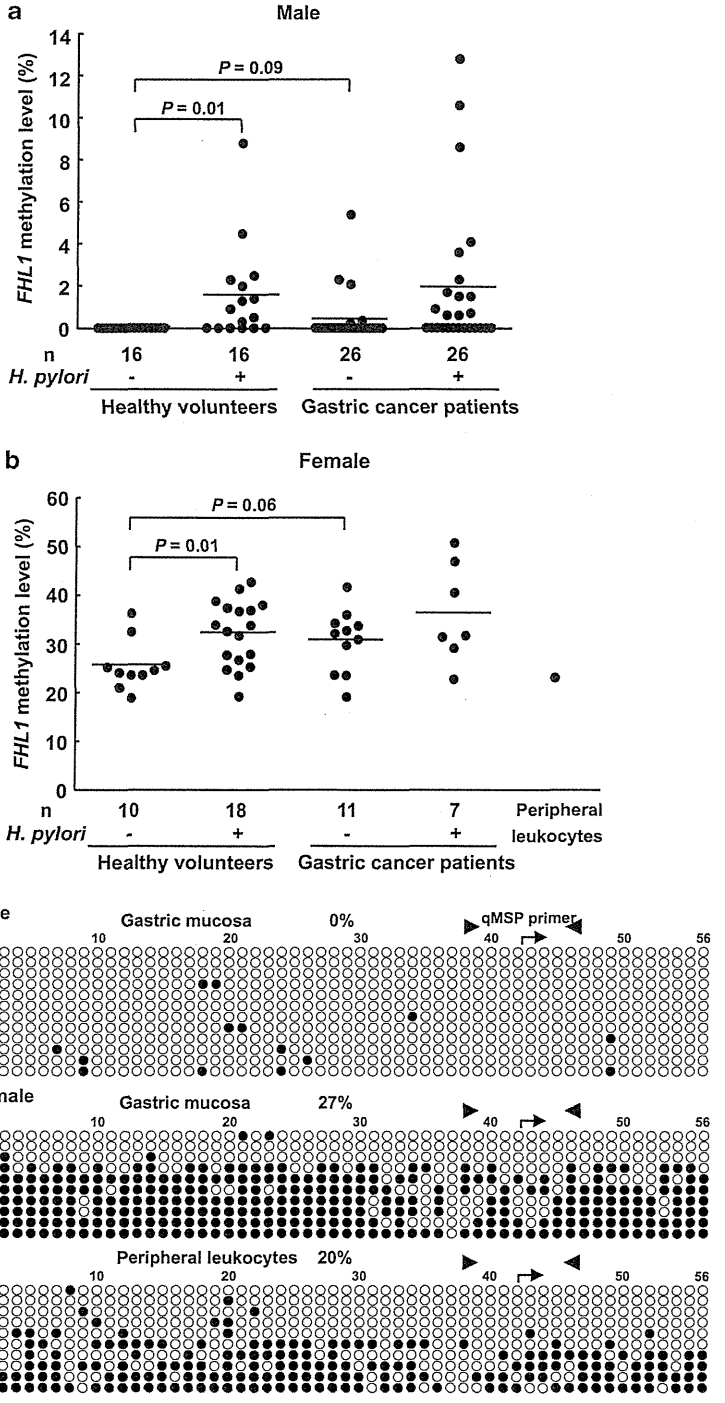

Figure 6. *FHL1* methylation levels in male and female gastric mucosae. (**a**) Methylation levels in male gastric mucosae of healthy volunteers and non-cancerous mucosae of gastric cancer patients. A horizontal line represents the mean methylation level for each group. Among healthy volunteers, *FHL1* 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.