TABLE 1 Patient and tumour characteristics

Variable	All patients ($n = 715$)	Patients with cancer ($n = 257$)	TR12-positve cancer ($n = 204$)	TR12-negative cancer ($n = 53$)	P
Age (years)	66 (61–71)	66 (63–72)	68 (63–72)	67 (63–73)	0.914
PSA level (ng/mL)	6.1 (4.7–8.5)	7.0 (5.1–9.6)	7.2 (5.1–9.8)	6.8 (5.2–8.7)	0.370
Prostate volume (mL)	35 (27–47)	29 (23–39)	29 (22–37)	32 (27–48)	0.003
% Abnormal DRE	16	25	29	11	0.009
Number of positive cores, *n* (range)	–	5 (2–8)	6 (3–9)	2 (1–2)	<0.001
Biopsy GS, *n* (%)					
5–6	–	83 (32)	52 (25)	31 (58)	<0.001
3 + 4	–	69 (27)	56 (27)	13 (25)	
4 + 3	–	44 (17)	39 (19)	5 (9)	
8 – 10	–	61 (24)	57 (28)	4 (8)	

Continuous variables are expressed as the median (interquartile range). GS, Gleason score; TR12-positive, transrectal 12-core biopsy-positive; TR12-negative, transrectal 12-core biopsy-negative

Transperineal sampling site*	% Cancer detection	P
A1	47	
A2	28	
AL	28	
P1	24	
P2	19	
PL	19	
TZ	19	
A1 + A2	62	0.020†
P1 + P2	40	
A1 + A2 + AL	75	0.009†
P1 + P2 + PL	51	

TABLE 2 TR12-negative cancer (n = 53) detection rates in each transperineal sampling site

**See Fig. 1. †According to a chi-squared test (A1 + A2 vs P1 + P2, A1 + A2 + AL vs P1 + P2 + PL). TR12-negative, transrectal 12-core biopsy-negative.*

Cochran–Armitage test was used to test for trends. $P < 0.05$ was considered statistically significant.

RESULTS

CHARACTERISTICS OF TR12-NEGATIVE CANCERS: ANALYSES ON BIOPSY SPECIMENS

Prostate cancers were detected through 3D26PBx in 257 (35.9%) of the 715 men. Of these 257 cancers, 53 (21%) were identified as TR12-negative cancers; in other words, the addition of the TP14PBx sites to the TR12PBx sites improved the cancer detection rate by 26%. Patient and tumour characteristics of TR12-positive and TR12-negative cancers are shown in Table 1. Compared to patients with TR12-positive cancers, patients with TR12-negative cancers had significantly larger prostates and a lower incidence of abnormal DRE. The frequency of cancers with biopsy GS ≤6 and that of cancers with biopsy primary Gleason grade ≤3 were higher in TR12-negative cancers, at 58% and 83%, respectively, than in TR12-positive cancers, at 25% ($P < 0.001$) and 53% ($P < 0.001$), respectively.

In the 53 TR12-negative cancers, cancer-positive rates within the TP14PBx sampling sites are shown in Table 2. The farthest anterior sampling site (A1; Fig. 1) had the highest cancer-positive rate of 47%. There were six cores from the anterior sampling sites (A1, A2 and AL; Fig. 1) that detected significantly ($P = 0.009$) more cancers than six cores from the posterior sites (P1, P2 and PL; Fig. 1), indicating that TR12-negative cancers are located more frequently in the anterior region than in the posterior region.

CHARACTERISTICS OF TR12-NEGATIVE CANCERS: ANALYSES ON RP SPECIMENS

In total, 120 of the 257 (47%) patients underwent RP [16]. Characteristics of TR12-positive and TR12-negative cancers are shown in Table 3. Among the pathological variables analyzed in the RP cohort, the TR12-positive and TR12-negative groups differed with respect to cancer location: specifically, TR12-negative cancers were located less frequently in the posterior region than the TR12-positive cancers.

SUBGROUPS IN WHICH TR12PBX IS INSUFFICIENT FOR CANCER DETECTION

To identify patient subgroups in which TR12PBx would be entirely insufficient for cancer detection and in which more sampling would be needed, we compared the incidence of TR12-negative cancers in subgroups defined by age, PSA level, prostate volume or DRE findings (Fig. 2). Although cancer detection rates of 3D26PBx were significantly higher in patient subgroups with higher age, higher PSA level, smaller prostates or abnormal DRE findings, the incidence of TR12-negative cancers did not differ significantly between any of the subgroups.

COMPARISON OF CANCER CHARACTERISTICS BETWEEN NORMAL AND ABNORMAL DRE GROUPS

PSA screening has significantly increased the proportion of men who undergo prostate biopsy based on PSA findings alone. To evaluate the efficacy of TR12PBx in men with normal DRE in more detail, the cancer characteristics of TR12-negative cancers were analyzed according to DRE findings (Table 4). TR12-positive cancers in the normal DRE group tended to have lower biopsy cancer grade and fewer positive cores compared to those in the abnormal DRE group. By contrast, in TR12-negative cancers, biopsy cancer grade and the number of positive cores did not differ significantly between the groups.

DISCUSSION

In the present study, we evaluated the characteristics of cancers missed by initial TR12PBx, more precisely, cancers missed by TR12PBx in patients who underwent 3D26PBx (TR12-negative cancers), and thus

showed the diagnostic performance of TR12PBx. Initial TR12PBx missed 21% of cancers that were detectable through 3D26PBx; however, it should be noted that more than half of TR12-negative cancers had a biopsy GS ≤6, and most of them had a biopsy primary Gleason grade ≤3. Furthermore, the median number of positive cores in TR12-negative cancers was only two out of 26, suggesting that a substantial number of TR12-negative cancers can be expected to be low-grade and low-volume diseases.

Although our RP cohort is highly selective, most TR12-negative cancers treated with RP were significant cancers. Yet 87% were organ-confined disease and 75% were primary Gleason grade 3 cancers with favourable prognosis. Combined with the biopsy findings, this indicates that TR12-negative cancers have lower malignant potential than TR12-positive cancers, and that most of them can be expected to be organ confined, although a small number of TR12-negative cancers appear to be significant cancers that would exhibit biological aggressiveness.

The characteristics of the location of TR12-negative cancers are clearly shown in the present study. Our analysis of positive transperineal sites in TR12-negative cancers confirmed that TR12-negative cancers were located in the anterior portion of the gland rather than the posterior portion. The cancer location of TR12-negative cancers in RP specimens also supports this notion, suggesting that TR12PBx would be insufficient to detect anterior cancers. The results obtained in the present study are similar to those obtained by Moussa *et al.* [11], who reported that the addition of only two extreme anterior apical cores to TR12PBx transrectal sampling improved cancer detection by 7.5% and that these two cores achieved the highest rate of unique cancer detection. They therefore introduced the 14-core biopsy scheme (TR12PBx biopsy plus two extreme apical cores) as an initial biopsy to detect more anterior apical cancers.

On the basis of these findings, simply adding more transrectal sampling sites from the bottom of the prostate gland to TR12PBx would not increase its cancer detection rate efficiently. Indeed, several studies have tested transrectal extended biopsy methods using more than 12 cores; however, most of these so-called 'saturation' transrectal biopsy protocols did not outperform TR12PBx [17–19]. The transrectal 21-core biopsy [10] can identify more cancers than TR12PBx can, although the 9.8% increase in cancer detection that results from the nine additional samplings appears to be inefficient. In the present study, the addition of two far-anterior transperineal sampling to the TR12PBx improved its cancer detection rate by 11%. From these results, we consider that a few additional samplings in the anterior apical portion are effective for detecting cancers missed by TR12PBx.

In recent analyses on men with a PSA level <20 ng/mL without locally advanced tumours on DRE findings, there were no significant differences among subgroups defined by age, PSA level, prostate volume or DRE in the incidence of TR12-negative cancers. Therefore, we could not identify any subgroup in which TR12PBx would be entirely insufficient for cancer detection and in which more sampling would therefore be needed. There is the potential concern that TR12PBx may probably miss anterior aggressive cancers in men with normal DRE, although our analysis of cancer characteristics according to DRE findings shows that a large proportion of TR12-negative cancers probably consist of low-grade and low-volume disease, regardless of DRE findings. We consider that these results are sufficient grounds to eliminate such concerns.

Recently, the value of MRI for detecting prostate cancers and determining their location has been extensively studied [20]. Lawrentschuk *et al.* [21] retrospectively analyzed patients with anteriorly predominant tumours on MRI who had undergone prostate biopsy and reported that MRI would be useful in the detection of

TABLE 3 Patient and tumour characteristics of 120 men undergoing radical protectomy

Variable	TR12-positive cancer (n = 104/204)	TR12-negative cancer (n = 16/53)	P
Clinical			
Age (years)	67 (62–71)	64 (60–70)	0.245
PSA level (ng/mL)	6.7 (5.2–9.3)	6.4 (4.7–8.4)	0.362
Prostate volume (mL)	28 (22–34)	29 (20–38)	0.817
% Abnormal DRE	24	25	0.666
Biopsy			
Number of positive cores, *n* (range)	5 (3–9)	2 (1–3)	<0.001
GS, *n* (%)			
5–6	17 (16)	8 (50)	<0.001
3 + 4	32 (31)	5 (31)	
4 + 3	27 (26)	1 (6)	
8–10	28 (27)	2 (13)	
Radical protectomy			
GS, *n* (%)			
5–6	26 (25)	3 (19)	<0.001
3 + 4	49 (47)	9 (56)	
4 + 3	16 (15)	3 (19)	
8–10	13 (13)	1 (6)	
% Organ-confined disease	73	87	0.178
% Tumour volume ≥0.5 mL	80	75	0.437
% Significant cancer	92	87	0.396
Significant cancer			
% Located posteriorly	83	50	<0.001
% Located anteriorly	75	71	0.500
% Located apically	86	79	0.331

Continuous variables are expressed as the median (interquartile range). GS, Gleason score; TR12-positive, transrectal 12-core biopsy-positive; TR12-negative, transrectal 12-core biopsy-negative.

anterior tumours that are difficult to detect using transrectal biopsy. It appears that MRI is a promising tool for detecting anterior cancers, although its cost is high.

Pathological evaluation in the present study is based on the 2005 International Society of Urologic Pathology Consensus [13,14]. Fused glands, ill-defined glands with poorly formed glandular lumina and most of the cribriforms are previously categorized into Gleason pattern 3 but, in the 2005 consensus, are categorized into Gleason pattern 4. Furthermore, two new modifications to the Gleason scoring system are recommended in the evaluation of biopsy specimens. One is that any high-grade pattern, no matter how small quantitatively, should be incorporated into the GS, although any secondary grade that occupies <5% of the specimen would not have been reported under the previous system. The other modification is that all higher tertiary grade components of the tumour, which were previously ignored, should be incorporated into the GS. Accordingly, the rates of high-grade patterns scored according to the 2005 consensus are higher than those scored under the previous system. This phenomenon has been confirmed in a study by Billis *et al.* [22] showing that GS that had been scored under the previous system were upgraded by re-evaluation under the 2005 consensus in 26.7% of the biopsy specimens. Similarly, the GS of some of the patients in the present study would have been lower if they had been evaluated under the previous system. We consider that these findings strengthen our view that many TR12-negative cancers can be expected to be low-grade diseases.

The present study does not indicate that we should aim to actively detect TR12-negative cancers in all candidates for initial prostate biopsy because a substantial number of TR12-negative cancers are low-grade and low-volume diseases. Overdiagnosis and

FIG. 2. Bar graphs show detection rates of cancers of all types through 3D26PBx. The overlying line plots show detection rates of TR12-negative cancers (i.e. not detected through transrectal 12-core biopsy but detected through transperineal 14-core biopsy). Statistical analyses were performed using the Cochran–Armitage trend test.

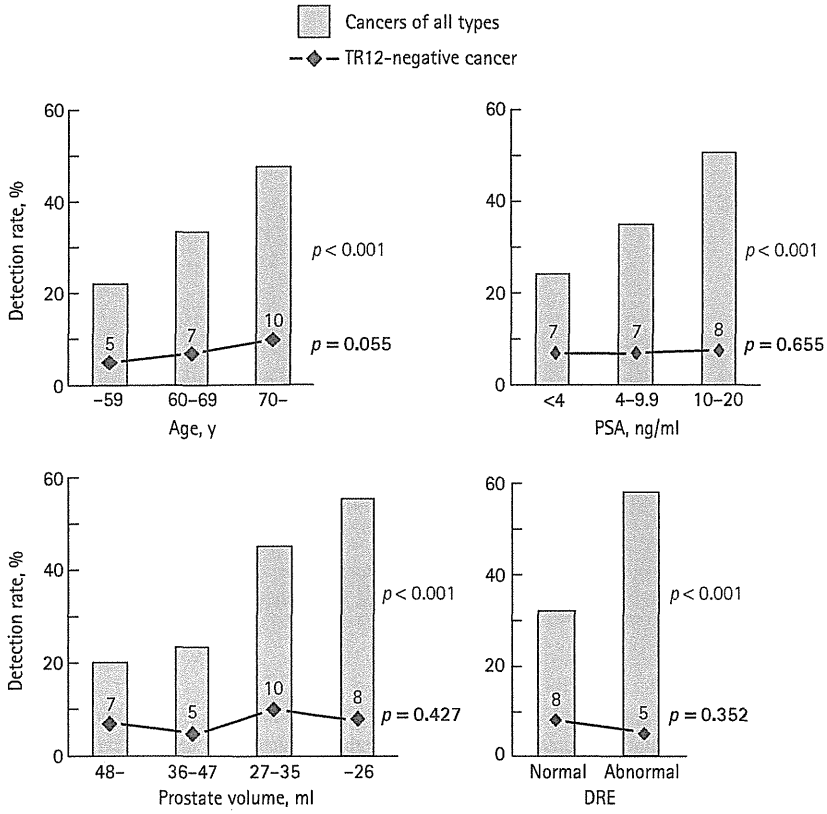

TABLE 4 Comparison of patients and tumour characteristics between normal and abnormal DRE cohorts

Variable	All patients			Patients with cancer			TR12-positive cancer			TR12-negative cancer		
DRE	Normal ($n = 602$)	Abnormal ($n = 113$)	P	Normal ($n = 192$)	Abnormal ($n = 65$)	P	Normal ($n = 145$)	Abnormal ($n = 59$)	P	Normal ($n = 47$)	Abnormal ($n = 6$)	P
Age (years)	65	67	0.010	68	67	0.226	69	67	0.261	67	65	0.508
PSA level (ng/mL)	6.0	6.7	0.022	6.9	7.4	0.217	7.0	7.4	0.270	6.8	7.3	0.715
Prostate volume (mL)	36	32	0.032	30	28	0.435	29	30	0.671	34	26	0.035
Number of positive cores	–	–		3	6	<0.001	5	7	<0.001	2	2	0.251
Biopsy GS ≤6 (%)	–	–		36	22	0.031	28	19	0.152	60	50	0.488
Biopsy primary Gleason grade ≤3 (%)	–	–		66	48	0.010	60	44	0.037	83	83	0.733

Continuous variables are expressed as the median. GS, Gleason score; TR12-positive, transrectal 12-core biopsy-positive; TR12-negative, transrectal 12-core biopsy-negative.

overtreatment are now issues of major concern in the management of prostate cancer. Draisma *et al.* [23] have reported that the rate of overdiagnosis of prostate cancer has been estimated at 23–66% of screening-detected cancers. The main purpose of prostate biopsy is not only to detect more prostate cancers, but also to detect more life-threatening cancers. Even if we missed a case of prostate cancer at an initial biopsy, we would be able to determine the need for a repeat biopsy through the PSA test in most cases, and most cancers detected by repeat biopsy are manageable and not life-threatening [24]. If the goal of screening were simply to detect life-threatening cancers, the addition of sampling sites to the TR12PBx protocol would not be essential in all candidates for initial biopsy.

Another important purpose of biopsy, however, is to accurately characterize any tumours to allow for a more informed treatment decision-making process. If a custom treatment is to be devised for each individual patient, more sampling is required to generate more information, although more sampling leads to a greater detection of indolent cancers. This clinical dilemma makes it difficult to determine the optimal biopsy scheme. We now consider that the addition of anterior sampling sites to the initial TR12PBx would be a reasonable option for younger men with a long life expectancy or for men with suspected anterior cancer as assessed by MRI. On the basis of the analysis in subgroups divided by DRE findings, the finding that a DRE was normal does not mean that additional sampling of the anterior prostate should be performed. At repeat biopsy after negative TR12PBx, however, anterior samplings are highly recommended.

The present study has several limitations that should be considered. Given that 3D26PBx does not identify all cancers, it is possible that TP12PBx may fail to detect an even greater percentage of cancers than reported in the present study. We recommend transperineal sampling for the detection of TR12-negative cancers, although we realize that the transperineal approach may be unfamiliar to many urologists. A recently reported technique for simple and effective local anaesthesia would render transperineal extended biopsy more feasible [12]. Furthermore, because of the limited duration of the follow-up in the present study, we could not report the oncological outcome of TR12-negative cancers. Long-term observation will be required to acquire a better understanding of the diagnostic performance of TR12PBx.

In conclusion, TR12PBx missed 21% of cancers that were detected by 3D26PBx on initial biopsy. Although many of the undetected cancers were expected to be low-grade and low-volume diseases, it should be noted that the initial TR12PBx has a small but definite risk of missing anterior significant cancers.

CONFLICT OF INTEREST

None declared.

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Abbreviations: **3D26PBx**, three-dimensional 26-core prostate biopsy; **GS**, Gleason score; **RP**, radical prostatectomy; **TR12PBx**, transrectal 12-core prostate biopsy; **TP14PBx**, transperineal 14-core prostate biopsy.

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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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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.

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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

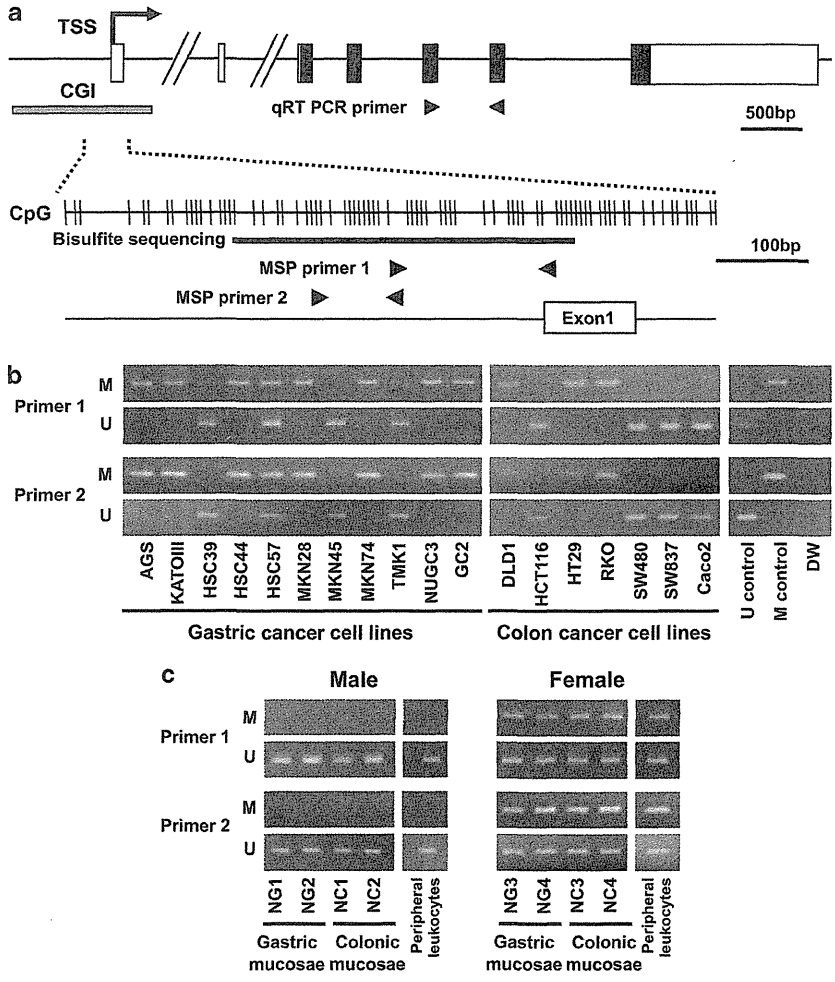

Figure 1. *Genomic structure of FHL1 and its methylation status in cancer cell lines, normal-appearing mucosae and peripheral leukocytes.* (**a**) Genomic structure of *FHL1* and a CpG map of its promoter CGI. Open box, non-coding exon; closed box, coding exon; arrow, transcription start site (TSS);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

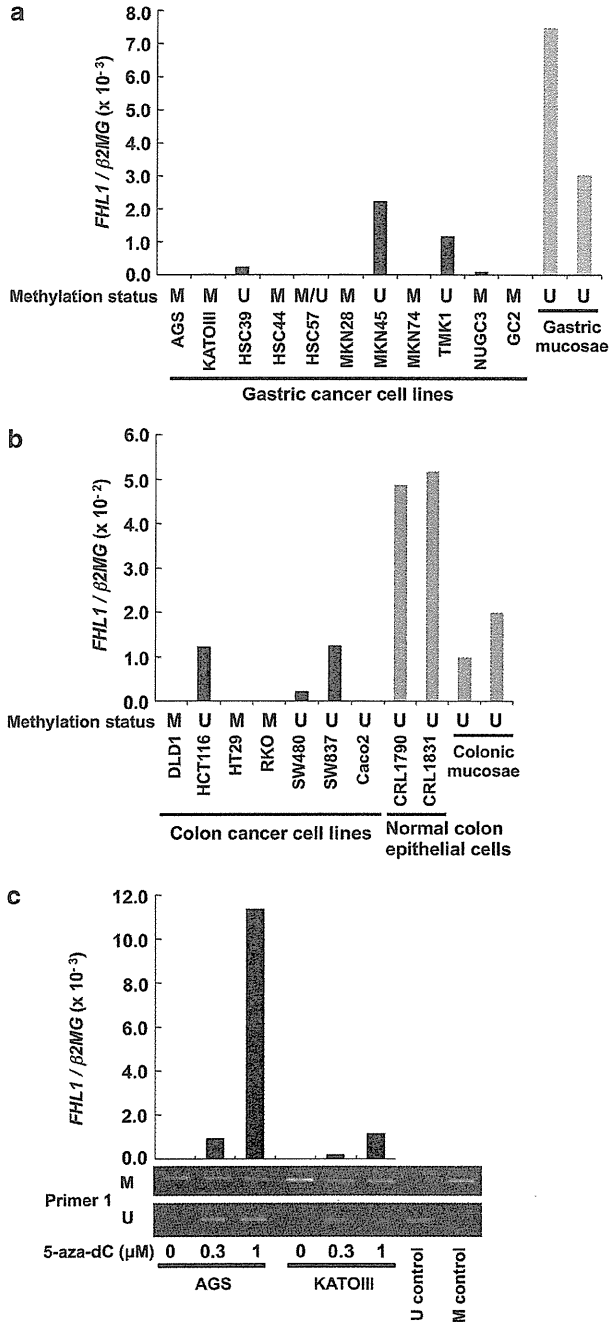

Figure 2. Methylation-silencing of *FHL1* in gastrointestinal cancer cell lines. (**a**) qRT–PCR of *FHL1* in gastric cancer cell lines and normal-appearing gastric mucosae. Results of MSP in Figure 1b are shown by M, M/U and U. M, only methylated DNA detected; M/U, both methylated and unmethylated DNA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

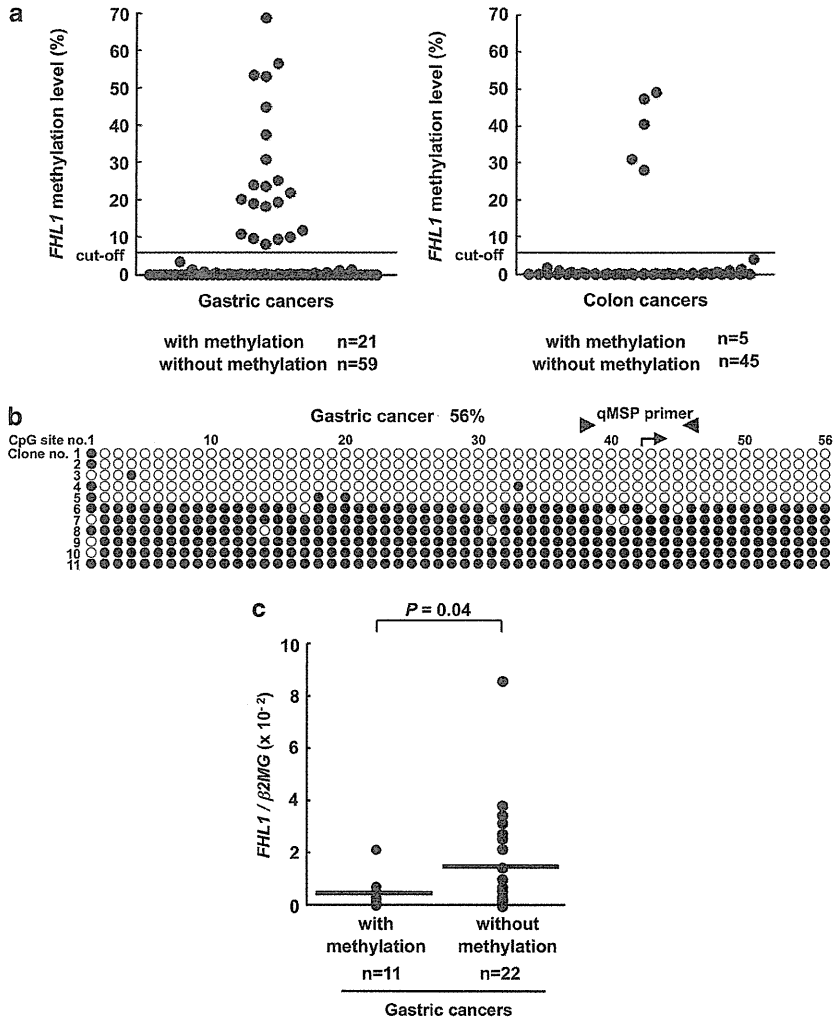

Figure 3. Methylation of *FHL1* in surgical gastrointestinal cancer specimens and its effect on expression. (**a**) Methylation levels in gastric (left) and colon (right) cancers derived from male patients. A horizontal line shows a cutoff value of 6%. *FHL1* was methylated in 21 of 80 primary gastric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

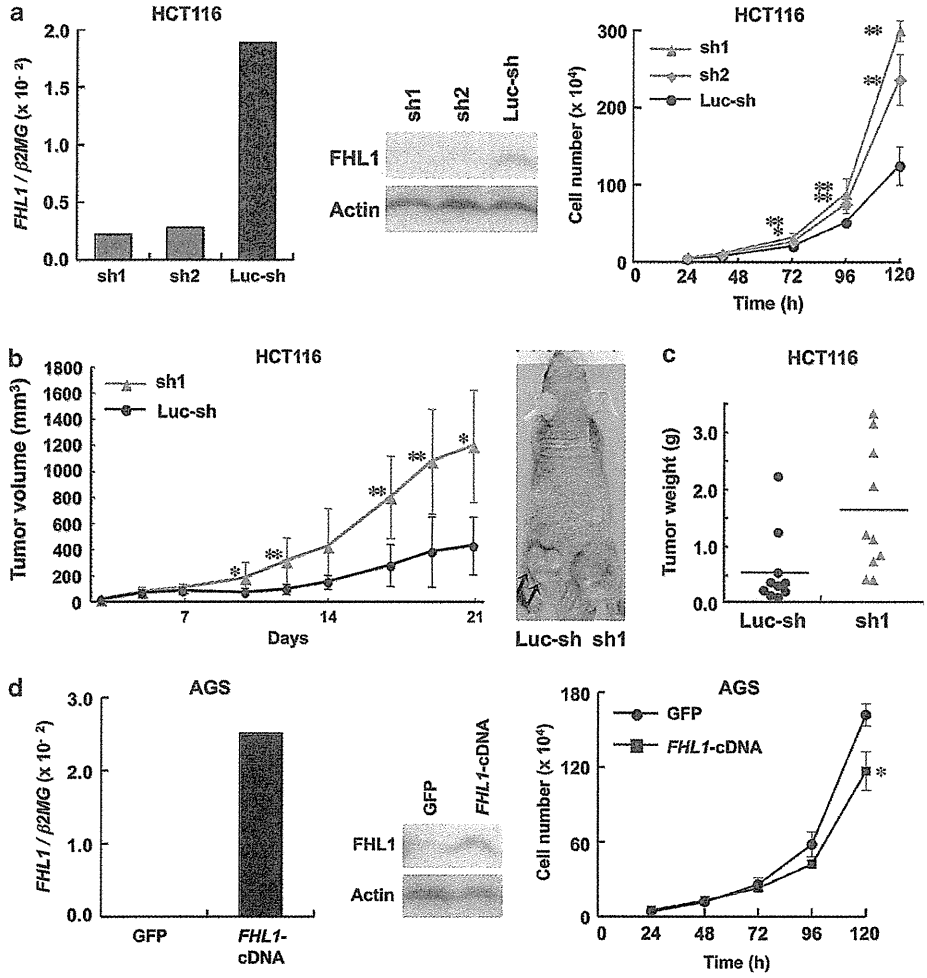

Figure 4. Growth-suppressive activity of *FHL1 in vitro* and *in vivo*. (**a**) *FHL1* knockdown and the resultant increased growth of HCT116 cells. Decreased expression of *FHL1* by its knockdown was confirmed by qRT–PCR (left) and western blot (middle). Growth rates of cells with *FHL1* knockdown were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

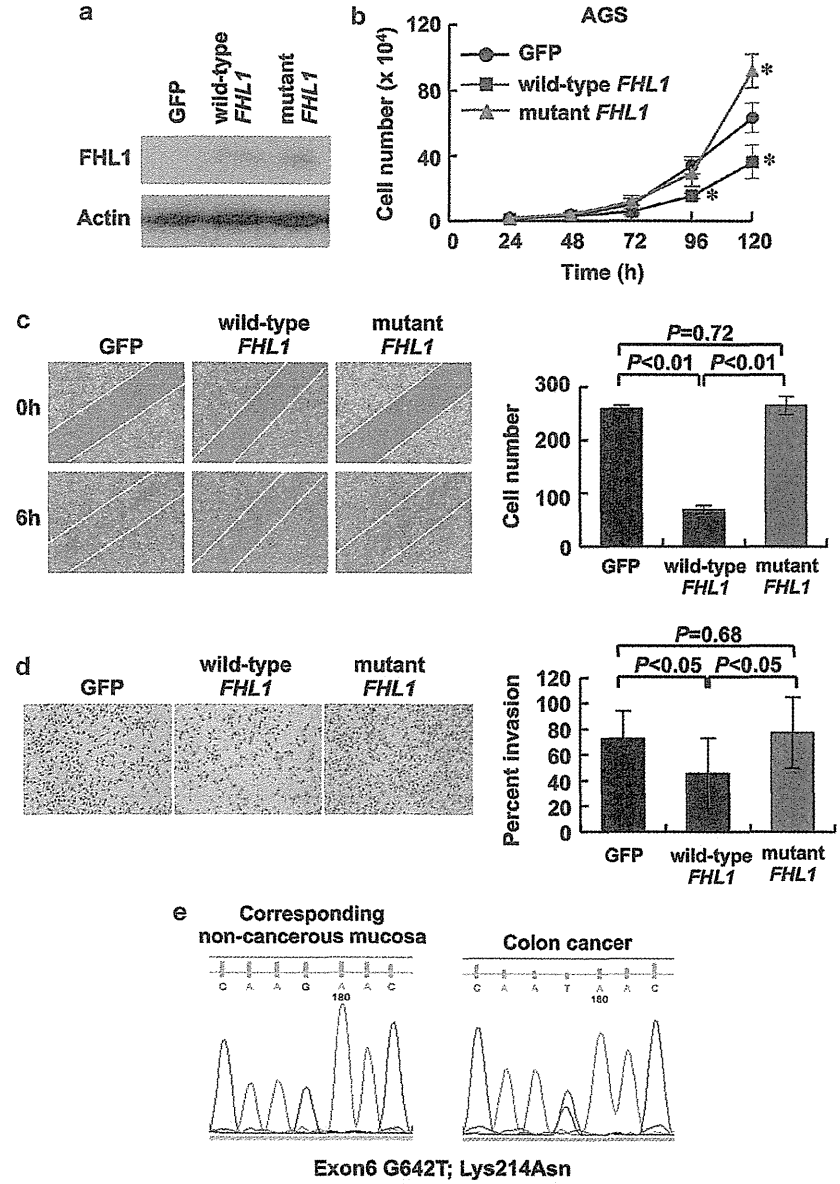

Figure 5. Inhibitory effects of *FHL1* on migration and invasion, and the lack of such functions in *FHL1* with the G642T mutation in AGS. (**a**) Expression levels of exogenous wild-type and mutant *FHL1* detected by western blot. (**b**) The growth-suppressive effect of the wild-type *FHL1*, and the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

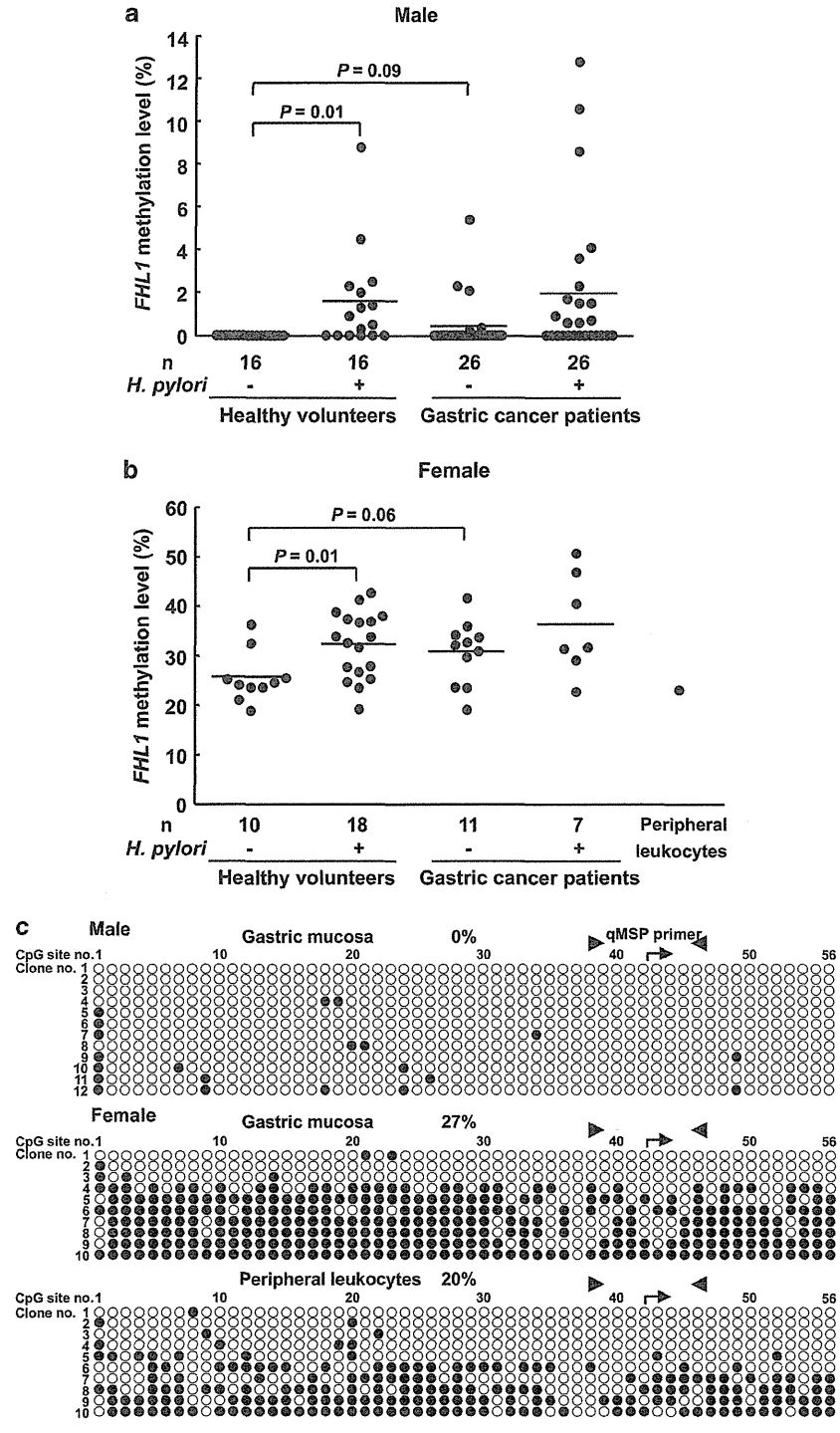

Figure 6. *FHL1* methylation levels in male and female gastric mucosae. (**a**) Methylation levels in male gastric mucosae of healthy volunteers and non-cancerous mucosae of gastric cancer patients. A horizontal line represents the mean methylation level for each group. Among healthy volunteers, *FHL1*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

ACKNOWLEDGEMENTS

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Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc)

Original Article

Methylation of Breast Cancer Susceptibility Gene 1 (*BRCA1*) Predicts Recurrence in Patients With Curatively Resected Stage I Non-Small Cell Lung Cancer

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BACKGROUND: Even after early detection and curative resection of early stage non-small cell lung cancer (NSCLC), a significant fraction of patients develop recurrent disease. Molecular biomarkers that can predict the risk of recurrence thus need to be identified to improve clinical outcomes. **METHODS:** Using the methylation-specific polymerase chain reaction assay, promoter methylation of the breast cancer susceptibility gene 1 (*BRCA1*) was assessed in cancer tissues from 70 patients with curatively resected stage I NSCLC. The clinical relevance of *BRCA1* methylation status was evaluated in terms of outcome of the disease. **RESULTS:** Methylation of the *BRCA1* promoter was detected in 13 of 70 patients (18.6%). Multiple logistic regression analysis revealed that *BRCA1* methylation was an independent risk factor for recurrence ($P = .0197$) and that patients with *BRCA1* methylation demonstrated significantly poorer recurrence-free survival compared to those without ($P = .0139$). Cox's proportional hazard regression analysis revealed that *BRCA1* methylation was an independent risk factor for recurrence-free survival ($P = .0155$). **CONCLUSIONS:** Methylated *BRCA1* can be a potential biomarker that predicts the prognosis after curative resection of stage I NSCLC. Considering that *BRCA1* plays a role in chemotherapy-induced apoptosis, it is plausible that identification of methylated *BRCA1* could provide information that is clinically relevant to tailored adjuvant therapy. **Cancer 2013;119:792–8.** © *2013 American Cancer Society.*

KEYWORDS: *BRCA1*, methylation, epigenetics, prognosis, non-small cell lung cancer.

INTRODUCTION

Lung cancer has become the leading cause of cancer deaths in many countries. The treatment strategy chosen for patients with lung cancer is generally guided by tumor–node–metastasis (TNM) classification. Surgery with curative intent is the standard treatment of choice for patients with stage I non–small cell lung cancer (NSCLC). However, even after complete resection of stage I NSCLC, there is a wide spectrum of outcomes.[1,2] The possibility that molecular biomarkers might better predict biological characteristics and outcomes than TNM classification should be investigated.[3]

A growing body of evidence indicates that aberrant methylation of cytosine-guanine dinucleotide (CpG) islands in the promoter regions of tumor suppressor genes silences these genes by blocking transcription.[4–7] Promoter methylation in various tumor suppressor genes has been demonstrated to be involved in the development and/or progression of lung cancer[3,8–10] and has thus been used as a molecular biomarker to accurately predict the outcome of disease.[11]

Germline mutations in the breast cancer susceptibility gene 1 (*BRCA1*) elevate the risk of breast and ovarian cancer development;[12] hypermethylation of the *BRCA1* promoter has been demonstrated in sporadic breast and ovarian cancers.[13,14] It is now known that *BRCA1* is a tumor suppressor gene that encodes a multifunctional protein that is involved in both breast and ovarian cancers.[15] In addition, germline mutations in *BRCA1* are a statistically significant risk factor in the prognosis of pancreatic and cervical cancers.[16] Several studies have investigated the clinical relevance of messenger RNA expression[17] and methylation of the *BRCA1* promoter[18,19] in lung cancer, with emphasis primarily on responses to chemotherapy.[20] The relationship between epigenetic modifications of *BRCA1* and the prognosis of curatively resected stage I NSCLC has not been elucidated.

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In this study, we examined the methylation status of the *BRCA1* promoter in 70 patients with curatively resected stage I NSCLC. Using methylation-specific polymerase chain reaction (PCR), we investigated the possible association of *BRCA1* methylation with the outcome of this disease.

MATERIALS AND METHODS

Study Population

A total of 70 patients who underwent complete resection for stage I NSCLC at National Hospital Organization Kure Medical Center and Chugoku Cancer Center in Japan between June 2005 and March 2009 were enrolled in this study. The end of the follow-up period was defined as December 2011. No patients received any preoperative chemotherapy or radiation therapy. Patients who underwent surgery for Noguchi Type A or B tumors were excluded from this study. The demographic data and clinicopathological features of enrolled patients were collected using an institutional database. Written, informed consent was obtained from all enrolled patients, and the protocol for this study was approved by the institutional review board.

Sample Preparation

Surgically resected lung tissue samples were immediately snap-frozen and subsequently stored in liquid nitrogen. Genomic DNA was extracted from both primary tumors and adjacent normal lung tissue, through use of proteinase K digestion and phenol-chloroform extraction followed by ethanol precipitation, as described.[21]

Bisulfite Modification and Methylation-Specific PCR

For sodium bisulfate modification, DNA was digested using *Bam*HI (New England Biolabs, Ipswitch, Mass), and 1 μg of the digested DNA was denatured in 0.3 N NaOH at 37°C for 15 minutes. The samples underwent 15 cycles of 30-second denaturation at 95°C and 15-minute incubation at 50°C in 3.1 N sodium bisulfite (pH 5.0) and 0.5 mM hydroquinone. The product was desalted with the Wizard DNA cleanup system (Promega), and desulfonated in 0.6 N NaOH. The sample was ethanol-precipitated and dissolved in 20 mL of TE (Tris plus ethylenediamine tetraacetic acid) buffer. Methylation-specific PCR was carried out using 1 μL of the sodium bisulfite–modified DNA; primers for amplification were specific for methylated or unmethylated sequences, as described.[22–24] The primer sequence and PCR conditions for *BRCA1* were as follows: unmethylated forward primer (5′-TGGTAGTTTTTTGG TTTTTGTGGTAATG), unmethylated reverse primer (5′-TCAACAAACTCACACCACACAATCA), methylated forward primer (5′-CGGTAGTTTTTTGGT TTTCGTGGTAACG), methylated reverse primer (5′-TCAACGAACTCACGCCGCGCAATCG), for 37 cycles and annealing at 66°C. All procedures were repeated at least 3 times for each sample.

Immunohistochemical Analysis

Paraffin blocks of resected tumors were cut into 5 -μm slices, then processed using standard deparaffinization and rehydration techniques. A monoclonal antibody against *BRCA1* (1:500; Calbiochem, Billerica, Mass) was used as the primary antibody for detecting protein expression. Immunodetection was performed by incubation with a specific biotinylated secondary antibody followed by use of the Vectastain ABC kit (Vector Laboratories, Burlingame, Calif). 3,3′-Diaminobenzidine (BD Biosciences, Franklin Lakes, NJ) was used as the developing reagent followed by a hematoxylin counterstain.

Statistical Analysis

Associations between clinicopathological characteristics and *BRCA1* methylation were determined using the Fisher's test. Logistic regression analysis was performed to estimate the odds ratios of independent factors for recurrence. The effect of promoter methylation on time to death or recurrence was estimated using the Kaplan-Meier method, and the log-rank test was used to analyze differences between groups. Overall survival was calculated from the date of surgery to the date of death or the last follow-up. Recurrence-free survival was calculated from the date of surgery to the date of recurrence or the last follow-up. Cox's proportional hazard regression analysis was used to analyze the hazard ratios of independent factors for survival. Differences were considered statistically significant with a P value $< .05$.

RESULTS

DNA Methylation Profiles of Tumor Tissues in the Evaluation Set

To identify a prognostic marker associated with recurrence, DNA methylation in 14 genes (*p16*, Cyclin-dependent kinase inhibitor 2A; *APC*, Adenomatous polyposis coli; *CDH1*, Cadherin 1; *RARB*, Retinoic acid receptor beta; *BRCA1*, Breast cancer susceptibility; *TIMP3*, Tissue inhibitor of metalloproteinase-3; *RASSF1A*, Ras association domain family member 1; *p27*, Cyclin-dependent kinase inhibitor 1B; *FHIT*, Fragile histidine triad; *MGMT*, O6-methylguanine-DNA methyltransferase; *TERC*, Telomerase RNA component; *MLH1*, Human mutL homolog 1; *GSTP1*, Glutathione

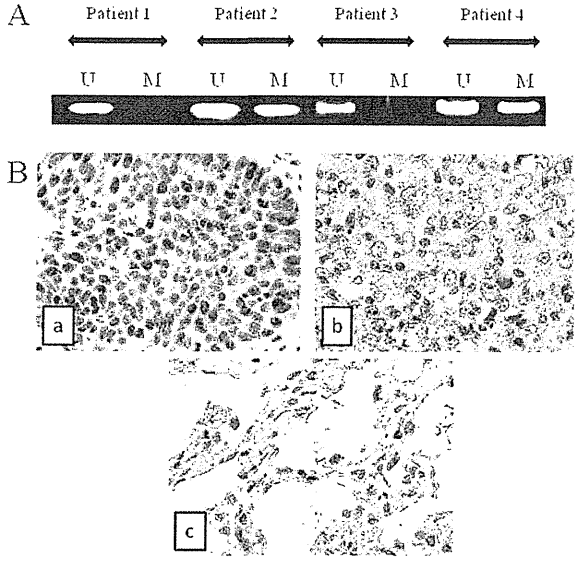

Figure 1. (A) Analysis of *BRCA1* gene methylation is shown in stage I non-small cell lung cancer (NSCLC). Methylation-specific polymerase chain reaction of *BRCA1* was performed using primers specific for unmethylated (U) and methylated (M) forms of *BRCA1* in tumor tissue. *BRCA1* methylation was detected in tumor tissues of patients 2 and 4 (arrow). (B) Immunohistochemical analysis of *BRCA1* proteins is shown in resected NSCLC specimens. Positive immunoreactivity is visible as a brown precipitant in cell nuclei. Representative expression of *BRCA1* protein in the patient without *BRCA1* methylation is shown (panel a), and a concordant lack of immunoreactivity is found in the patient with *BRCA1* methylation (panel b). Normal adjacent lung tissue with expression of *BRCA1* protein is shown as the positive control (panel c). Original magnification, ×400.

S-transferase P 1; and *MASPIN*, Serpin peptidase inhibitor) was analyzed in selected patients, who were matched with respect to sex, age, histology, stage, and differentiation as the evaluation set. In this evaluation set, patients with recurrence showed a significantly higher frequency of *BRCA1* methylation compared with patients without recurrence, and the methylation status in any of the remaining genes did not show statistically significant association with recurrence in this evaluation set (data not shown).

BRCA1 Methylation in Resected Stage I NSCLC

Figure 1A shows representative results of methylation-specific PCR for *BRCA1*. The overall frequency of methylation of the *BRCA1* promoter was 18.6% (13 of 70 patients, Table 1). The proportion of patients with methylated *BRCA1* was quite similar to that observed in previous studies.[25] Five of 13 patients with *BRCA1* methylation in tumor tissues had *BRCA1* methylation in their matched noncancerous tissues as well (Table 1). Three of these 5 patients developed recurrent tumors

TABLE 1. Comparison of *BRCA1* Methylation Status Between Cancerous and Matched Noncancerous Adjacent Tissue

Status		Cancerous Tissue (n = 70)	Matched Noncancerous Tissue (n = 70)
BRCA1	Methylated	13 (18.6%)	5[a]
	Unmethylated	57 (81.4%)	65

[a] These 5 patients showed *BRCA1* methylation in both cancerous and matched noncancerous tissues.

(multiple lung metastases were diagnosed at 2 years after surgery in 1 patient; bone metastasis was diagnosed at 7 months after surgery in 1 patient; and brain metastasis was diagnosed at 7 months after surgery in 1 patient).

Images showing representative immunohistochemical staining of *BRCA1* protein are shown in Figure 1B. Staining within nuclei of tumor cells was considered positive. Immunohistochemical analysis confirmed that all tumors with *BRCA1* promoter methylation showed an absence of or marked reduction in *BRCA1* expression; tumor cells without methylation contained considerably more *BRCA1* protein in their nuclei. As described in previous studies, *BRCA1* expression levels were associated with promoter hypermethylation status.[19,26]

Correlation Between Disease Outcome and BRCA1 Methylation Status

Demographics and clinical characteristics according to *BRCA1* methylation status are shown in Table 2. There was no statistically significant relationship between *BRCA1* methylation status and any of the clinicopathological features that we analyzed.

Table 3 shows the odds ratio for the risk of recurrence in clinicopathological variables by univariate logistic regression analysis. The degree of differentiation, vessel infiltration, and *BRCA1* methylation status were statistically significant as predictors for recurrence ($P = .0347$, $P = .0300$, and $P = .0369$, respectively). Multiple logistic regression analysis of clinicopathological variables that were significant by univariate analysis revealed that *BRCA1* methylation status was an independent risk factor for recurrence ($P = .0197$, Table 4).

Prognostic Impact of BRCA1 Methylation

The median follow-up time of patients in this study population was 1350 ± 458 days. Patients without event survived for at least 900 days after surgery by the end of the follow-up period. Three-year survival rates were 89.5% for overall survival and 83.9% for recurrence-free survival.

Recurrence-free survival rates in patients with *BRCA1* methylation were significantly poorer than in those without *BRCA1* methylation ($P = .0139$, Fig. 2). Overall survival did not reach statistical significance between groups assigned by methylation status ($P = .2588$, data not shown). Cox's proportional hazard regression analysis revealed that *BRCA1* methylation was an independent risk factor for recurrence-free survival ($P = .0155$, Table 5). These data indicate that *BRCA1* methylation could be used as a biomarker for predicting the disease outcome after curative resection of stage I NSCLC. Of the 13 patients with *BRCA1* methylation, 5 developed recurrent tumors. Two of these 5 patients received chemotherapy (paclitaxel and carboplatin in 1 patient; gefitinib in 1 patient) after diagnosis of the recurrent tumors. Two patients were followed without any intensive therapy, and 1 patient received radiation therapy for brain metastasis. Two patients (paclitaxel and carboplatin, 1; best supportive care, 1) survived more than 3 years after the diagnosis of recurrence. In contrast, 7 patients without *BRCA1* methylation developed recurrent tumors. One of these 7 patients survived more than 3 years after surgical resection of pulmonary metastases that had been diagnosed 1 year

TABLE 2. Analysis of Baseline Demographics and Clinical Characteristics of Study Population by *BRCA1* Methylation Status

Characteristic		*BRCA1* Methylated (n = 13)	*BRCA1* Unmethylated (n = 57)	*P*
Age, y		65.2 ± 2.6	68.7 ± 1.2	.2193
Sex	Female	5	12	
	Male	8	45	.2801
Histology	AD	10	39	
	SQ	2	13	
	Others	1	5	.8204
Differentiation	Well	6	24	
	Moderately, poorly	7	33	.7901
Lymphatic duct infiltration	+	6	17	
	–	7	40	.3297
Vessel infiltration	+	7	20	
	–	6	37	.2258
Pathological stage	IA	8	36	
	IB	5	21	.9132
Smoking	Current, former	9	46	
	Never	4	11	.5141
Chronic lung disease	+	8	24	
	–	5	33	.2323
Any prior tumor	+	0	13	
	–	13	44	.1074

Abbreviations: AD, adenocarcinoma; SQ, squamous cell carcinoma.

TABLE 4. Multiple Logistic Regression Analysis of Clinicopathological Variables Found to Be Significant by Univariate Analysis

Variables		OR	95% CI	*P*
BRCA1 methylation status	(+/–)	6.3450	1.3503–33.4334	.0197
Differentiation	(Well/moderately, poorly)	0.1729	0.0226–0.8019	.0232
Vessel infiltration	(+/–)	1.5430	0.3648–6.7321	.5500

Abbreviations: CI, confidence interval; OR, odds ratio.

TABLE 3. Univariate Logistic Regression Analysis of Clinicopathological Valuables for Recurrence

Factors		Recurrence +; Event n = 12	Recurrence –; Unevent n = 58	OR	95% CI	*P*
Age > 70 y	Yes/no	7/5	23/35	2.1304	0.6079–7.9791	.2346
Sex	Female/male	2/10	15/43	0.5733	0.0820–2.5028	.4844
Histology	AD/SQ, others	6/6	43/16	0.3488	0.0948–1.2697	.1083
Differentiation	Well/moderately, poorly	2/10	28/30	0.2143	0.0311–0.9030	.0347
Lymphatic duct infiltration	+/–	5/7	18/40	1.5873	0.4207–5.6656	.4820
Vessel infiltration	+/–	8/4	19/39	4.1053	1.1448–17.0146	.0300
Pathologic stage	IA/IB	6/6	38/20	1.8999	0.5310–6.8335	.3177
Smoking	Current, former/never	11/1	44/14	3.4999	0.5970–66.7958	.1861
Chronic lung disease	+/–	8/4	24/34	2.8333	0.7974–11.6248	.1084
Any prior tumor	+/–	3/9	10/48	1.6000	0.3142–6.5550	.5417
BRCA1 methylation	+/–	5/7	8/50	4.4643	1.0996–17.8341	.0369

Abbreviations: AD, adenocarcinoma; CI, confidence interval; OR, odds ratio; SQ, squamous cell carcinoma.

after the resection of his primary lung cancer. Four of these 7 patients survived less than 4 months after diagnosis of their recurrent tumors and died of cancer; 2 patients survived less than 6 months, with recurrent tumors by the end of the follow-up period.

Correlation Analysis of Combination Genes With BRCA1

Because the methylation status of *p16*, *APC*, *CDH1*, *RASSF1A*, and *FHIT* were reported to predict the risk of recurrence in curatively resected stage I NSCLC,[3,8] the association between methylation of these genes and recurrence was assessed in all 70 study patients. Promoter methylation was detected in 20.0% for *p16*, 34.3% for *APC*, 52.9% for *CDH1*, 30.0% for *RASSF1A*, and 27.1% for *FHIT*. Among these genes, p16 methylation, which was demonstrated to have association with recurrence by Brock et al,[3] was not significantly but marginally associated with recurrence in our patients (odds ratio, 3.89; 95% confidence interval (CI), 0.972-15.170; $P = .0546$).

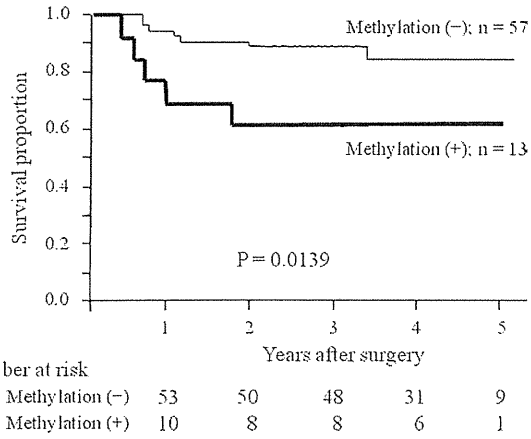

Figure 2. Patient survival is classified by *BRCA1* methylation status. Recurrence-free survival differed significantly between patients with *BRCA1* methylation and those without ($P = .0139$). *P* value was calculated using the log-rank test.

Methylation of the other 4 genes did not show statistically significant association with recurrence. After combination analyses, methylation of either *p16* or *BRCA1* was associated with an increased risk of recurrence (odds ratio, 13.13; 95% CI, 3.052-91.744; $P = .0003$), and significantly poorer recurrence-free survival ($P = .0004$). Methylation of either *FHIT* or *BRCA1* was associated with an increased risk of recurrence (odds ratio, 6.16; 95% CI, 1.628-30.228; $P = .0067$), and significantly poorer recurrence-free survival ($P = .0070$).

DISCUSSION

Hypermethylation of CpG islands in the promoter region of various genes has been reported in lung cancer. Gene silencing through epigenetic alterations has been widely implicated in a variety of pathologic processes, including cancer induction and progression.[3,8–10] Although many investigators have studied various clinicopathological features that might predict the outcome of curatively resected lung cancer, the clinical relevance of aberrant promoter methylation to the outcome of disease has been investigated by only a few groups. These investigations have identified aberrant methylation of specific genes or cohypermethylation of several genes as risk factors for recurrence. Recent reports have identified *p16*, H-cadherin 13 (*CDH13*), *RASSF1A*, *APC*, and *FHIT* as risk factors for recurrence in patients with stage I NSCLC treated by curative surgery.[3,8] Results of multivariate analysis in this study indicate that *BRCA1* methylation had significant effects on the outcome of the disease, even in curatively resected stage I NSCLC. Moreover, correlation analysis demonstrated that the combination of *BRCA1* with *p16* or *FHIT* seemed to be the better predictor of the outcome of disease; however, because the cohort of this study was small, future prospective studies using larger sample sizes are needed to investigate how methylation of *BRCA1* contributes to the outcome of curatively resected stage I NSCLC.

TABLE 5. Cox's Proportional Hazard Regression Analysis of Prognostic Variables Found to Be Significant by Univariate Analysis

		Univariate Analysis			Multivariate Analysis		
Variables		HR	95% CI	*P*	HR	95% CI	*P*
BRCA1 methylation status	(+/−)	3.6934	1.0923–11.5869	.0366	4.7256	1.3780–15.0518	.0155
Differentiation	(Well/moderately, poorly)	0.2320	0.0356–0.8807	.0302	0.1864	0.0283–0.7219	.0129

Abbreviations: CI, confidence interval; HR, hazard ratio.