stimulated NF-κB activation and mRNA expression of several inflammatory factors in a dose-dependent manner, and prevented degradation of IκB-α and phosphorylation of p65 subunit. To evaluate the effects of CAPE on *H. pylori*-induced gastritis, Mongolian gerbils were inoculated with *H. pylori*, fed diets containing 0.1 % CAPE, and sacrificed after 12 weeks. Infiltration of neutrophils and mononuclear cells and expression of NF-κB p50 subunit and phospho-IκB-α were significantly suppressed by CAPE treatment in the antrum of *H. pylori*-infected gerbils. Labeling indices for 5′-bromo-2′-deoxyuridine both in the antrum and corpus were markedly reduced at the highest dose, suggesting a preventive effect of CAPE on epithelial proliferation. Furthermore, in the pyloric mucosa, mRNA expression of inflammatory mediators including tumor necrosis factor-α (TNFα), interferon-γ, interleukin (IL)-2, IL-6, KC (IL-8 homologue), and iNOS was significantly reduced. These results suggest that CAPE has inhibitory effects on *H. pylori*-induced gastritis in Mongolian gerbils through the suppression of NF-κB activation, and may thus have potential for prevention and therapy of *H. pylori*-associated gastric disorders [117].

Statin, a 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor

Statins are commonly used lipid-lowering drugs that reduce the risk of cardiovascular morbidity and mortality. Although recent studies have pointed to chemopreventive effects of statins against various cancers, their efficacy for gastric cancer is unclear. Thus, pitavastatin, a lipophilic statin, was examined on *H. pylori*-associated stomach carcinogenesis and gastritis using Mongolian gerbil and mouse models. The incidences of *H. pylori*-associated gastric adenocarcinomas and degrees of chronic gastritis were not decreased by pitavastatin compared with those of control values. Expression of IL-1β and TNFα mRNAs in the pyloric mucosa was markedly upregulated in pitavastatin-treated animals. Furthermore, in the *H. pylori*-infected groups, serum total cholesterol, triglyceride, and low-density lipoprotein levels were significantly increased by pitavastatin treatment, contrary to expectation. In the short-term study, *H. pylori*-infected gerbils and mice also showed significant upregulation of serum triglyceride levels by pitavastatin, whereas total cholesterol was markedly reduced and low-density lipoprotein exhibited a tendency for decrease in non-infected animals. These findings indicate pitavastatin to be ineffective for suppressing gastritis and chemoprevention of gastric carcinogenesis in *H. pylori*-infected gerbils. Our serologic results also suggest that the *H. pylori* infection and consequent severe chronic gastritis interfere with the cholesterol-lowering effects of pitavastatin [116].

Intestinal metaplasia and intestinalization of gastric cancer

Human gastric-and-intestinal-mixed-type intestinal metaplasia: aberrant expression of transcription factors

H. pylori plays a causative role in the development of chronic atrophic gastritis and intestinal metaplasia as well as stomach neoplasms. Although intestinal metaplasia has long attracted attention as a putative preneoplastic lesion for stomach cancers, its clinicopathologic significance has yet to be clarified in detail. Using gastric and intestinal epithelial cell markers, intestinal metaplasia was here divided into two major types: a gastric-and-intestinal-mixed type and a solely intestinal type [31, 121]. In the former, gastric and intestinal phenotypic markers appeared not only within the glandular but also at the same cellular level [66]. Furthermore, neuroendocrine cells also showed intestinalization along with their exocrine counterparts [74]. The molecular mechanisms of intestinal metaplasia include the ectopic expression of CDX1/CDX2 [2, 56, 88], OCT-1 [32], and members of the Erk pathway. Suppression of the expression of gastric transcription factors such as SOX2 [121], genes that are involved in the Sonic hedgehog pathway, and RUNX3 [71], a tumor suppressor gene, could be additional relevant alterations. The expression of PDX1 may also be associated with pseudopyloric gland metaplasia and intestinal metaplasia [84]. Detailed analysis of gene regulation may shed light on the molecular bases of gastric lesions, leading to strategies for chemoprevention [122].

Intestinal metaplasia in experimental animals

Experimentally, a phenotypic shift from gastric-and-intestinal-mixed-type intestinal metaplasia to solely intestinal type could be clearly observed on sequential observation of rat stomach treated with X-rays [138]. In Mongolian gerbil model, gastric-and-intestinal-mixed-type intestinal metaplasia was found to appear first, followed by the solely intestinal type with appearance of Paneth cells during the overall course of *H. pylori* infection in the HPGs [69].

Summarizing these data, it was suggested that intestinal metaplasia might be caused by the gradual intestinalization of stem cells from the gastric-and-intestinal-mixed type to the solely intestinal type.

Intestinalization of adenocarcinoma

Human gastric adenocarcinomas have been classified by Lauren into two major groups, the "intestinal" and "diffuse" types [45], which respectively nearly correspond to the "differentiated" and "undifferentiated" types [65, 91]. However, the above-mentioned classifications are inadequate for

studies of histogenesis of gastric carcinomas and phenotype expression at the cellular level because they confuse intestinal phenotypic cancer cells with a "diffuse" structure and gastric phenotypic ones with the "intestinal" type of Lauren [113]. The phenotypic expression of gastric cancer cells of each histological type can be clearly classified into gastric and intestinal epithelial cell types by immunohistochemistry using gastric and intestinal epithelial cell markers such as MUC5AC, MUC6, MUC2, and villin, independent of the histological type (Table 2) [16, 37, 40, 57–59, 98, 113, 135]. Gastric cancers comprising epithelial elements presenting only gastric or intestinal phenotypic expression are classified as of gastric, or intestinal phenotypes, respectively. Those with both gastric type cells and intestinal type cells have a gastric-and-intestinal-mixed phenotype, while the remainder exhibiting neither are grouped as a null type [37, 57, 98, 108, 135].

It has been suggested that "intestinal" type carcinomas arise in intestinalized mucosa, whereas the "diffuse" type develops from the gastric mucosa proper [11, 13, 45, 65] and a number of authors have proposed that intestinal metaplasia is a precancerous lesion for differentiated type gastric cancers [11, 12, 63, 89, 136, 137]. However, this hypothesis is based on morphological similarities between cancers and intestinal metaplasia in the surrounding mucosa and previous studies on phenotypic expression of each intestinal metaplastic or stomach cancer cells have pointed to several contradictions [16, 24, 37, 44, 57, 59, 83, 105–108]. In both experimental animals and humans, gastric cancers at early stages, independent of the histological type, mainly consist of gastric type cancer cells, and a phenotypic shift from gastric to intestinal phenotypic expression is clearly observed with progression [4, 104–106, 109, 113, 131]. When the phenotypic classification is compared in early and advance stomach cancers, shift from gastric toward intestinal, and then null phenotypes was observed [57, 59] (Fig. 4a).

Regarding the histogenesis of gastric cancers, it would be logical if those originating from intestinal metaplasia should be of the intestinal type. Even if the phenotypic expression of intestinal type gastric cancer cells is unstable, the incidence of intestinal type cancer cells in small gastric cancers should then be higher than in large gastric cancers, the opposite from the actual case, and expression in fact appears to be stable [106]. In addition, on analysis of microsatellite instability, Tamura et al. [102] found that the majority of differentiated adenocarcinomas of the stomach may develop through a de novo pathway from the viewpoint of the microsatellite alterations. Endoh et al. [17] also clarified that genetic backgrounds of differentiated type tumors were quite different among cellular phenotypes. Thus, it has been proposed that intestinal metaplasia is important not as a precancerous lesion but as a paracancerous phenomenon [16, 54, 108]. Therefore, many questions remain regarding its pathogenesis as well as the actual relationship to gastric cancers.

Gastric and intestinal phenotypic expression in stomach cancers in carcinogen-treated and *H. pylori*-infected Mongolian gerbils

The *H. pylori*-infected *Mongolian gerbil* has been established as an appropriate animal model for studies of stomach cancer development. However, there have hitherto been no data on the phenotypic classification of glandular stomach cancers in *H. pylori*-infected and non-infected Mongolian gerbils. Thus, the phenotypes of 50 and six advanced glandular stomach cancers in *H. pylori*-infected and non-infected gerbils, respectively, were analyzed using several gastrointestinal epithelial phenotypic markers. The lesions were divided phenotypically into 21 gastric, 24 gastric-and-intestinal mixed, four intestinal and one null types, with 90.0 % of the lesions harboring gastric elements and

Table 2 The phenotypic markers for gastrointestinal epithelial cells

Tissue types	Cell types	Markers for human tissues	Markers for Mongolian gerbils
Gastric	Foveolar	MUC5AC	Human gastric mucin (HGM)
		Periodic acid-Schiff staining (PAS)	PAS
	Pyloric	MUC6 Paradoxical concanavalin A staining (PCS)	PCS
Intestinal	Goblet	MUC2	Small intestinal mucinous antigen (SIMA)
		Alcian blue CDX2	Alcian blue
	Absorptive	Villin	Intestinal type alkaline phoshate (I-ALP)
		CD 10 CDX2	CD 10

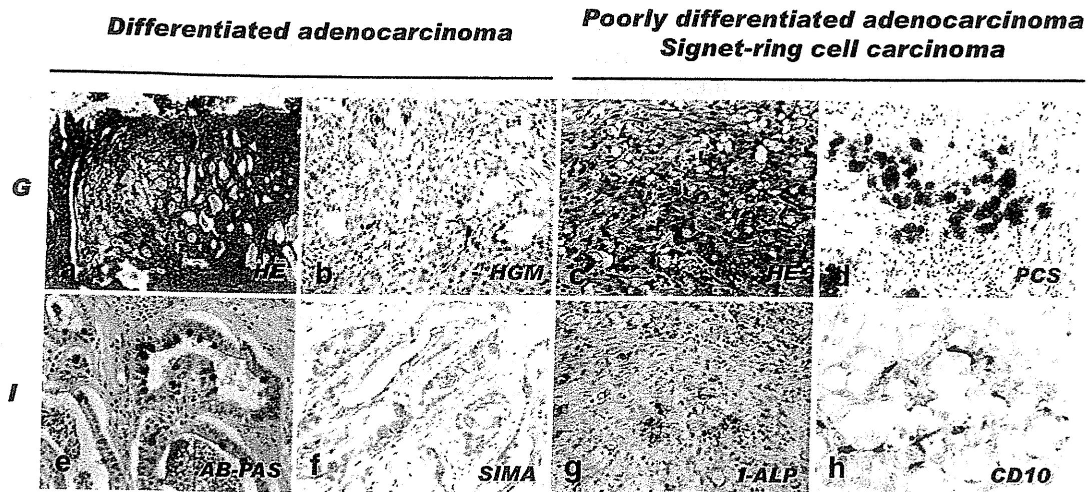

Fig. 3 Adenocarcinomas with gastric or intestinal phenotypic expression in MNU-induced *H. pylori*-infected Mongolian gerbils. **a**, **b**, **e**, **f** Differentiated adenocarcinoma. **c**, **d**, **g**, **h** Poorly differentiated adenocarcinoma/signet-ring cell carcinoma. **a**, **b**, **c**, **d** Gastric type (*G*, *colored red*). **e**, **f**, **g**, **h** Intestinal type (*I*, *colored blue*). **a**, **c** HE staining. **d** Paradoxical concanavalin A staining (*PCS*). **e** Alcian blue-periodic acid-Schiff (*AB-PAS*) staining. **b**, **f**, **g**, **h** Immunohistochemistry with antibodies against human gastric mucin (*HGM*) (**b**), small intestinal mucinous antigen (*SIMA*) (**f**), and intestinal-alkaline phosphatase (*I-ALP*) (**g**), and CD10 (**h**) (Table 2). Reproduced from Ref. [60]with permission

56.0 % demonstrating intestinal phenotypic expression in *H. pylori*-infected Mongolian gerbils. All six lesions were classified as gastric type in non-infected gerbils. There was no clear correlation with the presence of intestinal metaplasia in surrounding mucosa. Most of the advanced adenocarcinomas retain a gastric cellular phenotype, suggesting intestinal metaplasia as a paracancerous phenomenon rather than a premalignant condition. On the other hand, more than half of the cancers harbor intestinal phenotypes only in *H. pylori*-infected group compared with none in uninfected cancers with statistical significance. *H. pylori* infection was considered to trigger intestinalization of both stomach cancers and non-neoplastic mucosa (Figures 3 and 4b, Table 2) [60].

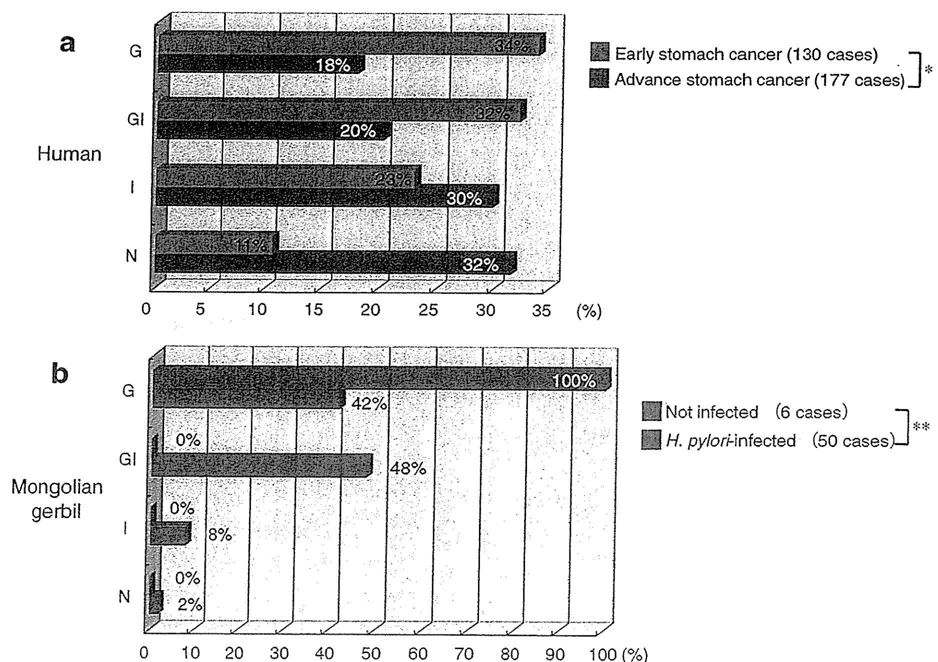

Fig. 4 Intestinalization of stomach cancers with *H. pylori* infection: **a** Phenotypic classification of human stomach cancers in early [59] (*orange bars*) and advanced cases [57] (*Burgundy bars*). Transition is apparent from gastric (*G*), gastric-and-intestinal mixed (*GI*), intestinal (*I*), and toward null (*N*) phenotypes in advanced cancers compared to early cases. *$P<0.0001$, χ^2 test for trend. **b** Phenotypic classification of stomach cancers in Mongolian gerbils. *H. pylori* infection (*red bars*) induced intestinalization of stomach cancers, whereas non-infected animals developed those with only gastric phenotype (*blue bars*)[59]. **$P<0.02$, χ^2 test for trend.

Conclusions

H. pylori infection is a very important factor for gastric carcinogenesis in human stomach. Since the discovery of *H. pylori*, the Mongolian gerbil has become one of the most important model animal for analysis of stomach carcinogenesis and trials of chemoprevention. As revealed by the experimental models described above, it was clarified that *H. pylori* itself only causes chronic inflammation and acts as promoter in stomach carcinogenesis. Further analyses need be conducted to determine mechanisms of carcinogenesis and contribute to chemopreventive methods.

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ORIGINAL ARTICLE

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

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

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INTRODUCTION

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

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

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

RESULTS

Screening of methylation-silenced genes on chromosome X

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

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

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

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

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

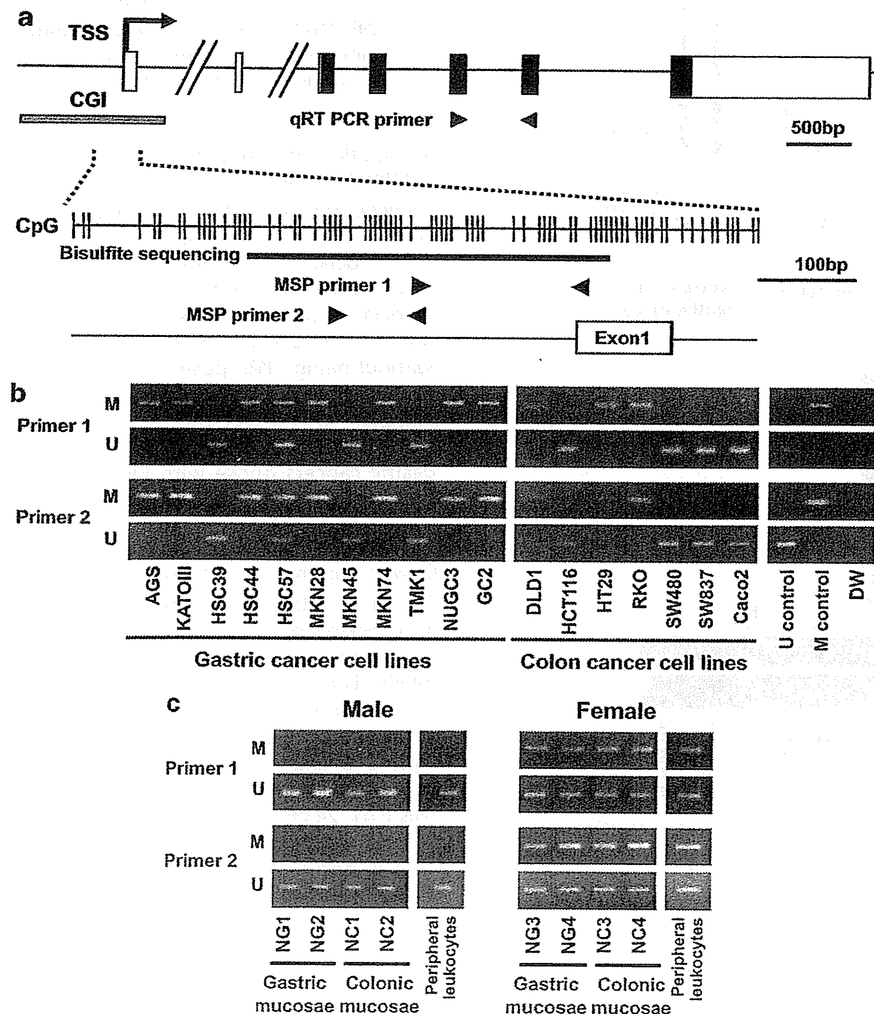

Figure 1. Genomic structure of *FHL1* and its methylation status in cancer cell lines, normal-appearing mucosae and peripheral leukocytes. (**a**) Genomic structure of *FHL1* and a CpG map of its promoter CGI. Open box, non-coding exon; closed box, coding exon; arrow, transcription start site (TSS);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

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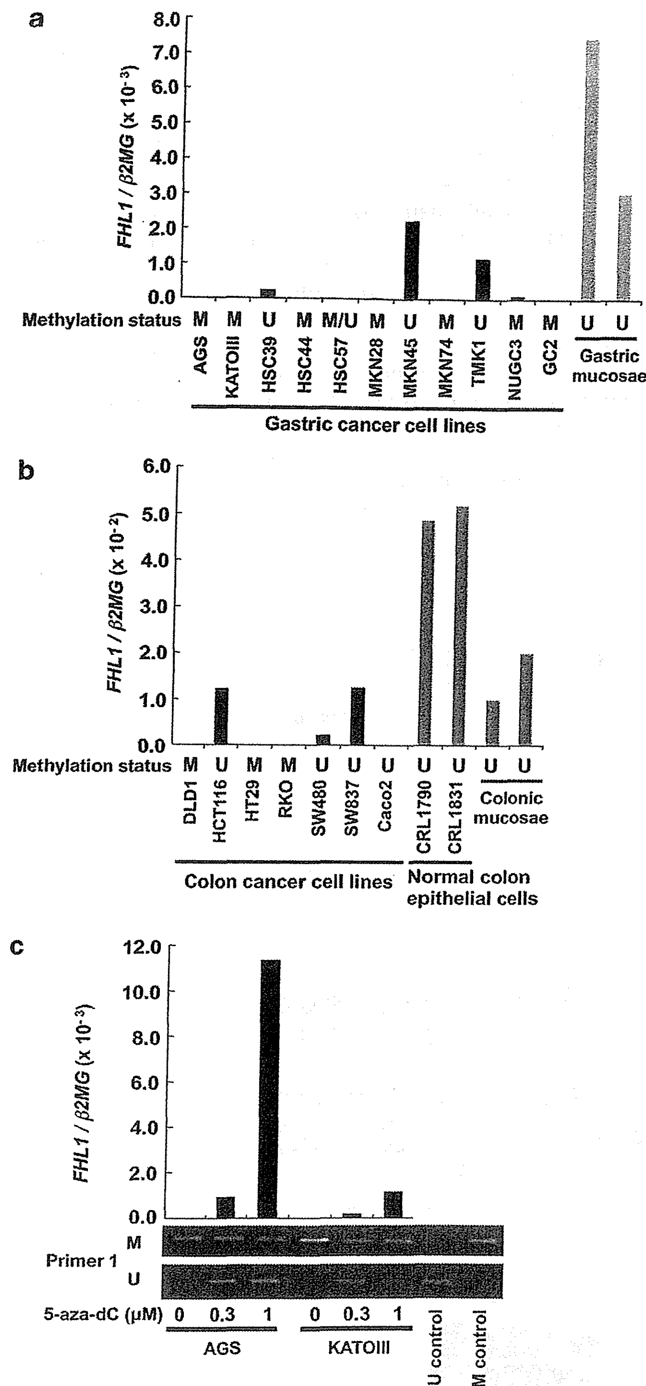

Figure 2. Methylation-silencing of *FHL1* in gastrointestinal cancer cell lines. (**a**) qRT–PCR of *FHL1* in gastric cancer cell lines and normal-appearing gastric mucosae. Results of MSP in Figure 1b are shown by M, M/U and U. M, only methylated DNA detected; M/U, both methylated and unmethylated DNA 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%; $P = 2.9 \times 10^{-6}$). *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

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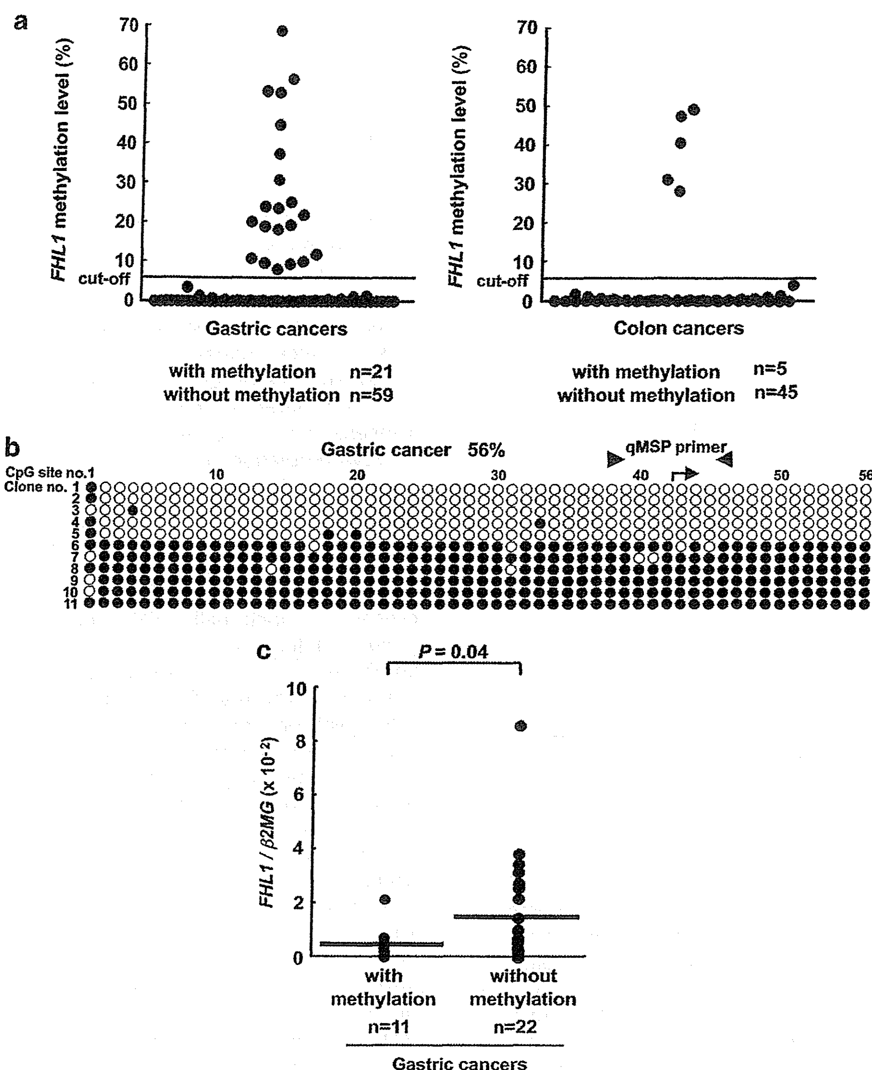

Figure 3. Methylation of *FHL1* in surgical gastrointestinal cancer specimens and its effect on expression. (**a**) Methylation levels in gastric (left) and colon (right) cancers derived from male patients. A horizontal line shows a cutoff value of 6%. *FHL1* was methylated in 21 of 80 primary gastric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

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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

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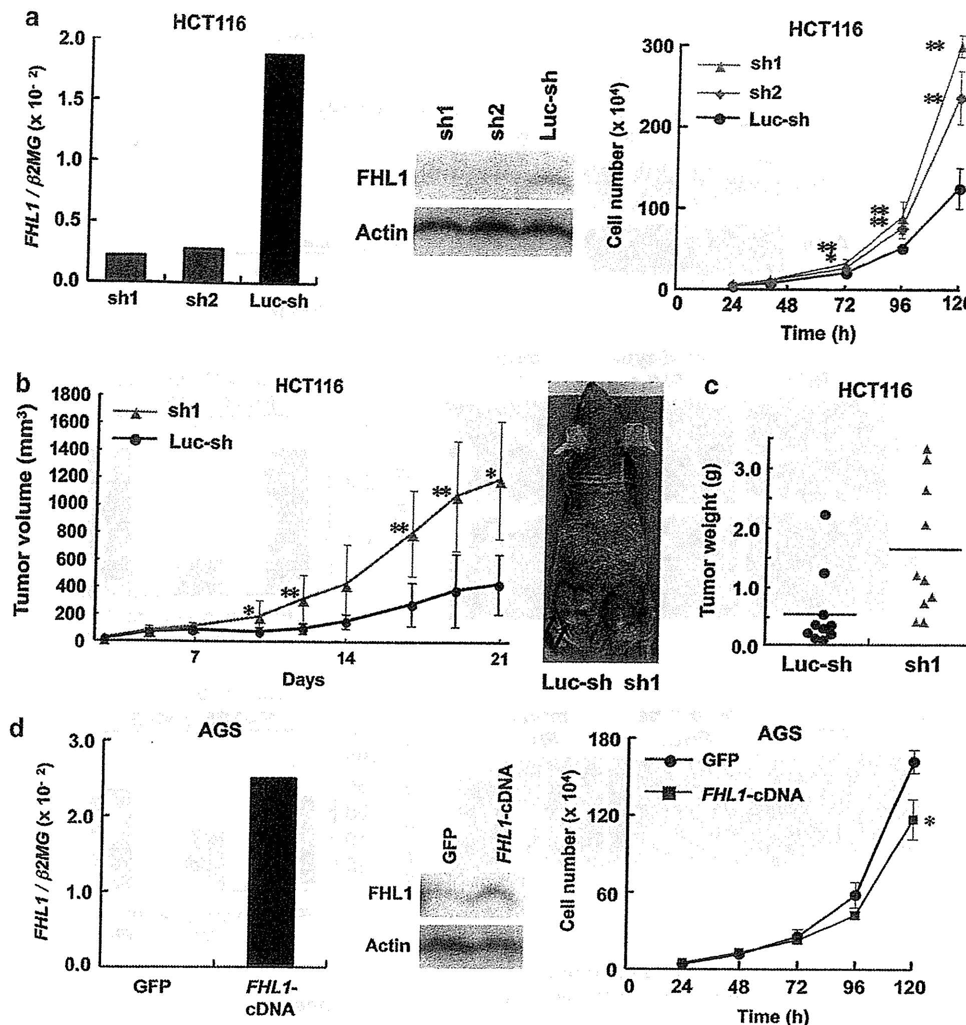

Figure 4. Growth-suppressive activity of *FHL1* *in vitro* and *in vivo*. (**a**) *FHL1* knockdown and the resultant increased growth of HCT116 cells. Decreased expression of *FHL1* by its knockdown was confirmed by qRT–PCR (left) and western blot (middle). Growth rates of cells with *FHL1* knockdown 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 (CRL 1790 and CRL 1831) 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

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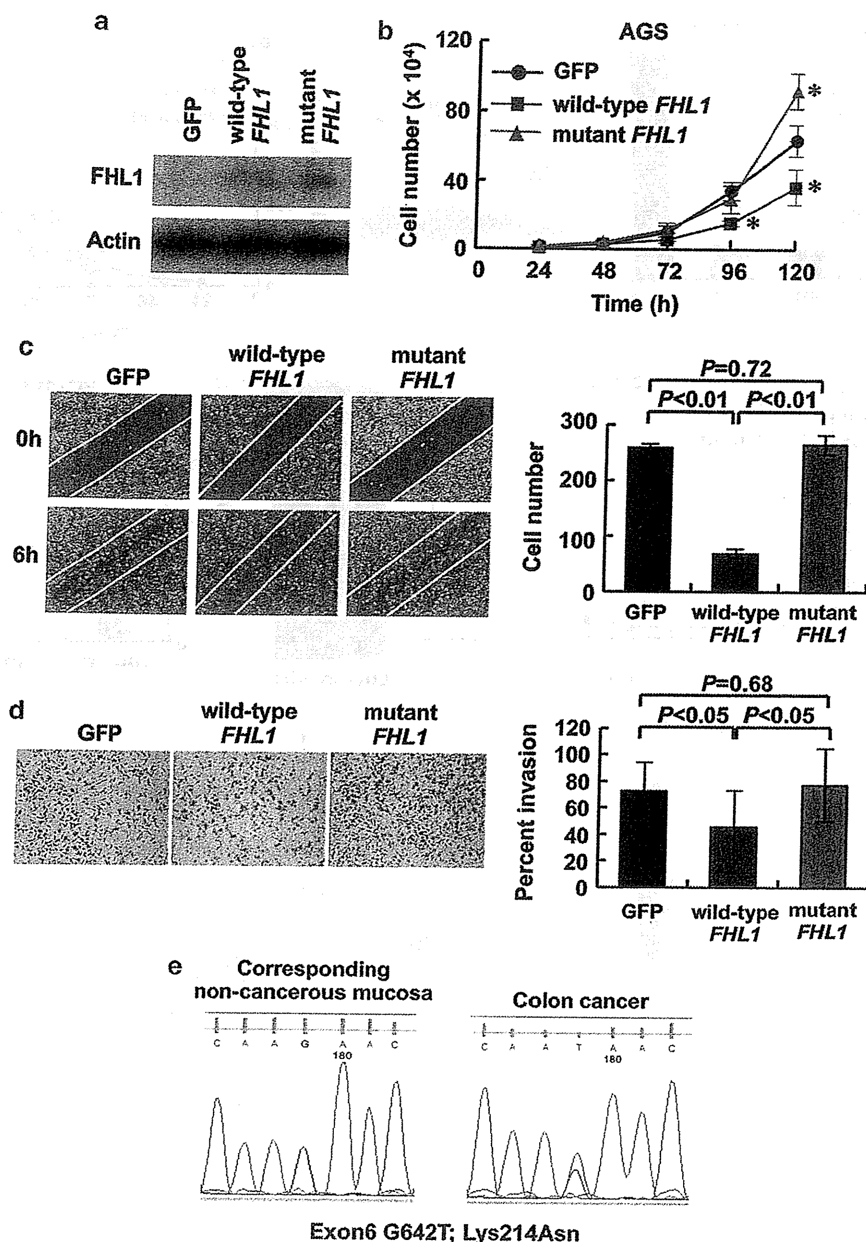

Figure 5. Inhibitory effects of *FHL1* on migration and invasion, and the lack of such functions in *FHL1* with the G642T mutation in AGS. (**a**) Expression levels of exogenous wild-type and mutant *FHL1* detected by western blot. (**b**) The growth-suppressive effect of the wild-type *FHL1*, and the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

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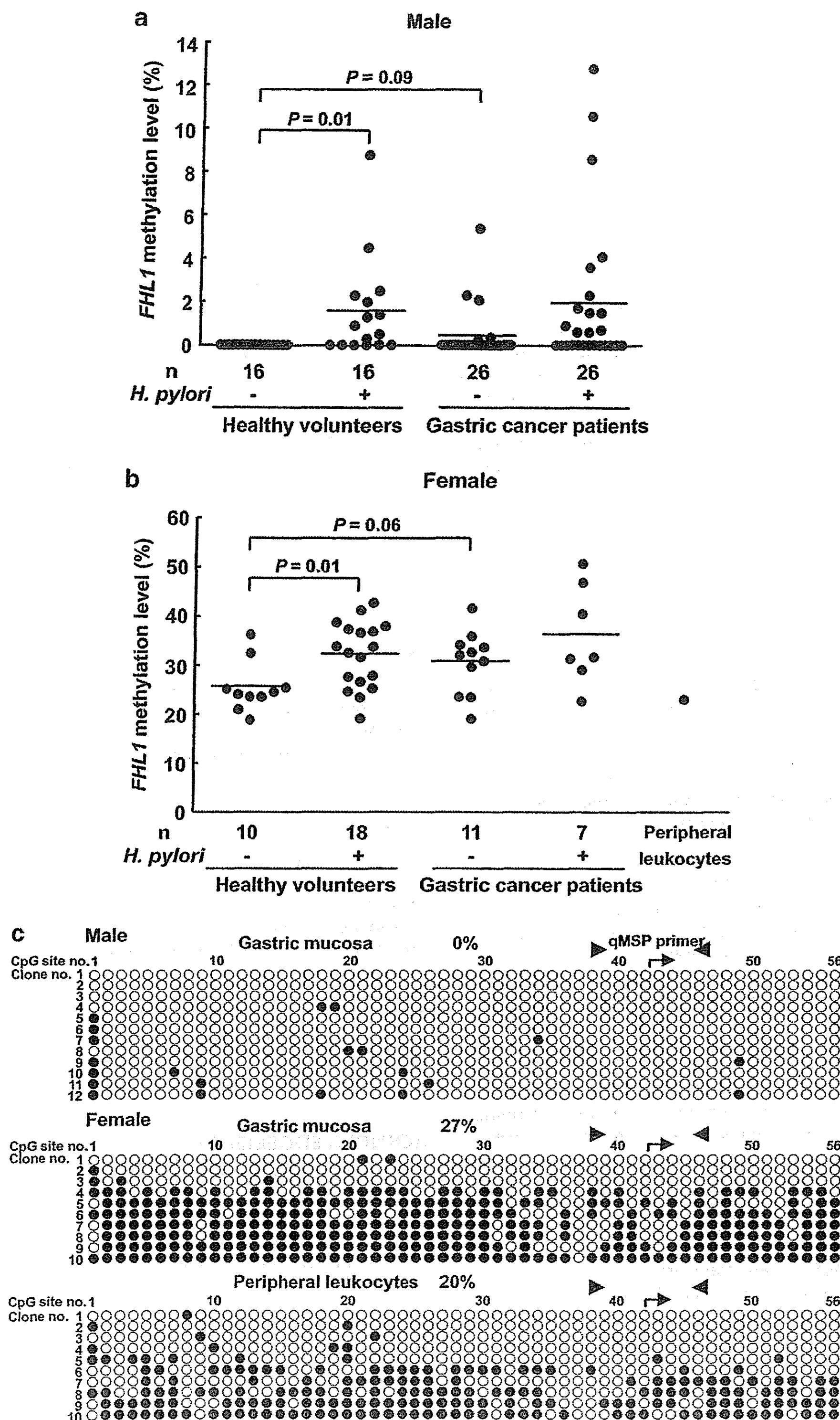

Figure 6. *FHL1* methylation levels in male and female gastric mucosae. (a) Methylation levels in male gastric mucosae of healthy volunteers and non-cancerous mucosae of gastric cancer patients. A horizontal line represents the mean methylation level for each group. Among healthy volunteers, *FHL1* methylation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

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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; 4×10^5 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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Protection from inflammatory bowel disease and colitis-associated carcinogenesis with 4-vinyl-2,6-dimethoxyphenol (canolol) involves suppression of oxidative stress and inflammatory cytokines

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Oxidative stress is associated with various pathological processes including inflammatory bowel disease, which is a major cause of colon cancer. Here, we examined the antioxidative and anti-inflammatory effects of 4-vinyl-2,6-dimethoxyphenol (canolol), a potent antioxidant compound obtained from crude canola oil. Oral administration of 2% dextran sulfate sodium (DSS) resulted in the progression of colitis with shortening of the large bowel length. Administering a diet containing canolol significantly suppressed pathogenesis; diarrhea markedly improved and the length of large bowel returned to almost normal. Pathological examination clearly revealed improvement of colonic ulcers. Production of inflammatory cytokines, i.e. interleukin-12 and tumor necrosis factor-α, was significantly increased during this pathological process; their production was markedly inhibited by canolol. In the azoxymethane/DSS-induced colon cancer model, mice receiving canolol had a reduced occurrence of cancer, to 60%, compared with control mice, 100% of which had colon cancer. The numbers of tumors in each mouse were also significantly reduced in mice receiving the canolol-containing diet (5.6±2.0) compared with azoxymethane/DSS control mice (10.8±4.2). No apparent toxicity of canolol was observed. Moreover, inflammatory cytokines (i.e. cyclooxygenase-2, inducible nitric oxide synthase and tumor necrosis factor-α) and oxidative responding molecules, i.e. heme oxygenase-1, in colon were suppressed during this treatment. In a mouse colon 26 solid tumor model, canolol significantly suppressed cyclooxygenase-2 expression; however, no significant tumor growth inhibition was observed, suggesting that canolol preferably shows chemopreventive effects during the stages of initiation/promotion. Canolol may, thus, be considered a potential cancer preventive agent or supplement.

Abbreviations: 8-OHdG, 8-hydroxydeoxyguanosine; AOM, azoxymethane; BHT, butylated hydroxytoluene; COX-2, cyclooxygenase-2; DAI, disease activity index; DSS, dextran sulfate sodium; ELISA, enzyme-linked immunosorbent assay; HO-1, heme oxygenase-1; IBD, inflammatory bowel disease; IL-12, interleukin-12; iNOS; inducible NO synthase; LPS, lipopolysaccharide; NO, nitric oxide; ROS, reactive oxygen species; SIN-1, 3-(4-morpholinyl)sydnonimine hydrochloride; TNF-α, tumor necrosis factor-α.

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Introduction

Inflammatory bowel disease (IBD) comprises a group of common diseases that manifest chronic inflammation of the colon and small intestine (1–3). The major types of IBD are Crohn's disease and ulcerative colitis. Although IBD itself is rarely fatal, it can greatly diminish the quality of life because of pain, vomiting, diarrhea and other socially unacceptable symptoms. More important, patients with IBD commonly have an increased risk of colorectal cancer, i.e. the risk of colon cancer in patients with ulcerative colitis begins to rise significantly above that of the general population approximately 8-10 years after diagnosis (1–4).

At present, a common therapeutic modality for IBD is use of anti-inflammatory agents, including sulfasalazine (Salazopyrin) and acetylsalicylic acid, steroid hormone and other immunosuppressive agents. Most of these treatments are symptomatic and palliative because the etiology of the disease is not yet established. As a result, the disease persists for a long time. Therefore, a therapeutic/preventive strategy that is based on the mechanism of IBD is an urgent necessity.

Although the exact cause of IBD must be determined, dysfunctional immunoregulation is thought to be the primary reason (1–4). Genetic, infectious, immunological, and psychological factors have also been implicated as influencing the development of IBD. Recently it was also reported that, similar to *Helicobacter pylori*-induced gastritis, bacterial infection may be involved in pathogenesis of IBD, and combination therapy with antibiotics produced a significant therapeutic effect (5–8).

Another possibility concerns reactive oxygen species (ROS): high levels were produced in IBD, which suggests that ROS may be implicated in the molecular etiology of IBD (9,10). The destructive effects of ROS on DNA, proteins and lipids, because of the highly reactive nature of ROS, may contribute to initiation and propagation of the disease (6,7). The investigation of antioxidant agents may, thus, help illuminate the etiology, treatment and prevention of IBD. Indeed, many researchers proved antioxidant treatment of IBD to be effective, not only in animal experiments but also in clinical settings (9,11).

In our laboratory, we identified a potent antioxidant phenolic compound in crude canola (rapeseed) oil, 4-vinyl-2,6-dimethoxyphenol (canolol), which exhibits a more potent alkylperoxyl (ROO$^•$) radical scavenging activity than many well-known antioxidants, such as α-tocopherol, vitamin C, β-carotene, rutin and quercetin (12). Recently canolol was also found in mustard seed oil (13). We previously reported a strong inhibitory capacity of canolol against the endogenous mutagen peroxynitrite ($ONOO^-$), which is a potent oxidizing and nitrating agent, and suppression by canolol of bacterial mutation, via protection from DNA damage (14,15). In related studies, we demonstrated a protective effect of canolol against gastritis and gastric ulcers and a preventive effect on gastric carcinogenesis in the *H.pylori*-infected, carcinogen-treated Mongolian gerbil, which is an excellent animal model of *H.pylori*-induced, chronic active gastritis similar to IBD and involving ROS (16).

Addition of dextran sulfate sodium (DSS) to the drinking water of mice induced acute colitis characterized by bloody diarrhea, ulceration and inflammatory infiltration of leukocytes in the colon, as a result of toxicity to gut epithelial cells and distortion of the integrity of the mucosal barrier (17). The DSS-induced colitis model, which we used in this study, is commonly utilized as a model of inflammatory colitis (5,6). Application of azoxymethane (AOM) together with DSS produces a model of chronic colitis and colitis-associated colon carcinogenesis (18). The purpose of our present study was to evaluate the effectiveness of canolol for inhibition of IBD and

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colitis-associated carcinogenesis using a DSS-induced mouse colitis model and AOM/DSS-induced colon carcinogenesis in mice, respectively. We also investigated the effect of canolol on oxidative stress and inflammatory cytokines during development of colitis and colon carcinogenesis. The toxicity of canolol and its effect on a mouse colon 26 solid tumor model were also examined.

Material and Methods

Chemicals

Canolol (molecular weight, 180 Da), with >95% purity, was synthesized by Junsei Chemical Co., Ltd. (Tokyo, Japan). Antioxidant 2,6-di-*tert*-butyl-4-methylphenol [butylated hydroxytoluene (BHT), Sigma, St Louis, MO] was added to canolol solution (in ethanol) at the concentration of 300 ppm. BHT at this concentration had no significant therapeutic effect on colitis and colon cancer prevention (16). The preparation in solid form or solution was sealed under helium or nitrogen, and stock solution in ethanol was kept at −80°C. DSS was purchased from Wako Pure Chemical (Osaka, Japan), and AOM was from Sigma. 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide was purchased from Dojindo Chemical Laboratory (Kumamoto, Japan).

Diets

The AIN93G diet containing canolol was used in this study with some modifications. Components of the modified AIN93G diet are as follows (g/kg): corn starch, 397; casein, 200; α-corn starch, 132; sucrose, 100; soybean oil, 70; cellulose, 50; AIN93G mineral mixture, 35; AIN93G vitamin mixture, 10; L-cystine, 3.0; choline bitartrate, 2.5; and BHT, 0.014. L-Cystine and BHT were purchased from Sigma; other components were from Oriental Yeast Co., Ltd (Tokyo, Japan). Canolol was first dissolved in soybean oil and then mixed into the diet to the concentration of 0.1 or 0.3%. The control diet contained the same components but no canolol. The diets were sealed under vacuum and were stored at −30°C; they were given daily after being thawed. Each day, leftovers from the previous day's feeding were measured, and new food was provided to replace the amount eaten.

Cell culture

Human embryonic kidney cells HEK293 and human colon cancer cells Caco-2 were cultured in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA), and mouse colon cancer cells colon 26 were cultured in Roswell Park Memorial Institute 1640 medium (Invitrogen), at 37°C in an atmosphere of 5% CO_2/95% air.

Animals and experimental protocol

Female ICR mice, 6 weeks old and weighing 20 to 25 g, and female BALB/c mice, 8 weeks old, were obtained from Kyudo (Tosu city, Saga, Japan). All animals were maintained under standard conditions and were fed water and murine chow *ad libitum*. All experiments were carried out according to the Guidelines of the Laboratory Protocol of Animal Handling, Sojo University, and were approved by the Animal Care Committee of Sojo University.

As to the experimental protocol for the DSS-induced colitis model, ICR mice of canolol treatment groups were fed with diet containing different concentrations of canolol during the entire experimental period (7 days). Control ICR mice were fed with the same diets but without canolol. Two hours after feeding canolol-containing diet, water containing 2% DSS was supplied to all groups except the healthy normal ICR mouse group, for entire 7 days (Supplementary Figure 1A, available at *Carcinogenesis* Online). Fresh diet was supplied daily, and the body weights of mice and amounts of consumed diet were determined each day. According to this protocol, symptoms indicating the severity of colitis obtained by macroscopic observation, such as characteristics of fecal pellets, diarrhea and hematochezia, were recorded. On day 7, the mice were killed, and specimens of blood, colon and liver were collected for biochemical and pathological examinations. After the length of each colon was measured, the colon specimen was fixed with 20% formalin solution and embedded in paraffin. Paraffin-embedded sections (6 μm thick) were prepared as usual for histological examination after hematoxylin and eosin staining, as well as for immunohistochemical staining as described below. Serum obtained from the blood collected was used to determine levels of tumor necrosis factor-α (TNF-α) and interleukin-12 (IL-12), as described below.

As to the experimental protocol for colon carcinogenesis in ICR mice induced by AOM/DSS, on day 1, AOM (at 10 mg/kg) dissolved in saline was administered intraperitoneally, and after 1 week, 2% DSS was given orally in the drinking water for 1 week. The diet was changed to the canolol-containing diet from 2 h before AOM administration and was continued for the entire experimental period of 6 weeks (Supplementary Figure 1B, available at *Carcinogenesis* Online). The amount of food consumed was calculated daily. Six weeks after the AOM injection, mice were killed, and colon and liver specimens were collected. The numbers of tumors in the colon of each mouse were measured.

Evaluation of colitis severity

We evaluated the colitis severity by measuring disease activity index (DAI) semiquantitatively, by measuring colon length as an indirect marker of inflammation, and by using histology after hematoxylin and eosin staining. The DAI was determined by scoring changes in animal weight, presence of occult blood, gross bleeding and stool consistency, as described in the literature (19). We used five grades of weight loss (0: either a gain of weight or no weight loss; 1: 1% to 5% loss; 2: 5% to 10% loss; 3: 10% to 20% loss; 4: more than 20% loss), three grades of stool consistency (0: normal; 2: loose; and 4: diarrhea) and three grades of occult blood (0: negative; 2: occult blood-positive; and 4: gross bleeding). Individual mice were graded, and the mean value for each experimental group was obtained.

Further, histological evaluation of ulcer was carried out to quantitate the degree of colitis. The numbers of ulcer regions were counted in whole-colon mucosa and divided by the total length of the evaluated colon specimens. The numbers of ulcers are expressed in unit length (mm).

Effect of canolol on colon 26 transplanted tumor

The effect of canolol on tumor was further investigated in a mouse colon cancer model. Cultured colon 26 cells (2×10^6) were implanted subcutaneously in the dorsal skin of Balb/c mice. Ten days after tumor inoculation, when tumor reached a diameter of 5–6 mm, canolol (dissolved in corn oil) was orally administration at the dose of 100 mg/kg (0.1 ml), and corn oil without canolol was used for control mice. Administration was carried out every second day, totally for three times. Growth of the tumors was monitored every 2–3 days by measuring tumor volume with a digital caliper, which was estimated by measuring longitudinal cross-section (L) and transverse section (W) according to the formula $V = (L \times W^2)/2$. On day 15 after the first canolol administration when tumor reached a diameter ~12–13 mm, mice were killed and tumor tissues were excised for histological examination and immunohistochemical analysis as described below.

Immunohistochemical analyses of cyclooxygenase-2

Expressions of cyclooxygenase-2 (COX-2) in colon mucosa of mice with DSS-induced colitis and in mice with AOM/DSS-induced colon carcinogenesis, and also in colon 26-implanted syngeneic solid tumor, were detected immunohistochemically as described previously (16), using a rabbit anti-mouse COX-2 polyclonal antibody (diluted 1:500, Cayman Chemical, Ann Arbor, MI) with 3,3′-diaminobenzidine (Wako Pure Chemical) for visualization. Images were analyzed with ImageJ software (National Institutes of Health, Bethesda, MD) for brown deposition of 3,3′-diaminobenzidine as COX-2 positive. One pathologist (T.T.) who was not informed about the samples examined the immunostained slides.

To quantitate the degree of staining, numbers of COX-2-positive cells were counted in whole-colon mucosa in DSS-induced colitis experiment, or counted in a distal quarter of colon mucosa, which is the target region of AOM/DSS in colon carcinogenesis experiment, and divided by the total length of the evaluated colon specimens to compare each sample equally. The numbers of COX-2-positive cells are illustrated in unit length (mm).

In the experiments using colon 26 solid tumor, three representative photographs were taken from each tumor using an AxioCam HRc digital camera and AxioVision v.4.8.2.0 software (Carl Zeiss, Oberkochen, Germany), and average positive areas in the each frame were compared between control and canolol groups.

Enzyme-linked immunosorbent assay for 8-hydroxydeoxyguanosine in the plasma of DSS-induced colitis mice with/without canolol treatment

Oxidative stress in the DSS-induced colitis mice with or without canolol treatment was examined by detecting 8-hydroxydeoxyguanosine (8-OHdG) in plasma, using an enzyme-linked immunosorbent assay (ELISA) kit (8-OHdG Check, JalCA, Fukuroi, Shizuoka, Japan). In brief, blood was drawn from the inferior vena cava after mice were killed, plasma samples were obtained by centrifugation (4°C, 5000*g* for 20 min) and DNA in each sample was then extracted using QuickGene DNA tissue kit (DT-S, Wako Pure Chemical), followed by hydrolysis using an 8-OHdG Assay Preparation Reagent Set (Wako Pure Chemical). The ELISA was then performed to detect 8-OHdG according to the manufacturer's instructions.

Effects of canolol on production of IL-12 and TNF-α in DSS-induced colitis

Serum samples from mice with DSS-induced colitis were obtained as described above, and levels of TNF-α and IL-12 were quantified by using an

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