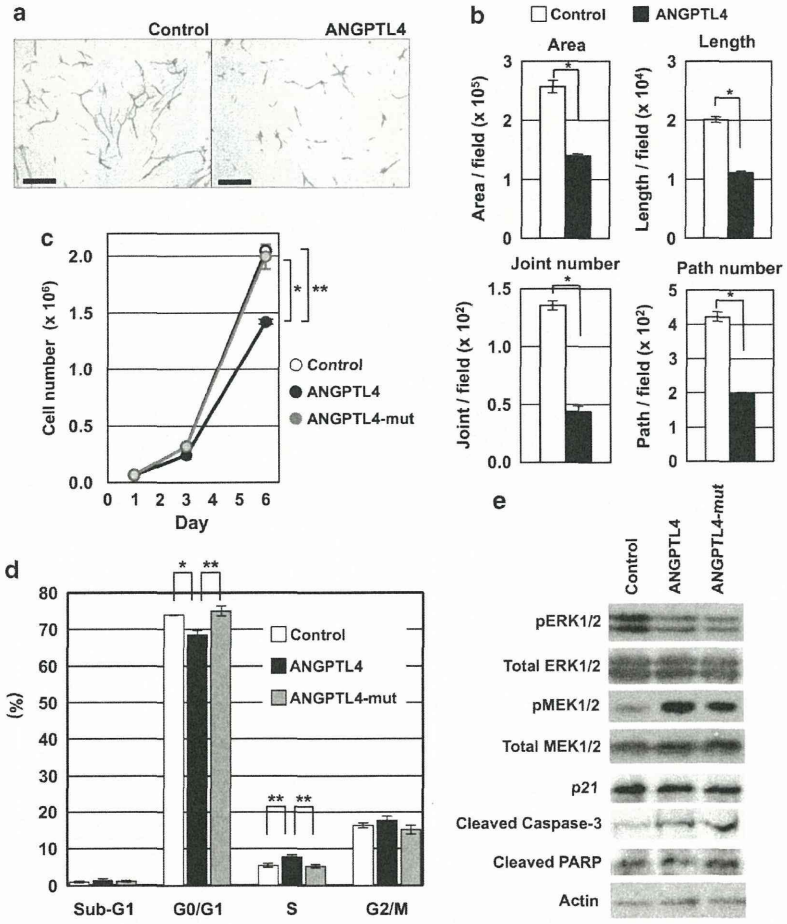

Figure 4. *In vitro* anti-angiogenic activity of tumor-derived ANGPTL4 and its molecular mechanisms. (**a**) Suppression of vascular tube formation by ANGPTL4. A conditioned medium was prepared by mixing the medium of subconfluent MKN28 cells expressing control or *ANGPTL4* and the HUVEC medium with VEGF-A (10 ng/ml) at 1:2. HUVECs on feeder neonatal normal human dermal fibroblast cells (Angiogenesis Kit, Kurabo, Osaka, Japan) arrived from the manufacturer on day 0, and the conditioned medium was supplemented on days 1, 4, 7 and 9. On day 11, cells were fixed and endothelial tubes were stained with anti-CD31antibody (BD Pharmingen, San Diego, CA, USA). The experiment was conducted in triplicate. Scale bars, 50 μm. (**b**) Quantification of the extent of vascular tube formation. Four parameters were scored in nine visual fields per well using the angiogenesis quantification software (Kurabo), and all the four parameters were shown to be suppressed by ANGPTL4. The results are shown as a mean ± s.d. $*P<0.001$ (Student's *t*-test). (**c**) Suppression of the HUVEC growth by ANGPTL4, but not by its mutant with the deletion. HUVECs were seeded at a density of 1×10^5 cells/10-cm dish on day 0, and the conditioned medium prepared as in **a** was supplemented on days 1, 3 and 5. The number of cells was counted on day 3 and day 6 by a Countess Automated Cell Counter (Invitrogen, Carlsbad, CA, USA). Each culture was carried out three times, and the result is shown as a mean ± s.d. $*P=8.4\times10^{-4}$, $**P=5.5\times10^{-5}$ (Student's *t*-test). (**d**) The effects of ANGPTL4 and its mutant with the deletion on the cell cycle of HUVECs. The HUVECs on day 6 of **c** were stained with propidium iodide, and cell populations in different phases of the cell cycle were determined by a FACS Caliber flow cytometer (Becton Dickinson, San Diego, CA). S-phase arrest was observed in HUVECs exposed to ANGPTL4, but not to its mutant with the deletion. $*P<0.05$, $**P<0.01$ (Student's *t*-test). (**e**) Immunoblot analysis of various signal molecules in HUVECs on day 6 of **c**. Decrease of pERK1/2 and increase of pMEK1/2 were induced by ANGPTL4 and also by its mutant with the deletion. This was considered because the deletion mutation affected mainly the interaction between ANGPTL4 and extracellular matrix, which was not necessary for this analysis. Primary antibodies used include anti-phospho-ERK1/2 (1:100, Cell Signaling Technology, Danvers, MA, USA), anti-total ERK1/2 (1:100, Cell Signaling Technology), anti-phospho-MEK1/2 (1:1000, Cell Signaling Technology), anti-total MEK1/2 (1:100, Cell Signaling Technology), anti-p21 (1:200, Cell Signaling Technology), anti-cleaved Caspase-3 (Asp175) (1:200, Cell Signaling Technology), anti-cleaved PARP (Asp214) (1:200, Cell Signaling Technology) and anti-actin (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Secondary antibodies conjugated to horseradish peroxidase were obtained from Cell Signaling Technology.

Figure 3. Inhibition of tumor angiogenesis by *ANGPTL4*, but not by its mutant with the deletion. (**a**) Macroscopic view of the Matrigel plugs recovered in the Matrigel plug assay. Matrigel (Matrigel basement membrane matrix high concentration, phenol red-free, BD Biosciences) was mixed with the MKN28 cells (5×10^6 cells) expressing control, *ANGPTL4*, or its mutant with the deletion. The Matrigel plug was subcutaneously injected into 5-week-old female athymic nude mice on day 1, and was recovered on day 10. Marked inhibition of tumor angiogenesis by *ANGPTL4*, but not by its mutant with the deletion, was observed. Brown color shows infiltration of blood vessels into the Matrigel plugs. (**b**) Hemoglobin content in the Matrigel plugs ($N=10$). The plugs were homogenized in red blood cell-lysing buffer (Sigma-Aldrich, St Louis, MO, USA), and the supernatants were measured with Drabkin's reagent (Sigma-Aldrich) to quantify the hemoglobin content in the plug. The content is shown as a mean ± s.d. ($N=10$). $*P=8.0\times10^{-4}$, $**P=1.2\times10^{-4}$ (Student's *t*-test).

demethylated the promoter and restored the *ANGPTL4* expression (Supplementary Figure S4). This showed methylation silencing of *ANGPTL4* in these cell lines.

Marked suppression of *in vivo* tumor growth by ANGPTL4, but not by a mutant with the deletion

The function of ANGPTL4, along with its mutant with the deletion, was examined by stably introducing wild-type or mutant *ANGPTL4* complementary DNA into MKN28 and AGS. The expression levels of the exogenous wild-type *ANGPTL4* and its mutant were kept in the range comparable to its physiological expression in gastric mucosae (Supplementary Figure S5a), and expression of *ANGPTL4* with the deletion mutation was confirmed by the amplification of a shorter size fragment (Supplementary Figure S5b).

Regarding *in vitro* effects, the *ANGPTL4* expression did not affect cell morphology, motility or cell growth (Supplementary Figures S6–S8). However, *in vivo*, sizes of engrafted tumors were strikingly suppressed by the wild-type *ANGPTL4*, markedly in AGS and almost completely in MKN28 (Figure 2). In contrast, when the mutant *ANGPTL4* was expressed in MKN28, it did not show any anti-tumorigenic effect. The presence of ANGPTL4 mRNA and protein expression in the transplanted tumors was confirmed (Supplementary Figures S9 and S10). The role of *ANGPTL4* in tumor development and progression has been highly controversial, but our data clearly showed that *ANGPTL4* suppresses tumor formation, at least in gastric cancers.

Inhibition of tumor angiogenesis as a mechanism for tumor suppression

As a mechanism for tumor suppression by *ANGPTL4*, anti-angiogenic activity of tumor-derived ANGPTL4 was examined. We performed an *in vivo* Matrigel plug angiogenesis assay to observe the vascularization that invades into a Matrigel[31] using MKN28 cells with control (*EGFP*), *ANGPTL4*, and its mutant with the deletion. Ten days after subcutaneous transplantation, the Matrigel plugs containing the control cells showed a high degree of blood vessel recruitment, as visualized by the high content of hemoglobin (Figure 3) and by the staining of CD31-positive vascular endothelial cells (Supplementary Figure S11). In contrast, the Matrigel plugs containing the *ANGPTL4*-expressing cells showed a marked suppression of the blood vessel recruitment. However, *ANGPTL4* with the deletion mutation did not have such activity. The lack of the suppressive effect was in accordance with a report that the CCD was essential for the interaction with the extracellular matrix and for its *in vivo* suppressive activity.[13] These results strongly indicated that the marked anti-angiogenic activity of tumor-derived ANGPTL4 was the cause of the marked suppression of tumor growth by ANGPTL4.

Mechanisms for the anti-angiogenic activity of the tumor-derived ANGPTL4

The mechanisms of how tumor-derived, secreted ANGPTL4 exerts its anti-angiogenic effect were analyzed. First, we conducted a vascular tube formation assay using human umbilical vein endothelial cells (HUVECs). A conditioned medium from *ANGPTL4*-expressing cells suppressed vascular tube formation of HUVECs, as visualized by staining with anti-CD31antibody (Figure 4a), and all the parameters to assess vascular formation were markedly suppressed (Figure 4b). This result showed that a large part of the anti-angiogenic activity of tumor-derived ANGPTL4 was mediated by the suppression of vascular tube formation in the tumor microenvironment.

The effect of the conditioned medium on the growth of HUVECs was then analyzed. The conditioned medium from cells expressing *ANGPTL4*, but not that from cells expressing its mutant with the deletion, suppressed the growth (Figure 4c). Cell cycle analysis showed that the conditioned medium from *ANGPTL4*-expressing cells significantly increased the number of cells in the S phase, suggesting that it induced an S-phase arrest (Figure 4d). However, the amount of p21, a potential inducer of the S-phase arrest,[32] was not increased (Figure 4e). No induction of apoptosis was observed by western blot analysis of apoptosis-related proteins, cleaved Caspase-3 and cleaved PARP (Figure 4e), or by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay (Supplementary Figure S8).

Finally, the effect of tumor-derived ANGPTL4 on the MAPK signaling was analyzed. The conditioned medium from the *ANGPTL4*-expressing cells clearly inhibited the phosphorylation of ERK1/2 (pERK1/2) (Figure 4e), and the phosphorylation of its immediate upstream mediator, pMEK, was in contrast increased. The conditioned medium from the cells expressing the mutant with the deletion showed a similar activity to that of the *ANGPTL4*-expressing cells. As CCD is not important for the delivery to target cells *in vitro* and the fibrinogen-like domain is important for inhibition of the Raf/MEK/ERK signaling,[11] it was considered that the deletion mutation did not affect the inhibition activity.

This study demonstrated that *ANGPTL4* is a mutated and methylation-silenced tumor suppressor whose product is secreted and inhibits angiogenesis. *ANGPTL4* mutation (loss-of-function) was identified for the first time in any type of cancers, and the anti-angiogenic activity of tumor-derived ANGPTL4 was shown here also for the first time. These data warrant further research into utilizing ANGPTL4 as a target of anti-angiogenesis cancer therapy.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

We are grateful to Dr Masabumi Shibuya, Tokyo Medical and Dental University, for his expert advice. This study was supported by the Third-term Comprehensive Cancer Control Strategy from the Ministry of Health, Labour and Welfare, Japan and by National Cancer Center Research and Development Fund. YN is a recipient of a Research Resident Fellowships from the Foundation for Promotion of Cancer Research.

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

Japanese Journal of Clinical Oncology Advance Access published April 25, 2013

JJCO Japanese Journal of Clinical Oncology

Jpn J Clin Oncol 2013
doi:10.1093/jjco/hyt058

Original Article

Stronger Prognostic Power of the CpG Island Methylator Phenotype than Methylation of Individual Genes in Neuroblastomas

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Received January 14, 2013; accepted March 30, 2013

Objective: The CpG island methylator phenotype is strongly associated with poor survival in neuroblastomas. Neuroblastomas with the CpG island methylator phenotype include almost all neuroblastomas with *MYCN* amplification, and, even among neuroblastomas without *MYCN* amplification, have worse prognosis. At the same time, methylation of individual tumor-suppressor genes is also reported to be associated with poor survival. The purpose of this study was to compare the prognostic power of the CpG island methylator phenotype with that of methylation of individual genes.

Methods: Methylation-specific polymerase chain reaction was performed for five individual genes (*CASP8*, *EMP3*, *HOXA9*, *NR1I2* and *CD44*) in 140 Japanese and 152 German neuroblastomas. Kaplan–Meier analysis and log-rank tests were conducted to compare the survival between groups defined by methylation status.

Results: Among the five individual genes, only *CASP8* methylation had a significant association with poor overall survival both in Japanese (hazard ratio = 3.1; 95% confidence interval = 1.5–6.4; $P = 0.002$) and German (hazard ratio = 4.8; 95% confidence interval = 2.1–11; $P = 0.0002$) neuroblastomas. *HOXA9* and *NR1I2* methylation were associated with poor survival only in German neuroblastomas. On the other hand, the CpG island methylator phenotype had a strong and consistent association in Japanese (hazard ratio = 22; 95% confidence interval = 5.3–93; $P = 1.5 \times 10^{-5}$) and German (hazard ratio = 9.5; 95% confidence interval = 3.2–28; $P = 4.7 \times 10^{-5}$) neuroblastomas.

Conclusion: The CpG island methylator phenotype is likely to have stronger prognostic power than methylation of individual genes in neuroblastomas.

Key words: neuroblastoma – methylation – CIMP – poor survival

INTRODUCTION

Neuroblastoma (NBL) is the most frequent extracranial pediatric tumor (1). The CpG island methylator phenotype (CIMP), methylation of multiple CpG islands (CGIs), was associated with poor survival with a hazard ratio (HR) of 22 [95% confidence interval (95% CI) = 5.3–93] in Japanese and 9.5 (95% CI = 3.2–28) in German NBLs, respectively (2,3). The prognostic significance of CIMP was further confirmed in Italian NBLs by a pyrosequencing assay (4). Notably, NBLs with CIMP included almost all NBLs with *MYCN* amplification (37/38 in Japanese and 23/23 in German NBLs), the strongest current prognostic marker (5–7). Even among NBLs without

MYCN amplification, CIMP was a significant and strong prognostic marker with an HR of 12 (95% CI = 2.6–59) in Japanese and 4.5 (95% CI = 1.3–16) in German NBLs.

CIMP is sensitively detected by methylation of marker CGIs, such as CGIs in gene bodies of the *PCDHB* gene family in NBLs. It is known that methylation of CGIs outside promoter regions (non-promoter CGIs) is not associated with loss of expression, and such non-promoter CGIs are more susceptible to methylation induction than promoter CGIs (8). As a model of the close association between methylation of non-promoter CGIs and poor survival, it was considered that CIMP consistently leads to methylation of non-promoter CGIs, such as CGIs of the *PCDHB* gene family in NBLs, and also to methylation of various promoter CGIs with low incidences, which causes poor survival.

At the same time, methylation of an individual gene has been also shown to be associated with poor survival. For example, methylation of *CASP8* was associated with poor survival with an HR of 5.3 (95% CI = 1.5–18; $P = 0.008$) (9). Methylation of *NR1I2*, *EMP3*, *HOXA9* and *CD44* was associated with poor survival with P values of 0.014, 0.03, 0.04 and 0.049, respectively (9–12). Functionally, *CASP8*, an apoptosis-related gene, has been reported to act as a tumor suppressor, and its loss is required for survival of NBL cells overexpressing *MYC* or *MYCN* (13). *NR1I2* (a nuclear receptor gene) and *EMP3* (a myelin-related gene) have been reported to have growth suppressive activity in NBL cells (10,11). However, the prognostic powers of methylation of these individual genes and of CIMP have never been analyzed in identical sets of NBLs.

In the present study, we aimed to compare the prognostic power of CIMP with that of methylation of individual genes.

PATIENTS AND METHODS

DNA Samples and Analysis of CIMP

The 140 Japanese and 152 German NBLs were identical with those analyzed in our previous studies (2,3). These samples were analyzed at the Division of Epigenomics, National Cancer Center Research Institute under the approval of institutional review boards. The presence of CIMP and *MYCN* amplification were determined as in our previous studies (2,3), and this information was used in the present study.

Sodium Bisulfite Modification and Methylation-specific PCR

Fully methylated DNA and fully unmethylated DNA were prepared by methylating genomic DNA with *Sss*I methylase (New England Biolabs, Beverly, MA, USA) and by amplifying genomic DNA with the GenomiPhi amplification system (GE Health Care, Buckinghamshire, UK), respectively. Bisulfite modification was performed using 1 μg of *Bam*HI-digested genomic DNA as previously described (14), and the modified DNA was suspended in 40 μl of Tris–ethylenediaminetetraacetic acid buffer (pH 8.0). An aliquot of 1 μl was used for methylation-specific polymerase chain reaction (PCR) (MSP).

MSP was performed using primers as previously published (11,13,15,16) (Supplementary data, Table S1). For the *NR1I2* gene, although the combined bisulfite restriction analysis was performed in the previous study (10), MSP targeting the same region was used in this study. Using fully methylated and unmethylated DNA, the annealing temperature that specifically amplified only methylated or unmethylated DNA was determined. Also, a minimum number of PCR cycles to obtain visible bands was determined using the (un)methylated DNA, and four cycles were added for the analysis of primary NBLs (Supplementary data, Table S1).

Statistical Analysis

Survival time was defined as the time between initial diagnosis and death, or time between diagnosis and last contact if no event had occurred. Kaplan–Meier analysis and log-rank tests were conducted to compare survival between the groups defined by methylation status. HRs were estimated by the Cox

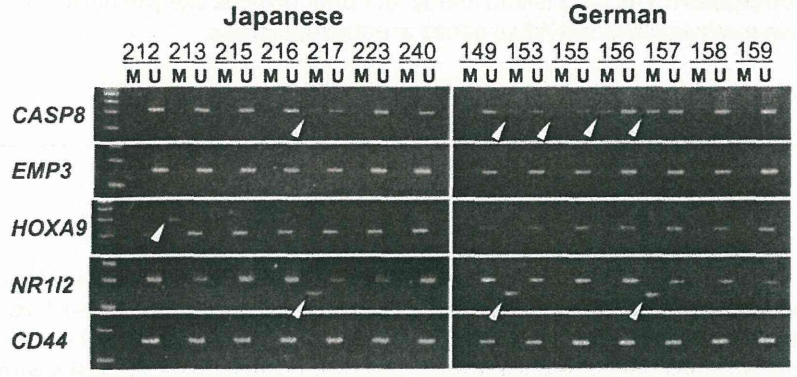

Figure 1. Methylation of promoter CpG islands (CGIs) of five individual genes (*CASP8*, *EMP3*, *HOXA9*, *NR1I2* and *CD44*) in Japanese and German neuroblastomas (NBLs). Representative results of methylation-specific PCR are shown. M and U, primers specific to methylated and unmethylated DNA, respectively. Arrowheads show the presence of methylated DNA molecules.

proportional hazard model. These statistical analyses were performed using the SPSS software, version 13.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

Methylation of Individual Genes and their Prognostic Power Compared with CIMP

CASP8, *EMP3*, *HOXA9*, *NR1I2* and *CD44* were methylated in 26, 4, 27, 15 and 3, respectively, of the 140 Japanese NBLs, and in 30, 2, 2, 13 and 2, respectively, of the 152 German NBLs (representative results shown in Fig. 1). The prognostic power of methylation of the five genes was analyzed in Japanese and German NBLs, respectively (Fig. 2A and Table 1). In Japanese NBLs, only *CASP8* methylation had a significant association with poor survival (HR = 3.1; 95% CI = 1.5–6.4; $P = 0.002$). Regarding CIMP, defined by methylation of multiple genes and detected by methylation of the *PCDHB* gene family (2), it had a strong association with poor survival (HR = 22; 95% CI = 5.3–93; $P = 1.5 \times 10^{-5}$), and its prognostic power was stronger than that of *MYCN* amplification (HR = 9.5; 95% CI = 4.4–21; $P = 4.0 \times 10^{-9}$) (Fig. 2B). In the identical set of Japanese NBLs, a stronger prognostic power of CIMP than methylation of an individual gene was clearly shown.

In German NBLs, *CASP8* methylation was also associated with poor survival (HR = 4.8; 95% CI = 2.1–11; $P = 0.0002$) (Fig. 2A and Table 1). In addition, *HOXA9* and *NR1I2* methylation were associated with poor survival with an HR of 14 for *HOXA9* (95% CI = 3.1–62; $P = 0.0006$) and 4.2 for *NR1I2* (95% CI = 1.6–11; $P = 0.003$), respectively. Regarding CIMP and *MYCN*, as shown in our previous study (3), CIMP had a strong association with poor survival (HR = 9.5; 95% CI = 3.2–28; $P = 4.7 \times 10^{-5}$) and it was comparable to that of *MYCN* (HR = 12; 95% CI = 4.9–29; $P = 4.8 \times 10^{-8}$) (Fig. 2B). The stronger prognostic power of CIMP was consistently shown in the identical set of German NBLs.

Association Between CIMP and Methylation of Individual Genes

Among the five individual genes analyzed in this study, two genes (*CASP8* and *NR1I2*) were methylated at a significantly higher incidence in NBLs with CIMP (Fig. 3). In Japanese NBLs with and without CIMP, *CASP8* methylation was found in 24/67 and 2/73, respectively ($P = 5.0 \times 10^{-7}$). *NR1I2* methylation was found in 15/67 and 0/73, respectively ($P = 3.2 \times 10^{-5}$). Also in German NBLs with and without CIMP, *CASP8* methylation was found in 28/50 and 2/95, respectively ($P = 2.6 \times 10^{-14}$). *NR1I2* methylation was found in 11/50 and 1/95, respectively ($P = 1.4 \times 10^{-5}$). These results showed that CIMP was associated with methylation of multiple promoter CGIs, mainly *CASP8* and *NR1I2*.

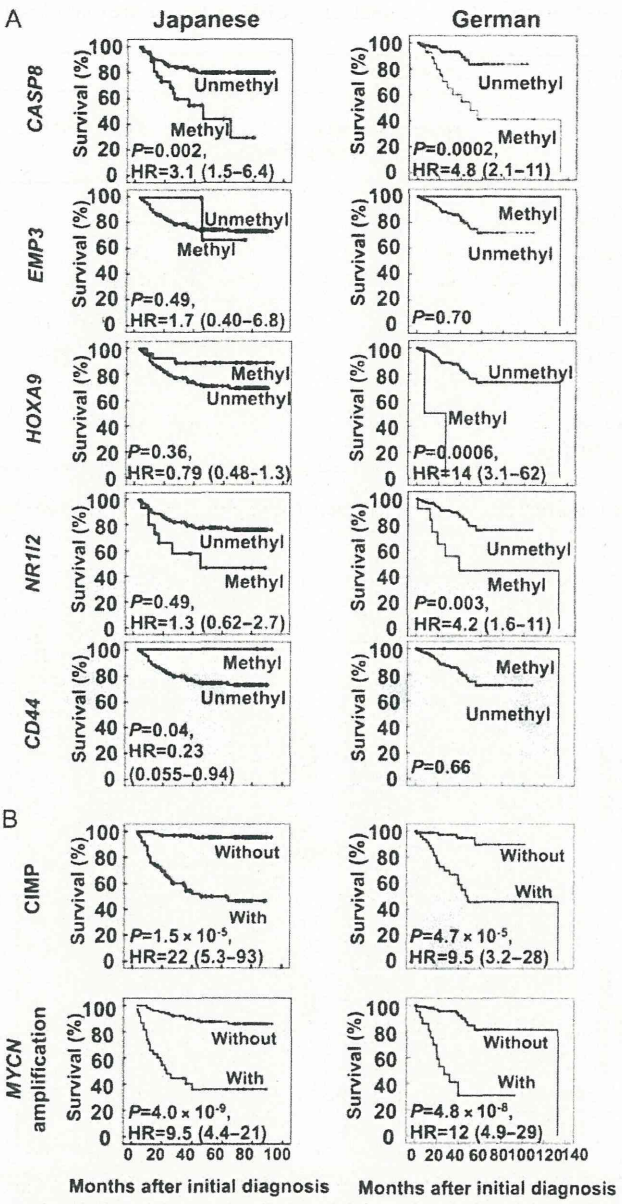

Figure 2. Prognostic power of (A) methylation of five individual genes (*CASP8*, *EMP3*, *HOXA9*, *NR1I2* and *CD44*), and (B) CpG island methylator phenotype (CIMP) and MYCN amplification in Japanese and German NBLs. Kaplan–Meier survival curves were drawn using the SPSS software. Among the five genes, only CASP8 methylation had a significant association with poor survival both in Japanese and German NBLs.

DISCUSSION

The stronger prognostic power of CIMP than methylation of individual genes was shown in this study. Also, the association between CIMP and methylation of multiple promoter CGIs was indicated. These results supported the idea that CIMP leads to a poor prognosis by induction of methylation of promoter CGIs of various tumor-suppressor genes with low incidences.

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

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

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

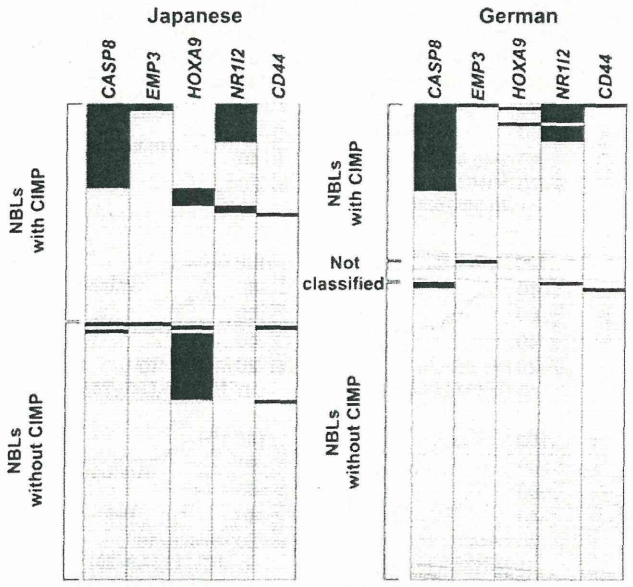

Figure 3. Methylation profiles of the five individual genes in NBLs with and without CIMP. Left panel, 140 Japanese NBLs; and right panel, 152 German NBLs. NBLs were classified by CIMP status determined as in our previous studies (2,3), and then aligned by methylation statuses of the five genes. In German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

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

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

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

Supplementary data

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

Funding

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

Conflict of interest statement

None declared.

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Research Article
See related commentary by Schneider and Peek, p. 253

Cancer Prevention Research

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

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Abstract

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

Introduction

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

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

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

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Note: Supplementary data for this article are available at Cancer Prevention Research Online (http://cancerprevres.aacrjournals.org/).

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doi: 10.1158/1940-6207.CAPR-12-0369

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

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

Materials and Methods

Animals and sample preparation

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

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

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

Histological analysis

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

Luminometric methylation assay

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

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

Quantitative methylation-specific PCR

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

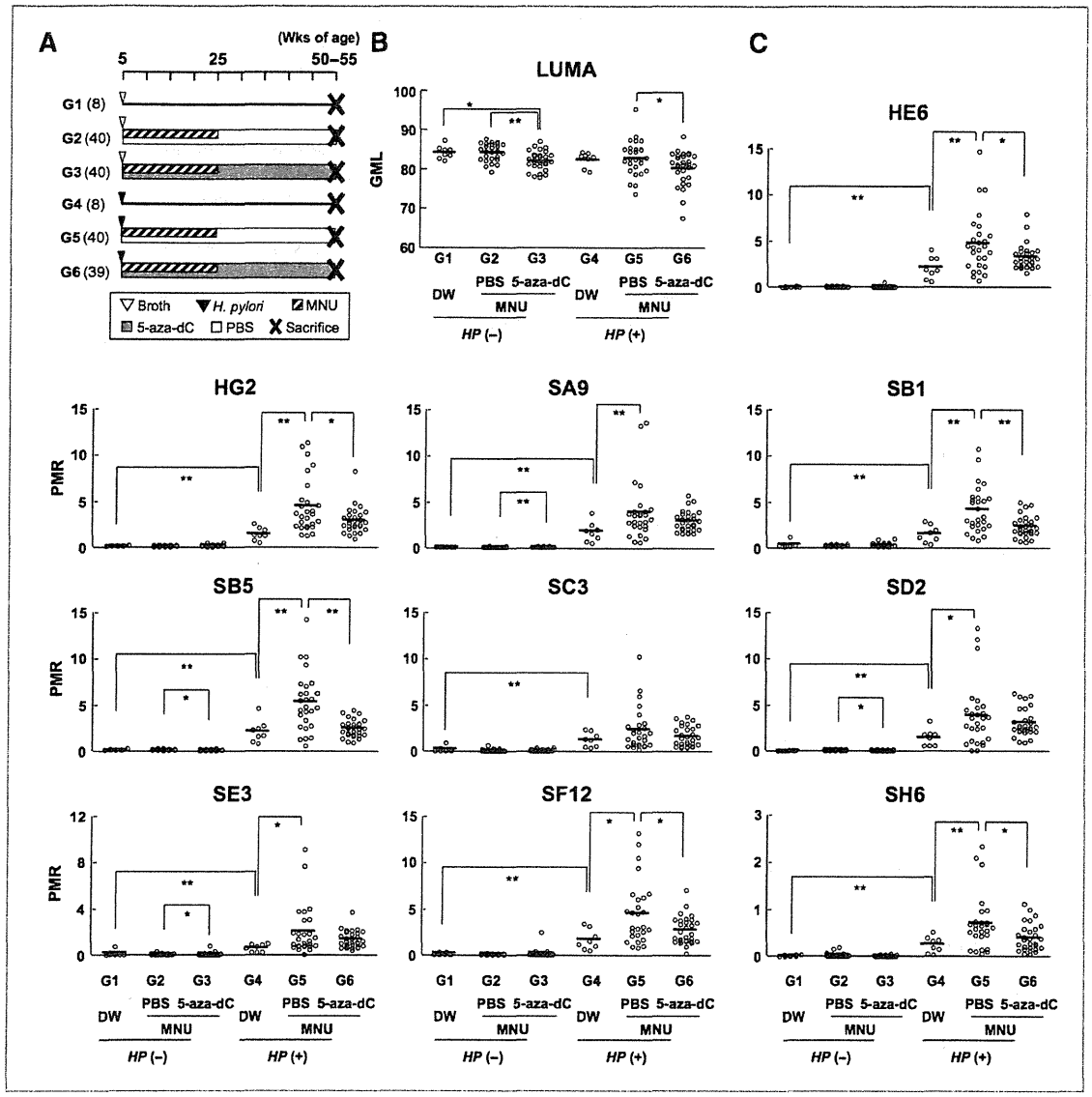

Figure 1. Suppression of DNA methylation by 5-aza-dC treatment. A, design of the carcinogenicity experiment. The number of animals is shown in parentheses. B, global DNA methylation levels analyzed by LUMA in GECs. C, methylation levels of 10 CGIs analyzed by qMSP in GECs. Both preexisting methylation and induction of aberrant methylation were suppressed by 5-aza-dC treatment. Bold horizontal bar, the average. *, $P < 0.05$; **, $P < 0.01$.

methylated fragments of a target CGI in *SssI*-treated DNA)/(number of the B2 repeat in *SssI*-treated DNA)] × 100.

Gene expression analyses

The number of cDNA molecules was quantified by quantitative reverse transcriptase-PCR (qRT-PCR) using gene-specific primers (*Il1b*, *Nos2*, and *Tnf*) as described (14). The number of cDNA molecules of a target gene was normalized to that of *Gapdh*.

Genomic PCR and sequencing

A forward primer (5′-AGATTCCTTGATGCCTGGGTGTC-3′) was designed in a region of the mouse *Tnf* promoter highly conserved with the human corresponding region. A reverse primer (5′-AGATTCCTTGATGCCTGGGTGTC-3′) was designed on the gerbil *Tnf* mRNA sequence (AB177841). The gerbil *Tnf* promoter was amplified using these primers, and the PCR product was directly sequenced with the same primers. The obtained sequence was

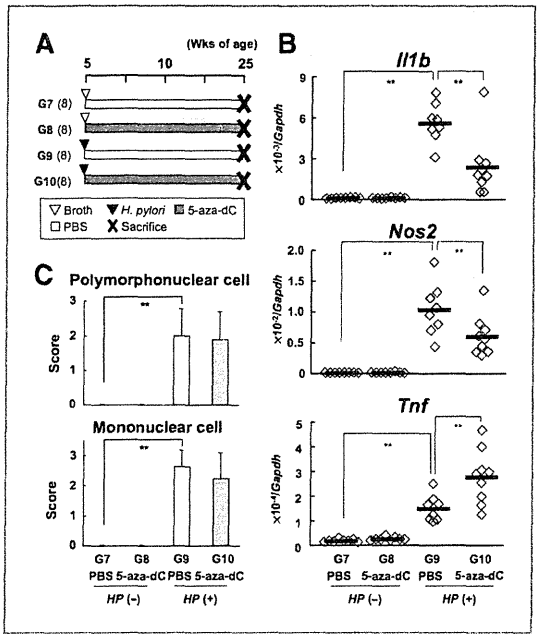

Figure 2. Dysregulation of inflammatory responses by 5-aza-dC treatment. A, design of the experiment for analysis of gastritis. The number of animals is shown in parentheses. B, expression levels of inflammation-related genes in gastric tissues containing submucosal layers. Bold horizontal bar indicates the average. C, infiltration score of polymorphonuclear cells and mononuclear cells in the stomach. Although the degree of *H. pylori*–induced infiltration of polymorphonuclear and mononuclear cells was not affected by 5-aza-dC treatment, expression of *Il1b* and *Nos2* was downregulated and that of *Tnf* was upregulated. Mean and SD are shown. **, $P < 0.01$.

registered in GenBank (AB762083.1). A CGI was searched by a EMBOSS CpG report program (40).

Statistical analysis

Statistical analyses were conducted by SPSS 13.0J (SPSS Japan Inc.). To evaluate significant difference between 2 independent groups of sample data, a Mann–Whitney *U* test was used. The difference of the proportion between 2 groups was evaluated by Fisher exact test.

Results

Suppression of *H. pylori*/MNU-induced gastric carcinogenesis by 5-aza-dC

To evaluate cancer prevention effects of 5-aza-dC, a carcinogenicity experiment was carried out (Fig. 1A). Among the gerbils with MNU treatment and *H. pylori* infection (G5 and G6), 5-aza-dC treatment decreased incidence of gastric cancers from 55.2% (G5) to 23.3% (G6, $P < 0.05$; Table 1). The incidence in G6 was similar to that in MNU-treated gerbils without *H. pylori* infection (G2, 20.7%). There were no significant differences in the tumor multiplicity and size among the groups. These results clearly showed that 5-aza-dC treatment suppressed *H. pylori*/MNU-induced gastric carcinogenesis in gerbils and suggested that it might have completely abrogated the cancer promotion effects of *H. pylori* infection. Among the MNU-treated gerbils without *H. pylori* infection (G2 and G3), 5-aza-dC treatment tended to decrease incidence of gastric cancers but it was not statistically significant.

Regardless of 5-aza-dC treatment, gerbils with MNU treatment (G2, G3, G5, and G6) showed low body weight than the gerbils without MNU treatment (G1 and G4), showing that the body weight loss was dependent upon MNU treatment, not upon 5-aza-dC treatment (Supplementary Fig. S2). Survival rates started to decrease from 25 weeks of age, and the decrease was dependent upon MNU treatment, not upon 5-aza-dC treatment (Supplementary Fig. S3). This showed that the dose of 5-aza-dC used in this study (125 μg/kg body weight) had no obvious effects on body weight and survival of gerbils.

Reduction of DNA methylation levels in GECs by 5-aza-dC

To confirm the demethylating effects of 5-aza-dC *in vivo*, methylation analyses were conducted in GECs. First, the global DNA methylation level was measured by LUMA, in which global CCGG methylation was measured by using

Table 1. Suppression of gastric cancers by 5-aza-dC

		Adenocarcinoma						
Group	Effective number	Well-differentiated	Poorly differentiated	Multiplicity (mean ± SD)	Diameter (mean ± SD), mm	Incidence (%)	Adenoma	Sarcoma
G1. *HP*(–) + DW	8	0	0	0	–	0/8 (0)	0	0
G2. *HP*(–) + MNU + PBS	29	3	3	1.0 ± 0	6.3 ± 1.6	6/29 (20.7)	1	2
G3. *HP*(–) + MNU + 5-aza-dC	33	0	2	1.0 ± 0	3.3 ± 3.3	2/33 (6.1)	1	0
G4. *HP*(+) + DW	8	0	0	0	–	0/8 (0)	0	0
G5. *HP*(+) + MNU + PBS	29	15	1	1.1 ± 0.3	5.7 ± 2.7	16/29 (55.2)[a]	0	2
G6. *HP*(+) + MNU + 5-aza-dC	30	4	3	1.0 ± 0	4.8 ± 1.7	7/30 (23.3)[b]	0	2

NOTE: 5-Aza-dC treatment decreased incidence of gastric cancers (adenocarcinomas) from 55.2% (G5) to 23.3% (G6).

[a]$P < 0.05$ compared with G3.

[b]$P < 0.05$ compared with G5.

a combination of methylation-sensitive and -insensitive restriction enzymes and pyrosequencing. Among the gerbils with MNU treatment and *H. pylori* infection (G5 and G6), 5-aza-dC treatment decreased the global methylation level from 83.0% ± 4.5% (mean ± SD)% (G5) to 80.3% ± 4.4% (G6, $P < 0.05$; Fig. 1B). Among the MNU-treated gerbils without *H. pylori* infection (G2 and G3), the methylation level was decreased by 5-aza-dC treatment (from 84.4% ± 2.3% in G2 to 82.2% ± 2.4% in G3, $P < 0.05$). No significant influence of *H. pylori* infection or MNU treatment was observed. These results indicated that 5-aza-dC worked as a DNA demethylating agent *in vivo* and decreased global methylation levels in GECs.

Next, methylation of 10 CGIs, where *H. pylori* infection was previously shown to induce aberrant methylation (Supplementary Fig. S1; refs. 14, 41), was analyzed by quantitative methylation-specific PCR (qMSP). 5-Aza-dC treatment reduced methylation levels in G6 to 46% to 80% of those in G5 for 6 CGIs (HE6, HG2, SB1, SB5, SF12, and SH6; $P < 0.05$; Fig. 1C). These results showed that 5-aza-dC treatment suppressed methylation induction by *H. pylori* infection and MNU treatment in GECs. The methylation levels in G5 were higher than those in G4 whereas those in G2 were not elevated compared with those in G1, indicating that MNU treatment had an augmenting effect on *H. pylori*–induced aberrant methylation.

Dysregulation of inflammation-related genes by 5-aza-dC

Among 10 inflammation-related genes whose expression was examined in the stomach, expression of 3 genes (*Il1b*, *Nos2*, and *Tnf*) has been shown to be associated with induction of methylation in GECs (14, 41). Therefore, we examined whether 5-aza-dC treatment affected expression of these 3 genes in the stomach after *H. pylori* infection using *H. pylori*–infected and -uninfected gerbils without MNU treatment (Fig. 2A). In the *H. pylori*–infected gerbils with 5-aza-dC treatment (G10), expression levels of *Il1b* and *Nos2* decreased to 42% and 58%, respectively ($P < 0.01$, respectively), of those in *H. pylori*–infected gerbils without 5-aza-dC treatment (G9; Fig. 2B). In contrast, *Tnf* was upregulated to 187% of G9 ($P < 0.01$). These results indicated that 5-aza-dC treatment caused up- and downregulation, namely dysregulation, of inflammation-related genes. As there was a possibility that upregulation of *Tnf* was due to demethylation of its promoter CGI, we sequenced its promoter region but found that there was no CGI (Supplementary Fig. S4). Because methylation of only promoters with CGIs can consistently silence their downstream genes (42), we considered that the upregulation of *Tnf* was unlikely to be due to demethylation by 5-aza-dC.

We also analyzed infiltration of inflammatory cells in the stomach. In gerbils with *H. pylori* infection (G9 and G10), 5-aza-dC treatment did not affect infiltration of mononuclear cells and polymorphonuclear cells (Fig. 2C). In gerbils without *H. pylori* infection (G7 and G8), there was little mononuclear cell and polymorphonuclear cell infiltration, and no effect of 5-aza-dC treatment was observed. These results showed that 5-aza-dC treatment did not affect infiltration of inflammatory cells.

Extra-gastric effects of 5-aza-dC treatment

To evaluate possible adverse effects of 5-aza-dC treatment, we first conducted macroscopic analysis. Although most organs did not show any abnormality, the testes were prominently atrophic in 5-aza-dC–treated gerbils [0.47 ± 0.05 (mean ± SD) g/2 testes in G3 and 0.50 ± 0.05 g/2 testes in G6] compared with gerbils in other groups (1.10 ± 0.11 g/2 testes in G2 and 1.09 ± 0.13 g/2 testes in G5).

We then conducted analysis of histopathologic abnormalities and global DNA methylation levels in the testes, small intestine, liver, and kidneys. In the testes of 5-aza-dC–treated gerbils, the numbers of spermatozoa and spermatids were markedly decreased regardless of *H. pylori*

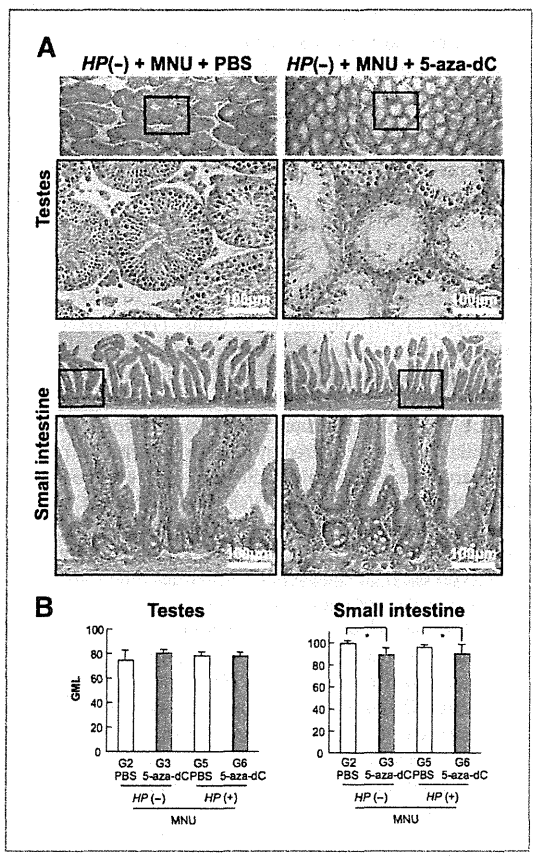

Figure 3. Adverse effects of 5-aza-dC treatment in extra-gastric tissues. A, tissue sections of the testes and small intestine. Bottom, is a magnified view of the region in the black rectangle in the top. Numbers of spermatozoa and spermatids were markedly decreased in the testes of 5-aza-dC–treated gerbil. B, global methylation levels in the testes and small intestine. Five gerbils in each group were randomly selected, and the methylation levels were measured by LUMA. Mean and SD are shown. *, $P < 0.05$. By 5-aza-dC-treatment, the global methylation level did not decrease in the testes but did in the small intestine.

infection status (Fig. 3A). Despite the presence of hypospermatogenesis, there was no significant decrease of the global methylation level (Fig. 3B). In contrast, in the small intestine, the global methylation level was reduced by 10.4% and 5.6% (G3 and G6, respectively Fig. 3B). However, no histologic changes compared with the untreated gerbils were observed (Fig. 3A). As for the liver and kidneys, there were no histologic abnormalities or reduction of global methylation levels in G3 and G6 (Supplementary Fig. S5).

Discussion

Our study using a gerbil model showed that 5-aza-dC treatment suppressed *H. pylori*/MNU-induced gastric cancers. This study showed for the first time that chemoprevention using a DNA demethylating agent is effective for chronic inflammation–associated cancers. As chronic inflammation contributes to about 25% of all cancer cases (43), and aberrant DNA methylation is frequently observed in tissues exposed to chronic inflammation (29), suppression of aberrant methylation might become an effective preventive approach for these types of cancers. This study also showed that induction of aberrant methylation is an important mechanism for gastric carcinogenesis by *H. pylori* infection.

As mechanisms of suppression of gastric cancers by 5-aza-dC, at least 2 modes of action were present. The first one was the DNA demethylating effect. 5-Aza-dC treatment decreased global methylation level in GECs and suppressed hypermethylation of CGIs. These results suggested that 5-aza-dC was capable of removing aberrant DNA methylation induced by *H. pylori* infection and thereby prevented cancer development. The second one was the effect on inflammation. It was previously shown that inflammation triggered by *H. pylori* infection is pivotal for aberrant methylation induction, and expression of inflammation-related genes (*Il1b, Nos2,* and *Tnf*) in the stomach is associated with the induction (14, 41). The present study showed that 5-aza-dC treatment dysregulated expression of these genes. Therefore, there is a possibility that altered balances among the related cytokines might have led to the reduced methylation induction.

Recently, in the stomach of hypergastrinemic INS-GAS mice, inhibition of *H. pylori* (*H. felis*)-induced global demethylation by folic acid supplementation was reported to suppress gastric dysplasia (44). The data are seemingly discordant with our data showing a cancer preventive effect by DNA demethylation. However, in the INS-GAS mice, global hypomethylation by *H. felis* was evident, suggesting that the demethylation plays important roles in the carcinogenesis. Hypermethylation by folic acid might exert the cancer preventive effect via suppression of global demethylation. In contrast, in our gerbil study, hypermethylation of CGIs by *H. pylori* infection, rather than global demethylation, was evident, suggesting that hypermethylation of CGIs rather than global demethylation was the major mechanism for the carcinogenesis. Thus, demethylation by 5-aza-dC was considered to have exerted the preventive effect via suppression of hypermethylation of CGIs.

No obvious adverse effect of 5-aza-dC treatment was observed besides hypospermatogenesis in the testes. Hypospermatogenesis due to 5-aza-dC treatment was reported in mice (24). As global hypomethylation was not detected in the testes of the 5-aza-dC–treated gerbils, the effect was speculated to be independent of its DNA demethylating activity. However, we cannot exclude the possibility that decrease of methylation was not detected due to immediate elimination of spermatozoa/spermatids with decreased DNA methylation. Regardless of the mechanism, the presence of this adverse effect precludes 5-aza-dC as a chemoprevention agent for the general population. However, chemoprevention by a DNA demethylating agent itself still might become a promising strategy if a DNA demethylating agent without such toxicity is developed.

Demethylating effects by 5-aza-dC were observed in the stomach and the small intestine, but not in the liver and the kidneys. Specific global demethylation in the stomach and intestine was also observed in the female $Apc^{min/+}$ mice administered a demethylating agent, zebularine (23). Turnover of epithelial cells in these tissues is known to be very rapid, being 3 to 4 days in mice (45, 46). As demethylating effects of 5-aza-dC and zebularine are exerted after their incorporation into gDNA and DNA replication, rapid cell turnover in the stomach and intestine could explain the organ-specific global demethylation.

Individuals with a severe epigenetic field defect, in contrast with the general population, can be considered as a target population for cancer prevention using 5-aza-dC after careful balancing of its preventive and adverse effects. In the case of gastric cancers, eradication of *H. pylori* is the primary strategy for prevention (47), but the incidence of gastric cancers remains high, even after *H. pylori* eradication, especially in persons with intestinal metaplasia and gastric atrophy (48). Notably, aberrant methylation in gastric mucosae decreases by *H. pylori* eradication, but it does not disappear completely (14, 49). The level of the remaining methylation reflects the risk of gastric cancers (8, 9). As DNA demethylating agents are likely to remove such accumulated methylation and suppress gastric cancers development, these individuals with a severe epigenetic field defect may benefit from epigenetic chemoprevention.

In summary, treatment with 5-aza-dC effectively prevented gastric cancers induced by *H. pylori* infection in gerbils, suppressed DNA methylation in GECs, and induced dysregulation of inflammation. Chemoprevention with a DNA demethylating agent is expected to become an effective strategy for prevention of chronic inflammation–associated cancers.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: T. Niwa, M. Tatematsu, T. Ushijima
Development of methodology: T. Niwa, T. Tsukamoto
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): T. Niwa, T. Toyoda
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): T. Niwa, T. Toyoda
Writing, review, and/or revision of the manuscript: T. Niwa, T. Ushijima
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): A. Mori
Study supervision: M. Tatematsu

Acknowledgments

The authors thank Ms. R. Shimada (Qiagen K.K.) for her technical support of LUMA.

Grant Support

This study was supported by Grants-in-Aid for The Third-term Comprehensive Cancer Control Strategy from the Ministry of Health, Labour, and Welfare, Japan (T. Ushijima); Grants-in-Aid for Young Scientists (#23701114) from the Japan Society for the Promotion of Science (T. Niwa); and Grants-in-Aid from the Foundation of Promotion of Cancer Research, Japan (T. Niwa).

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Received August 29, 2012; revised December 19, 2012; accepted January 8, 2013; published online April 4, 2013.

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Oncogene (2013) **32,** 2140–2149

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ORIGINAL ARTICLE

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

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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.

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

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

INTRODUCTION

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

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

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

RESULTS

Screening of methylation-silenced genes on chromosome X

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

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Received 20 September 2011; revised 25 April 2012; accepted 4 May 2012; published online 11 June 2012

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

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

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

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

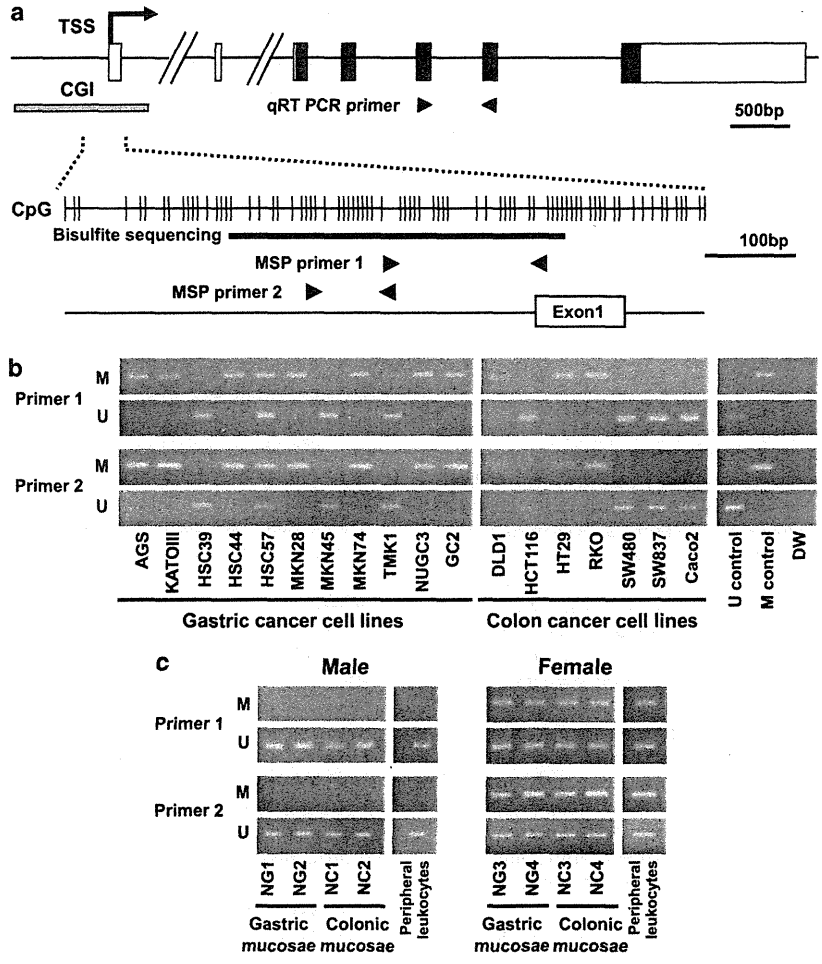

Figure 1. Genomic structure of *FHL1* and its methylation status in cancer cell lines, normal-appearing mucosae and peripheral leukocytes. (**a**) Genomic structure of *FHL1* and a CpG map of its promoter CGI. Open box, non-coding exon; closed box, coding exon; arrow, transcription start site (TSS);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

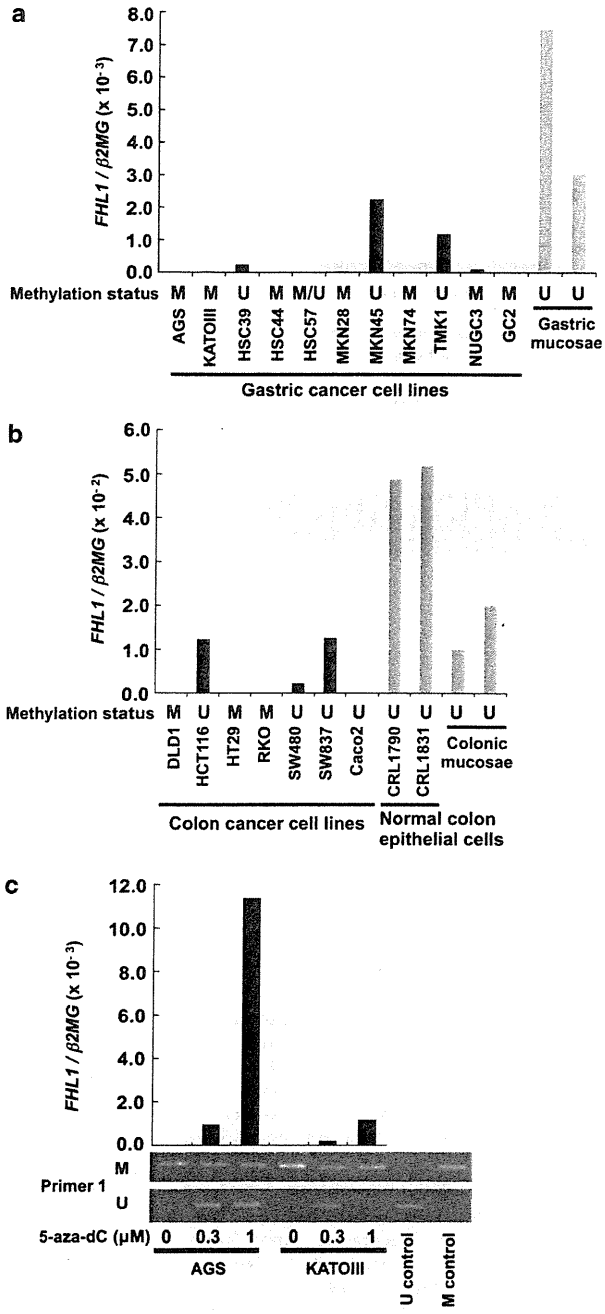

Figure 2. Methylation-silencing of *FHL1* in gastrointestinal cancer cell lines. (**a**) qRT–PCR of *FHL1* in gastric cancer cell lines and normal-appearing gastric mucosae. Results of MSP in Figure 1b are shown by M, M/U and U. M, only methylated DNA detected; M/U, both methylated and unmethylated DNA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

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

Methylation of *FHL1* in surgical gastrointestinal cancer specimens

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

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

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

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

Growth-suppressive activity of *FHL1*

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

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

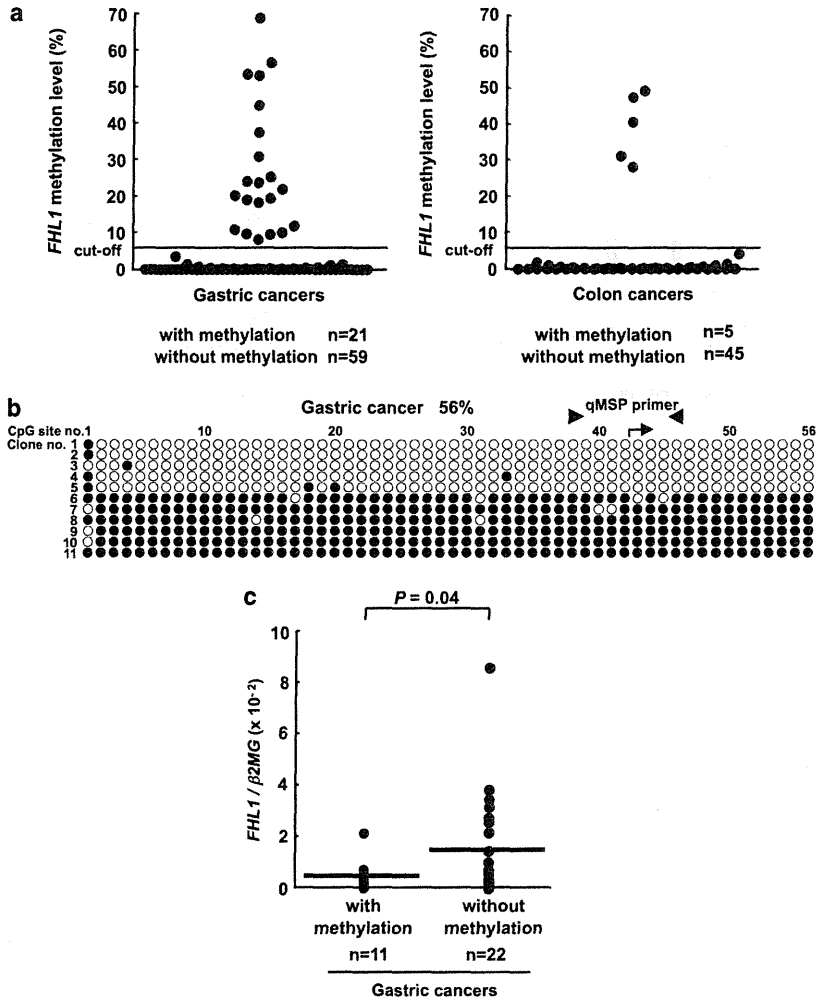

Figure 3. Methylation of *FHL1* in surgical gastrointestinal cancer specimens and its effect on expression. (**a**) Methylation levels in gastric (left) and colon (right) cancers derived from male patients. A horizontal line shows a cutoff value of 6%. *FHL1* was methylated in 21 of 80 primary gastric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

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

Inhibitory effects of *FHL1* on migration and invasion

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

An *FHL1* mutation and its loss of function

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

FHL1 methylation levels in non-cancerous gastric and colonic mucosae

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