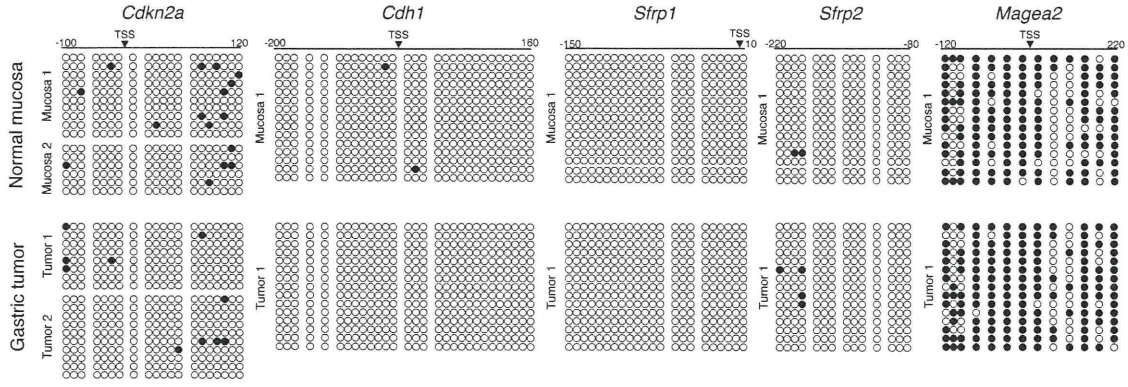

Fig. 6. Results of bisulfite DNA sequencing of the promoter CpG island region of *Cdkn2a*, *Cdh1*, *Sfrp1*, *Sfrp2* and *Magea2*. Numbers represent the positions relative to the transcription start site. TSS, transcription start site. Circles, CpG dinucleotide (potential target of methylation); closed circles, methylated CpG dinucleotide and open circles, unmethylated CpG dinucleotide.

tumors (Figure 6), indicating that the pattern of the site-specific aberrant methylations in mouse gastric tumors is distinctly different from that in human gastric cancers.

Discussion

Previous studies have revealed that a genetic reduction of the DNA methylation levels results in opposing effects on tumor development, depending on the tumor cell type and the stage of tumorigenesis. Consistent with earlier reports concerning tumorigenesis in the intestine (17,18) and the upper digestive tract (19), we herein demonstrated that the genetic reduction of DNA methylation levels suppresses gastric tumorigenesis in two different models of gastric cancer: the MNU-induced model and the $Apc^{Min/+}$ mouse model. Although further long-term observations would be required to determine whether DNA hypomethylation completely suppresses the progression to the invasive tumors or not, it is noteworthy that DNA hypomethylation inhibited the development of invasive adenocarcinoma, suggesting that DNA hypomethylation suppresses the malignant transformation of gastric tumors. This notion is also consistent with previous findings that genetic reduction blocked the development of invasive squamous cell carcinoma in the tongue and esophagus (19). These findings may shed some light on the involvement of epigenetic modification in the acquisition of invasive properties of tumor cells.

The presence of cancer-predisposed mucosa was initially described in the setting of oral carcinogenesis, and the concept of 'field cancerization' (28) has been widely accepted in regard to various organs, including the stomach (29,30). Recent studies suggest the involvement of epigenetic alterations in field cancerization in the human stomach (29–31). In the present study, the administration of MNU induced abnormal cell proliferation in non-cancerous gastric mucosa, which supported the concept of field cancerization. It is important that global DNA hypomethylation significantly suppressed the induction of abnormal proliferation in the MNU-exposed non-cancerous gastric mucosa. Because increased cell proliferation is obviously associated with the risk of cancer development, DNA hypomethylation suppresses the development of gastric tumors by preventing the gastric mucosa from being transformed into a favorable environment for tumor development.

In human gastric cancer, several tumor suppressor genes are inactivated more frequently by epigenetic silencing associated with site-specific DNA hypermethylation than by mutations (7). In addition, higher methylation levels at seven CpG islands in human gastric mucosa have also been reported to significantly correlate with a higher risk of gastric cancer development (29). Therefore, one may hypothesize that the genetic reduction of DNA methylation blocks the epigenetic silencing of the candidate genes that play a crucial role in MNU-induced gastric tumorigenesis. However, it remains controversial whether DNA hypermethylation plays a major role in gene silencing in rodent tumorigenesis. Although previous studies have indicated the presence of genomic hypermethylation in the tumor suppressor genes observed in rodent gastric tumors (32,33), another study revealed no involvement of DNA hypermethylation in the specific genes that are frequently hypermethylated in human gastric cancers (34). In the present study, we also examined the methylation status of the promoter regions of the genes that are frequently hypermethylated or hypormethylated in human gastric tumor (8–12,30) but found no altered DNA methylation patterns. Other epigenetic mechanisms, which are independent of the activation of the silenced genes, might therefore be associated with the suppression of gastric tumorigenesis by the genetic reduction of DNA methylation levels. Further analyses are therefore required to identify the target genes and/or sites of genomic hypomethylation responsible for the tumor suppression in this model.

Our data suggest that a pharmacological modification of the epigenetic status may be a potent strategy for the prevention and treatment of gastric cancers, and this idea is supported by the findings that *Helicobacter pylori*-infected human gastric mucosa harbors genomic hypermethylation (29,31) and several tumor suppressor genes are inactivated by promoter hypermethylation in human gastric cancer (7,9–12). However, possible side effects must be carefully taken into consideration for clinical application of DNA hypomethylating agents because the reduction of genomic methylation levels promotes the development of other type of tumors as previously reported (5,6,18).

In summary, we herein demonstrated that a reduction of the DNA methylation levels consistently suppresses gastric tumorigenesis, thus suggesting that DNA methylation is closely associated with gastric tumorigenesis.

Supplementary material

Supplementary Table 1 can be found at http://carcin.oxfordjournals.org/

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Fibroblasts associated with cancer cells keep enhanced migration activity after separation from cancer cells: A novel character of tumor educated fibroblasts

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Abstract. It is now clear that the association between cancer cells and recruited fibroblasts (cancer-associated fibroblasts; CAFs) leads to alteration of the biological properties of both types of cells and creates a specific microenvironment. Here we report a novel biological property of CAFs and its cellular mechanism using *in vivo* and *in vitro* model. Cultured CAFs derived from human lung cancer tissue displayed significantly higher migration activity in response to PDGF-BB than that of fibroblasts from corresponding non-cancerous tissue (NCAFs). Moreover, KM104^{GFP} (GFP-labeled human fibroblast cell line) co-cultured with human cancer cell line Capan-1 showed significantly higher migration activity than KM104^{GFP} alone. No such phenomenon occurred when KM104^{GFP} and Capan-1 were cultured separately. Even after KM104^{GFP} were sorted from co-cultured Capan-1, KM104^{GFP} retained their enhanced migration activity until passage-5 of culture in the absence of cancer cells. Despite a similar level of phosphorylation of ERK1/2 after exposure to PDGF-BB, the inhibitory effect of MEK inhibitor was significantly higher on migration of KM104^{GFP} that had been sorted from co-cultured Capan-1 than of KM104^{GFP} alone. This higher dependence on ERK1/2 signaling for cell migration was also seen in CAFs obtained from cancer tissue. The results of this study indicate that by association with cancer cells, CAFs can acquire enhanced migration activity which could be kept after separation from cancer cells and suggest the possibility that higher dependence on ERK1/2 signaling for enhanced migration activity would be one of the biological properties of CAFs.

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Key words: cancer associated fibroblast, MAP kinase, migration activity

Introduction

During the process of cancer cell invasion, the cancer cells associate with several types of stromal cells that together create the specific microenvironment of the cancer tissue. It is becoming clear that the microenvironment plays an important role in allowing the tumor to express its full neoplastic phenotype (1,2). Fibroblasts, which are the major component of the newly created stroma, are recruited from neighboring tissues and remote organs (3-7). This recruitment step is called the 'desmoplastic reaction' and is a kinetic sequence of events in the invasion process. Within the cancer tissue, the fibroblasts directly communicate with the cancer cells and other types of stromal cells and acquire a specific biological phenotype (8,9). The type of fibroblast in cancer tissue that has acquired a specific biological phenotype is called cancer-associated fibroblast (CAF), and such fibroblasts have been postulated to perform supportive roles that promote tumor progression and metastasis by secreting growth factors (10,11), chemokines (12,13), and matrix metalloproteinases (MMPs) (14,15). Although an increasing number of translational studies have emphasized the prognostic significance of the altered phenotype of CAFs (16-18), the cellular mechanisms of these alterations remain unclear.

It is noteworthy that expression of specific molecules on CAFs is maintained under *in vitro* conditions, which means that alterations of gene expression in CAFs are capable of being stably maintained even in the absence of continued exposure to cancer cells. Actually, in a comparison with cultured non-cancerous fibroblasts Nakagawa *et al* found that approximately 170 of 22,000 genes were up-regulated in cultured CAFs (fold change >2, $P<0.05$), and that the upregulated genes included many genes that encode cell

adhesion molecules and growth factors (19). However, the mechanisms by which specific gene expression is induced in CAF are controversial. In some studies, the frequency of LOH in CAFs has been reported to be similar to the frequency observed in the epithelial components (20-22). Qiu *et al*, on the other hand, reported that LOH and copy number alterations were extremely rare in CAFs from breast and ovarian cancers (23). Very little is known as to how genetic or epigenetic status can modify gene expression and cellular signaling(s) in CAFs.

The desmoplastic reaction during cancer progression centers on various functions of fibroblasts (24). One of the representative phenotypes involved in this reaction is migration activity, which is thought to be related to cancer progression (25). In this study we used *in vivo* and *in vitro* models to investigate the biological characteristics of CAFs, with a special focus on their migration activity and the cellular mechanism.

Materials and methods

Human fibroblast culture. Cancer-associated fibroblasts (CAFs) and non-cancerous tissue-associated fibroblasts (NCAFs) were obtained from the surgically resected lungs of lung cancer patients as previously reported (26). Briefly, approximately 5-mm^3 carcinoma tissue and non-cancerous lung tissue specimens were cut into about ten pieces and each was placed in α-MEM (Gibco, Grand Island, NY) culture medium with 10% heat-inactivated FBS and antibiotics (penicillin and streptomycin) (Sigma, St. Louis, MO). The medium was changed every other day until the tissue was surrounded by adherent fibroblasts. The tissue was then removed and cultured for two more days. When the cells reached 80% confluence they were harvested with 0.25% trypsin and 1 mmol/l EDTA and then replated at a density of $1x10^4$ cells/cm^2. The fibroblasts were separated from contaminating epithelial and macrophages by differential trypsinization and used between passages 3 and 7. All specimens were collected after the subjects had given their written informed consent and it was approved by the Institutional Review Board of the National Cancer Center.

Animals. Six-week-old female severe combined immunodeficient (SCID) mice (C.B-17 background) were purchased from CLEA Japan, Inc. (Tokyo, Japan) and maintained at the National Cancer Center Research Institute East (Chiba, Japan). All animals were maintained under specific-pathogen-free, temperature-controlled environmental conditions throughout this study, in accordance with the Institutional Guidelines. Written approval for all animal experiments (K03-011) was obtained from the local Animal Experiments Committee of the National Cancer Center Research Institute.

Cell lines and cell cultures. KM104 cells, SV-40 transformed human fibroblast cell line derived from bone marrow, were originally established from male patient as described previously (27). This cell line was cultured in RPMI-1640 medium (Sigma) with 10% heat-inactivated fetal bovine serum (FBS) and antibiotics (penicillin and streptomycin). Human pancreatic cancer cell line Capan-1 was purchased from the American Type Culture Collection (Rockville, MD). The Capan-1 cells were maintained in DMEM (Sigma) medium with 20% heat-inactivated FBS and antibiotics. All cells were maintained in a 5% CO_2 incubator at 37˚C.

Green fluorescent protein (GFP) labeling and cell sorting. KM104 were transfected with pEGFP-C1 vector encoding EGFP (Clonetech, Palo Alto, CA) by using Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA). GFP-positive KM104 ($KM104^{GFP}$) cells were sorted by using a FACSCalibur sorting system (Becton-Dickinson, San Jose, CA). After confirming that over 90% of cell line was GFP positive, they were used in the following experiment.

Intraperitoneal xenotransplantation of Capan-1 and $KM104^{GFP}$. Capan-1 cells ($5x10^6$ cells per animal) were injected into the peritoneal cavity of SCID mice as described previously, and 1 h later, $5x10^6$ $KM104^{GFP}$ were injected into each peritoneal cavity at a different injection site (28). The animals were sacrificed on day 21, and parapancreatic tumors were removed, minced, and cultured in 10% FBS RPMI-1640. Under subconfluent conditions, the adherent cells were harvested, and the GFP-positive cells were sorted with the FACSCalibur sorting system.

Co-culture system. $KM104^{GFP}$ ($1x10^6$) and Capan-1 ($2x10^5$) were co-cultured in RPMI-1640 medium with 10% FBS. After 48 h, cells were collected, and GFP-positive cells were sorted with the cell sorter, and the cultured. $KM104^{GFP}$ used in experiments were passaged by harvesting and distribution into five new plates. To obtain cell-conditioned medium, Capan-1 were cultured until semiconfluent conditions. The plate was then washed with sterile PBS, and the medium was changed to RPMI-1640+10% FBS. After incubation for 24 h, the Capan-1 culture supernatant was collected and added to the pre-seed $KM104^{GFP}$, and their chemotactic activity was assayed 48 h later.

Migration assays. In the preliminary study, we tested the ability of 10 synthetic chemoattractants and growth factors (b-FGF, CCL-21, EGF, IGF-1, IL-8, PDGF-AA, PDGF-BB, SDF-1, TGF-ß, VEGF-A), and found that PDGF-BB was the most powerful chemoattractant at the same protein concentrations (data not shown). PDGF-BB was therefore used as the chemoattractant in the subsequent experiments. The migration assay was performed by using 24-well culture chambers (Becton-Dickinson Labware, Bedford, MA) and a polycarbonate filter with an 8 μm pore size (Becton-Dickinson Labware) as previously described (28). Briefly, the lower chamber contained 0.6 ml of RPMI-1640 + recombinant PDGF-BB (final concentration; 0-100 ng/ml), or 0.6 ml of RPMI-1640 as a control. In the upper compartment, $2x10^4$ cells/well were placed in triplicate wells and incubated for 6 h at 37˚C in a humidified incubator under a 5% CO_2 atmosphere. At the end of the incubation period, the cells that had passed through the filter into the lower wells were stained with hematoxylin, and the cells in nine predetermined fields were counted under a microscope.

Western blotting. Western blot analysis was performed as follows. Cells were lysed in whole-cell extraction buffer

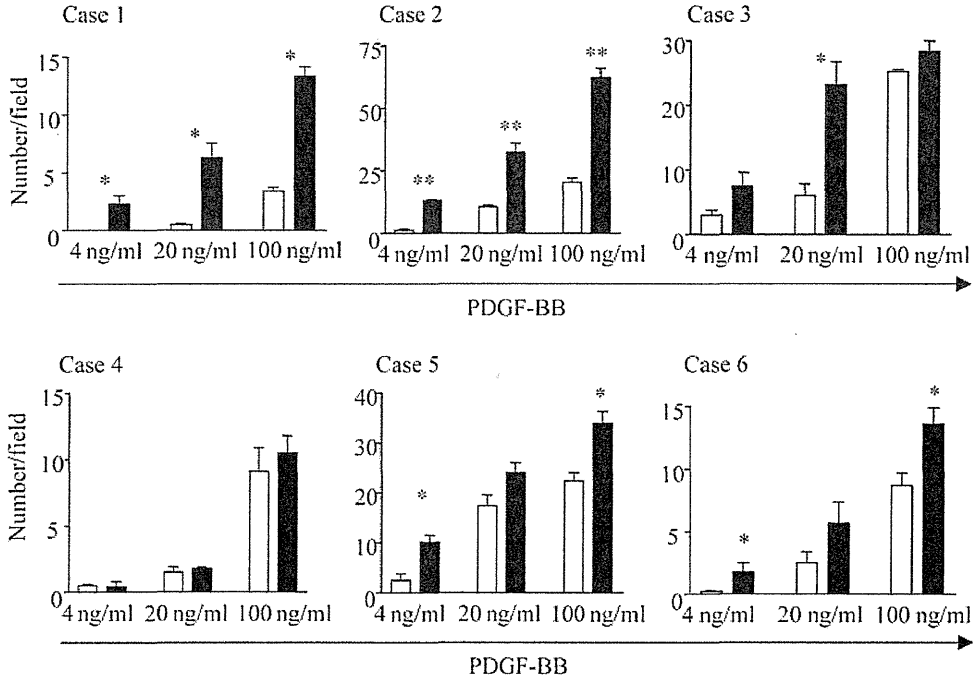

Figure 1. Cancer-associated fibroblasts (CAFs) isolated from human cancer tissue displayed enhanced migration activity in response to PDGF-BB. Fibroblasts isolated from human lung cancer tissue (cancer-associated fibroblasts; CAFs) and fibroblasts from corresponding non-cancerous lung tissues (non-cancerous-tissue-associated fibroblasts; NCAFs) were isolated and cultured, and their migration activity was compared. Except for case 4, migration activity of the CAFs in response to PDGF-BB was significantly higher than that of the NCAFs. (*P<0.05, **P<0.01).

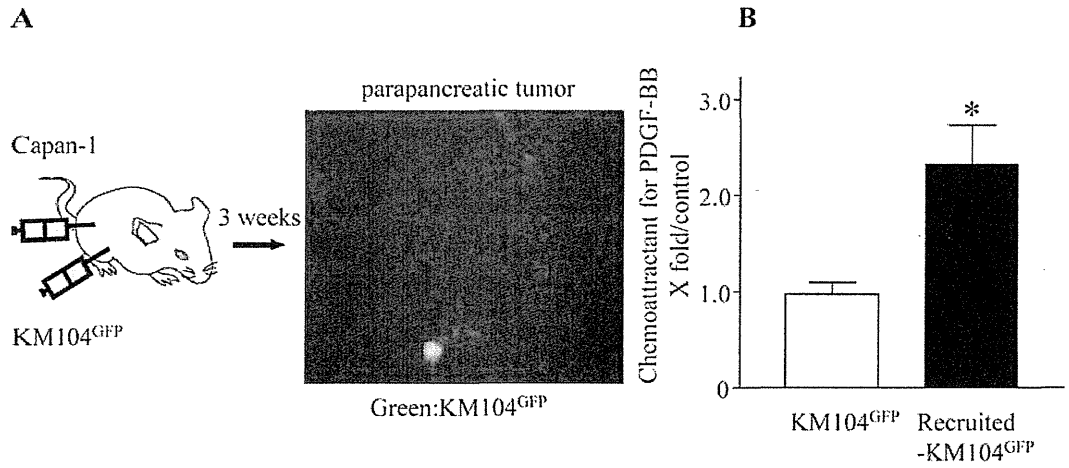

Figure 2. KM104^{GFP} recruited into cancer stroma *in vivo* display enhanced migration activity in response to PDGF-BB. (A) GFP-positive cells (KM104^{GFP}) are scattered within this abdominal tumor. (B) GFP (+) cells were sorted from the minced abdominal tumor and cultured *in vitro* (rec-KM104^{GFP}). Their migration activity in response to PDGF-BB was then compared with that of the parent cells (KM104^{GFP}). Rec-KM104^{GFP} exhibited a 2.4-fold higher migration activity than parent KM104^{GFP} (n=3, *P=0.03).

(20 mM Hepes-NaOH, 0.5% NP-40, 15% glycerol) containing a Complete protease inhibitor cocktail tablet (Roche Diagnostics, Mannheim, Germany). Proteins were separated on 12% SDS-polyacrylamide gels and then transferred to an Immobilon-P PVDF (polyvinylidene fluoride) membrane (Millipore, Billerica, MA). The blots were saturated with blocking buffer (5% skim milk in TBS-T) for 1 h at room temperature and then incubated for 1 h at 4°C with anti-phospho-ERK1/2 antibody (mouse monoclonal IgG, E10, phospho-p44/42 MAPK antibody-Thr 202/Tyr 204) and total ERK1/2 antibody (rabbit polyclonal, p44/42 MAPK antibody) according to the manufacturer's instructions (Cell Signaling Technology, Beverly, MA, USA). After washing in TBS-T, the membranes were incubated for 1 h at room

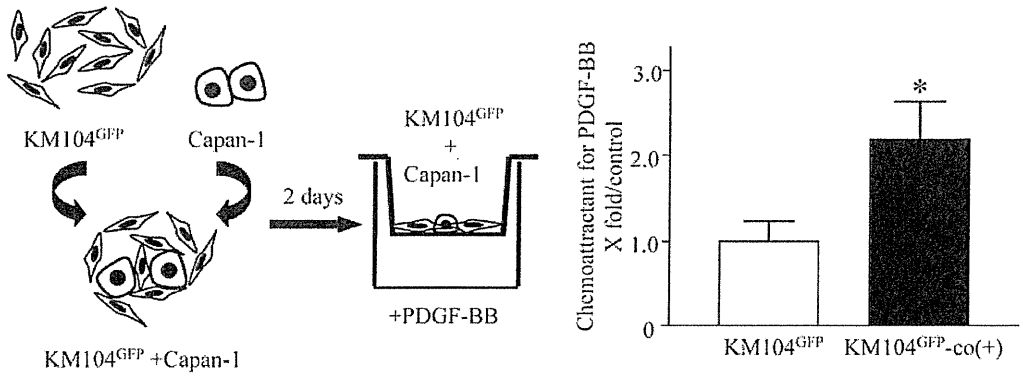

Figure 3. KM104^{GFP} co-cultured with a cancer cell line display enhanced migration activity in response to PDGF-BB. KM104^{GFP} were co-cultured with Capan-1 cells for two days [KM104^{GFP}-co(+)], and their migration activity in response to PDGF-BB was assayed. KM104^{GFP}-co(+) displayed 2.2-fold higher migration activity than KM104^{GFP} cultured in the absence of Capan-1 (n=9, *P=0.04)

temperature with HRP-Rabbit anti-mouse IgG or HRP-goat anti-rabbit IgG (Zymed, San Francisco, CA). Antibody binding was detected with an Amersham enhanced chemiluminescence system.

Methylated DNA immunoprecipitation-chip (MeDIP-chip) assay. A 5 μg sample of genomic DNA was sonicated to fragment lengths between 200 and 800 bp. After heat denaturation, the DNA was incubated with 5 μg of antibody against 5-methyl cytidine (Diagnode, Liège, Belgium) at 4°C overnight. Immuno-complexes were collected with Dynabeads Protein A (Invitrogen Dynal AS, Oslo, Norway), treated with Proteinase K, and purified by phenol and chloroform extraction and isopropanol precipitation. The chip assay was carried out with a human CGI oligonucleotide microarray (Agilent Technologies, Santa Clara, CA). Immunoprecipitated DNA from 4.5 μg of sonicated DNA and 1 μg of input DNA were labeled with Cy-5 and Cy-3, respectively, using an Agilent labeling kit (Agilent Technologies), and then hybridized with the microarray. The microarray was scanned with an Agilent G2565BA microarray scanner (Agilent Technologies). The methylation statuses of 8,528 promoter CGIs were evaluated by using Me values, which have been shown to have a higher correlation coefficient with the fraction of methylated DNA molecules (29,30), the methylation status of 8,528 promoter CGIs was evaluated.

MEK inhibitor treatment. Cells were pretreated with the MEK1/2 inhibitor compound UO126 (Promega, San Diego, CA) or with DMSO vehicle alone before harvesting. UO126 was used at a concentration of 25 μM, which was shown to maximally inhibit MEK in fibroblasts. In the upper compartment, $2x10^4$ cells/well were placed in medium containing 25 μM of U0126 and incubated for 6 h.

Statistical analyses. Three or four independent experiments were performed for each protocol. Results were expressed as the mean ± standard error of the mean (SEM). Statistical calculations were performed by using Prism 3.03 software. Differences in measured variables between experimental groups and control groups were assessed by using an unpaired t-test. $P<0.05$ was considered statistically significant.

Results

Cancer-associated fibroblasts (CAFs) isolated from human cancer tissue displayed enhanced migration activity in response to PDGF-BB. Fibroblasts isolated from human lung cancer tissue (cancer-associated fibroblasts; CAFs) and fibroblasts from corresponding non-cancerous lung tissues (non-cancerous tissue-associated fibroblasts; NCAFs) were isolated and cultured, and their migration activity was compared. Except for Case 4, migration activity of the CAFs in response to PDGF-BB was significantly higher than that of the NCAFs (Fig. 1).

Fibroblast cell line KM104^{GFP} recruited into cancer stroma displays enhanced migration activity in response to PDGF-BB. Capan-1 and KM104^{GFP} were injected intraperitoneally at a different site. Immunofluorescence examination of the parapancreatic tumor revealed the presence of KM104^{GFP} within the cancer tissue (Fig. 2A). GFP (+) cells were sorted from the minced parapancreatic tumor and cultured, and the chemoattractant response of the KM104^{GFP} to PDGF-BB was compared with that of the parent cells. KM104^{GFP} recruited into the tumor exhibited a 2.4-fold higher migration activity than parent KM104^{GFP} (n=3, Fig. 2B, P=0.03).

KM104^{GFP} co-cultured with the cancer cell line displayed enhanced migration activity in response to PDGF-BB. We co-cultured KM104^{GFP} and Capan-1 for two days and assayed the migration activity of KM104^{GFP} in response to PDGF-BB. No Capan-1 cells were able to migrate through the cell culture insert (8.0 μm pore size) under these conditions. KM104^{GFP} co-cultured with Capan-1 [KM104^{GFP}-co(+)] displayed 2.2-fold higher migration activity than KM104^{GFP} cultured in the absence of Capan-1 (n=9, P=0.04, Fig. 3).

Cell-cell contact is necessary for enhanced migration activity. To explore whether cell-cell contact is necessary for KM104^{GFP} to exhibit enhanced migration activity, we cultured KM104^{GFP} with KM104 cell conditioned medium (control) or Capan-1 cell conditioned medium for 2 days, and assayed their migration activity, but no significant difference in migration activity was found between the two groups (Fig. 4A). Next,

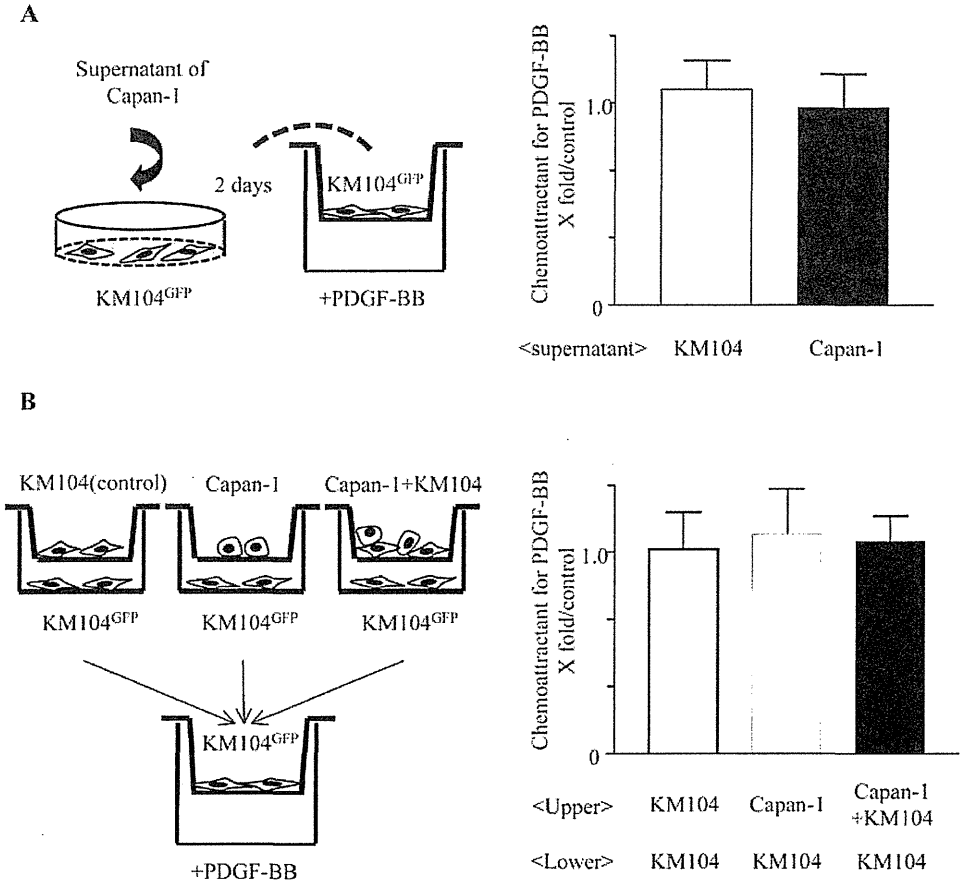

Figure 4. Cell-cell contact is necessary for enhanced migration activity. (A) KM104^{GFP} cells were cultured with conditioned medium from KM104 cell cultures (control) or Capan-1 cell cultures for 2 days, and their migration activity was assayed. (B) KM104^{GFP} were co-cultured in the 2-chamber system (upper chamber; KM104, Capan-1, or Capan-1 +KM104, lower chamber; KM104^{GFP}) for 2 days, and the migration activity of the KM104^{GFP} cultured in the lower chamber was assayed.

KM104^{GFP} were co-cultured in the 2-chamber system (upper chamber: KM104, Capan-1, or Capan-1+KM104; lower chamber: KM104^{GFP}) for 2 days, and the migration activity of the KM104^{GFP} cultured in lower chamber was assayed (Fig. 4B). Migration number of KM104^{GFP} exposed to Capan-1 cell supernatant (middle column) and KM104^{GFP} exposed to Capan-1+KM104 cells supernatant (right column) was almost the same as that of control group (left column). These findings suggested that direct KM104-Capan-1 contact is necessary for KM104^{GFP} to exhibit enhanced migration activity.

Enhanced migration activity of KM104^{GFP}-co(+) is kept after separation from cancer cells. GFP-positive KM104 were sorted (3.0x10^5) from cocultured Capan-1 and cultured (Fig. 5A). In a control experiment, KM104^{GFP} alone were cultured, and GFP-positive cells were sorted (sham-sorted) and cultured. Cells from subconfluent cultures (1.0x10^6) were then assayed for migration activity in response to PDGF-BB. The results showed that the sorted KM104^{GFP}-co(+) displayed 2.3-fold higher activity than sham-sorted KM104^{GFP} (P=0.02), indicating that the sorted KM104^{GFP}-co(+) displayed enhanced migration activity for almost 2 popu-lation doublings after separation from the cancer cells.

Sorted KM104^{GFP}-co(+) were then serially passaged by harvesting and distribution into five new plates, and migration activity was compared in each passage culture. Three independent experiments were performed, and the results are shown in Fig. 5B. The sorted KM104^{GFP}-co(+) kept their enhanced migration activity even after the passage-5 culture in every experiment. In experiments 2 and 3, the enhanced migration activity of the sorted KM104^{GFP}-co(+) returned to the levels of passaged sham-sorted KM104^{GFP} cells in the passage-6 culture. These results indicate that the sorted KM104^{GFP}-co(+) retained the enhanced migration activity for at least 13 population doublings after separation from the cancer cells *in vitro*.

MeDIP-chip analysis of CAFs. Because of the prolonged enhanced migration activity of CAFs, it was important to analyze the epigenetic changes that accompany this phenomenon. We used a methylation screening approach called MeDIP-chip to compare the DNA methylation profiles of the CAFs and corresponding NCAFs from two cases. The results of the MeDIP-chip analysis showed that 2.2% (188 promoter CGIs) (Case 1) and 1.1% (98 promoter CGIs) (Case 2) of the CGIs were hypermethylated in CAFs compared with NCAFs.

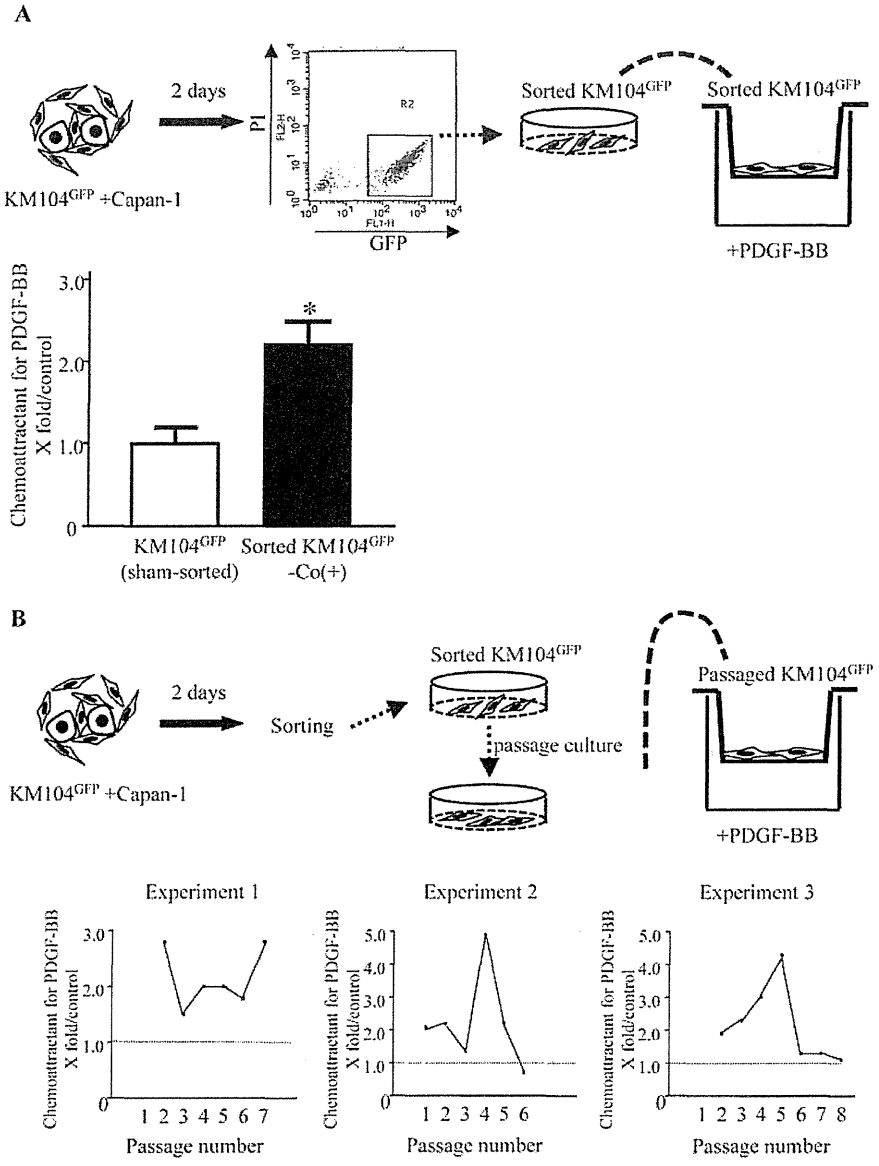

Figure 5. Sustained enhanced migration activity of KM104^{GFP}-co(+) after separation from the cancer cells. (A) After sorting from the co-cultured Capan-1, GFP-positive KM104 were sorted and cultured [Sorted KM104^{GFP}-co(+)]. In a control experiment, KM104^{GFP} alone were cultured, and the GFP-positive cells were sorted (sham-sorted) and cultured. Cells from a subconfluent culture were then examined for migration activity in response to PDGF-BB. Sorted KM104^{GFP}-co(+) displayed 2.3-fold higher activity than sham-sorted KM104^{GFP} (n=3, *P=0.02). (B) Sorted KM104^{GFP}-co(+) were serially passaged by harvesting and distribution into five new plates, and their migration activity was compared at each passage culture. Representative data of three independent experiments are shown.

0.5% (39 promoter CGIs) (Case 1) and 0.6% (50 promoter CGIs) (Case 2) were hypomethylated in CAFs compared with NCAFs (Table I). Ten promoter CGIs (*ALDH1L2*, *AP1G2*, *CENPJ*, *KBTBD6*, *MAB21L1*, *MED4*, *RCBTB2*, *SH2B3*, *TBC1D4* and *UBL3*) were hypermethylated in the CAFs from both cases (Table II), but no promoter CGIs were hypomethylated in the CAFs from either of the cases.

Role of extracellular signal-regulated kinase (ERK) 1/2 in PDGF-BB-induced migration of sorted KM104^{GFP}-co(+) and CAFs. Activation of extracellular signal-regulated kinase (ERK) 1/2 has been reported to play crucial role in PDGF-induced cell migration. We analyzed the effects of PDGF-BB

Table I. Aberrantly methylated promoter CGIs in CAF to be compared with NCAF.

	Pair	No. of genes
Hypermethylated genes		
	Case 1	188
	Case 2	98
Hypomethylated genes		
	Case 1	39
	Case 2	50

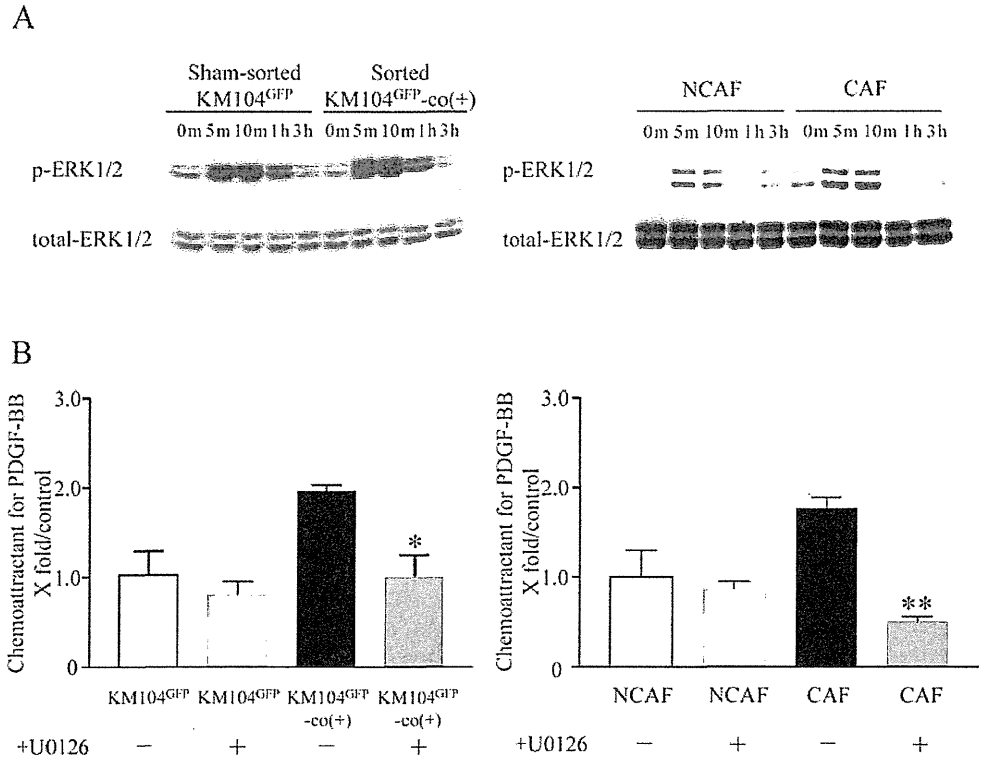

Figure 6. Role of extracellular signal-regulated kinase (ERK) 1/2 in PDGF-BB-induced migration by sorted KM104^{GFP}-co(+) cells and CAFs. (A) Western blotting of phosphorylated ERK1/2. PDGF-BB increased the level of ERK1/2 phosphorylation in sham-sorted KM104^{GFP} cells, sorted KM104^{GFP}-co(+) cells, NCAFs, and CAFs in a time-dependent manner, reaching a maximum at 300-600 sec, and the phosphorylation level decreased slightly thereafter. (B) Inhibitory effect of treatment with UO126 (25 μM) on cell migration. Treatment of the sham-sorted KM104^{GFP} and KM104^{GFP}-co(+) with UO126 decreased migration to 80.0±15.3% and 50.1±10.8% of untreated group, respectively (P=0.01). UO126 decreased the migration of NCAFs and CAFs to 86.7±8.9% and 28.1±1.6% of untreated group, respectively (P=0.001).

Table II. Genes hypermethylated in both CAFs.

Gene ID	Gene name
160428	Aldehyde dehydrogenase 1 family, member L2 (ALDH1L2)
8906	Adaptor-related protein complex 1, γ 2 subunit (AP1G2)
55835	Centromere protein J (CENPJ)
89890	Kelch repeat and BTB (POZ) domain containing 6 (KBTBD6)
4081	Mab-21-like 1 (C. elegans) (MAB21L1)
29079	Mediator complex subunit 4 (MED4)
1102	Regulator of chromosome condensation and BTB domain containing protein 2 (RCBTB2)
10019	SH2B adaptor protein 3 (SH2B3)
9882	TBC1 domain family, member 4 (TBC1D4)
5412	Ubiquitin-like 3 (UBL3)

Hypomethylated genes in both CAFs were not found.

on ERK1/2 activation of sorted KM104^{GFP}-co(+). As shown in Fig. 6A, PDGF-BB increased ERK1/2 phosphorylation in both sham-sorted KM104^{GFP} and sorted KM104^{GFP}-co(+) in a time-dependent manner up to a maximum at 300-600 sec, and the phosphorylation level decreased thereafter. The levels of phosphorylated ERK1/2 in both sham-sorted KM104^{GFP}and sorted KM104^{GFP}-co(+) was similar, and this phenomenon was also observed in CAFs and NCAFs isolated from lung cancer tissue.

Treatment of the sham-sorted KM104^{GFP} with UO126 (25 μM), a highly selective inhibitor of both MEK1 and MEK2, decreased migration to only 80.0±15.3% of untreated group, whereas it decreased migration of the sorted KM104^{GFP}-co(+) to 50.1±10.8% of untreated group (P=0.01) (Fig. 6B).

Furthermore, although U0126 decreased the PDGF-BB-induced migration of NCAFs to only 86.7±8.9% of untreated group, it decreased the migration of CAFs to 28.1±1.6% of untreated group (P=0.001).

Discussion

In the current study we demonstrated that fibroblasts that have associated with cancer cells retain their enhanced migration activity for a while after separation from the cancer cells. Furthermore, we first report the possibility that one of the biological characteristics of CAFs would be higher dependence on ERK1/2 signaling for their enhanced migration activity even in the absence of cancer cells.

It is now clear that activation of ERK1/2 is important for PDGF-induced cell migration (31-33). We examined the levels of ERK1/2 phosphorylation of sorted-KM104^{GFP}-co(+) and CAFs after PDGF-BB treatment, but there was no significant difference from the level in the control cells. However, the inhibitory effect of a selective inhibitor of both MEK1 and MEK2 on migration was significantly higher in sorted-KM104^{GFP}-co(+) and CAFs than in control cells. We also investigated the involvement of other MAPK family, including the JNK and P38, however, phosphorylation of these molecules in sorted-KM104^{GFP}-co(+)and CAFs were almost the similar level as those of control cells with or without PDGF-BB stimulation. Moreover, the inhibitory effect of their specific inhibitor SP600125 or SB203580 on cell migration in sorted-KM104^{GFP}-co(+) and CAFs and in untreated cells was almost equal (data not shown). This finding indicated that the fibroblasts that had associated with cancer cells were more dependent on the ERK1/2 pathway for cell migration. Activated ERKs regulate membrane protrusions and focal adhesion dynamics via myosin light chain kinase (MLCK), calpain, paxillin, and focal adhesion kinase (FAK) activation (32), however, the MeDIP-chip analysis did not reveal any promoter CGIs involved in these molecules. After association with cancer cells, pairs of factors that have a synthetic relationship in CAFs may differ from those in naive fibroblasts, thus increasing the dependence of cells on the downstream ERK1/2 pathway. Alternatively, the downstream ERK1/2 signaling pathway may play a more essential and qualitatively different role in a given pathway in CAFs than in naive fibroblasts, making them much more dependent on the activity of a specific signaling pathway. The direct association with cancer cells may cause 'signaling addiction' to occur in fibroblasts, which would be a similar condition explained by the concept of 'oncogene addiction' that some cancer cells are dependent on or addicted to one or a few genes for both maintenance of their malignant phenotype and cell survival (34,35).

DNA methylation is one of the molecular mechanisms that have long-term effects on gene expression. Since the enhanced migration activity of fibroblasts continues after being separated from cancer cells, we speculated that the association with cancer cells might change the methylation status of certain DNA(s) involved in cell migration. MeDIP-chip analysis identified that 10 promoter CGIs (*ALDH1L2, AP1G2, CENPJ, KBTBD6, MAB21L1, MED4, RCBTB2, SH2B3, TBC1D4* and *UBL3*) that were hypermethylated, no promoter CGIs were hypomethylated in both CAFs, and we were unable to identify any well-known methylated genes that were directly involved in cell migration. The cause of the persistent enhanced migration activity remains unknown, and additional research on chromatin modifications, such as histone deacetylation, will be necessary to understand the mechanisms underlying this phenomenon in CAFs.

Jiang *et al* detected global hypomethylation of genomic DNA by means of a methylation-sensitive SNP array analysis (MSNP), a restriction enzyme-based analysis, in the stromal myofibroblasts of gastric carcinomas (36). By contrast, when we focused on promoter CGIs in the current study, the results showed a higher number of hypermethylated genes than hypomethylated genes in CAFs (hypermethylated: 188 CGIs in Case 1 and 98 CGIs in Case 2; hypomethylated: 39 CGIs in Case 1 and 50 CGIs in Case 2). This difference might be caused by CGIs to be focused on. Our data showed for the first time that a few hundred genes were aberrantly hypermethylated in CAFs, suggesting that some regulators can be epigenetically targeted by the cancer microenvironment (37,38).

Cultured fibroblasts from idiopathic pulmonary fibrosis (IPF) were found to migrate faster than those from control fibroblasts, indicating that they also retained enhanced migration activity *in vitro* (39). Since there are many parallels between cancer and chronic active inflammation, as described in the saying, 'tumours are wounds that never heal', enhanced migration may be a common characteristic of fibroblasts recruited into fibrotic lesions.

Our current results may imply that by using this special property of CAFs, strategies that are directed to block the recruitment and tumor-associated functions of CAFs could be a possible method against tumors. Although the exact mechanisms of this phenomenon are not fully understood, cellular and molecular studies on the role of 'educated fibroblasts' should provide novel insights into the pathogenesis of a unique microenvironment of cancers.

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ORIGINAL ARTICLE—ALIMENTARY TRACT

Persistence of a component of DNA methylation in gastric mucosae after *Helicobacter pylori* eradication

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Abstract

Background *Helicobacter pylori* (*HP*) infection potently induces aberrant DNA methylation in gastric mucosae, and its accumulation is associated with gastric cancer risk. Cross-sectional analysis of methylation levels (fraction of methylated DNA molecules) and temporal analysis of methylation incidence suggested that methylation levels decrease after *HP* infection discontinues. We aimed to demonstrate the decrease in methylation levels.

Methods Thirty-five patients with *HP* infection who had undergone curative endoscopic resection and 11 healthy volunteers were recruited. Methylation levels were quantified by real-time methylation-specific PCR. Histology was evaluated according to the updated Sydney System.

Results In the 20 patients with successful eradication, the *FLNc* methylation level, along with infiltration of inflammatory cells, decreased from 0.6 to 0.4% at 6 weeks ($P = 0.049$) and remained low at 1 year. The *THBD* methylation level (30.1%) remained high at 6 weeks, but decreased to 19.0% at 1 year ($P = 0.0032$). Nine healthy volunteers with successful eradication tended to show a decrease of both *FLNc* and *THBD* at 6 weeks. However, the methylation levels after the decrease were still higher than those of healthy individuals without *HP* infection. In the 15 patients with persistent infection, the methylation levels remained the same. Before eradication, the *THBD* methylation level correlated with the degree of inflammatory cell infiltration ($P < 0.05$).

Conclusions Methylation levels in gastric mucosae decreased to certain levels after *HP* eradication in profiles unique to individual markers. Involvement of chronic inflammation in methylation induction was suggested.

Keywords DNA methylation · Epigenetics · *Helicobacter pylori* · Eradication · Molecular epidemiology

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Introduction

Gastric cancer is one of the most common cancers in the world, especially in eastern Asia [1]. To reduce its morbidity and mortality by early detection and early treatment, identification of individuals with high risk is important. Also, endoscopic resection (ER) is becoming a standard treatment for early gastric cancer [2, 3], and this

is leading to the problem of a high frequency of secondary gastric cancer after ER [4]. Therefore, risk diagnosis in the general population and patients who have undergone ER is indispensable for efficient surveillance of primary and secondary gastric cancers. For accurate risk diagnosis, we have to develop a risk marker that reflects the current accumulation level of genetic and epigenetic alterations in normal-appearing tissues. This is because exposure levels to carcinogenic factors and the degree of response to them are highly variable among individuals [5].

Infection by *Helicobacter pylori* (*H. pylori*) is the major causative agent of gastric cancers in Asian countries, and it is associated with accumulation of epigenetic alterations, namely aberrant DNA methylation, in gastric mucosae [6]. Among individuals without current *H. pylori* infection, a fraction of DNA molecules with methylation (DNA methylation level) of specific genes in gastric mucosae correlates with gastric cancer risk [6, 7]. Methylation levels retain their predictive power, even when adjusted by the extent of gastric atrophy [7]. Based upon these findings, DNA methylation levels in non-cancerous gastric mucosae are expected to provide a useful cancer risk marker that reflects past exposure to carcinogens and the degree of the "field defect" resultantly formed.

However, temporal profiles of DNA methylation levels in gastric mucosae during the course of *H. pylori* infection and its discontinuation have not been clarified yet. Quantification of methylation levels in cross-sectional groups suggested that the presence of *H. pylori* infection induces high levels of aberrant DNA methylation and that the high methylation levels will decrease to certain levels when the infection discontinues [6]. Non-quantitative studies showed that the incidence of methylation of the *CDH1* tumor-suppressor gene in gastric mucosae decreases after eradication of *H. pylori* [8, 9] and that incidences of multiple tumor-suppressor genes also decrease [10]. In a specific type of *H. pylori*-induced gastritis, enlarged fold gastritis, a quantitative study showed that *CDH1* methylation levels decrease at 3 months after eradication in *H. pylori* [11]. The decreased incidence in non-quantitative studies could be due to disappearance of foci with methylation-positive cells in some individuals or due to a decrease of methylation levels to below detection limits.

In this study, to clarify the temporal profiles of aberrant DNA methylation in gastric mucosae, we analyzed time trends of methylation levels in gastric mucosae before, and 6 weeks and 1 year after *H. pylori* eradication by a high-sensitivity quantitative method. To explore the possible role of chronic inflammation in methylation induction, we also analyzed the association between methylation levels and histological findings.

Materials and methods

Subjects, tissue samples and DNA extraction

Thirty-five patients with *H. pylori* infection who had undergone curative endoscopic resection (ER) of a well-differentiated early gastric adenocarcinoma [12, 13] were recruited from June 2006 to November 2006 at the National Cancer Center Hospital (Tokyo, Japan) under approval of the institutional review board and with written informed consents. None of the patients had received *H. pylori* eradication therapy prior to this study or regularly used proton pump inhibitors. The average age of the patients was 65.3 ± 7.4 (range 51–75), and the male-to-female ratio was 4 to 1 (28 men and 7 women). Eleven healthy volunteers with *H. pylori* infection who had no past history of gastric cancer (average age 59.9, male to female 6 to 5) were also recruited.

H. pylori infection status was analyzed by the culture test (Eiken, Tokyo, Japan) and rapid urease test (Otsuka, Tokushima, Japan). *H. pylori* was eradicated by 1-week administration of Lansoprazole (LPZ) 30 mg b.i.d, Amoxicillin (AMPC) 750 mg b.i.d and Clarithromycin (CAM) 200 mg b.i.d. Successful eradication was established by negative results for both the culture and rapid urease tests at multiple time points. *H. pylori* was successfully eradicated in 20 of 26 patients who received eradication therapy. The remaining six patients in whom eradication failed and nine patients who did not want to receive eradication therapy were treated as a group of persistent infection. There was no significant difference in the average age between the group of successful eradication and group of persistent infection (65.2 ± 6.9 and 65.4 ± 8.1, respectively, $P = 0.94$). Among the 11 healthy volunteers who received *H. pylori* eradication therapy, 9 individuals were successfully eradicated (average age 60.4, male to female ratio: 4 to 5).

Endoscopic biopsy materials were collected from three standard sites of non-cancerous gastric mucosae (upper gastric body, middle gastric body and the antrum in the lesser curvature) at three time points: the start point (before the eradication), 6 weeks after eradication and 1 year after eradication. In individuals without eradication therapy, biopsy materials were also collected at corresponding time points. Two biopsy samples were obtained from each site, and used for methylation and histological analyses. High molecular weight DNA was extracted by the standard phenol/chloroform method. Fasting blood samples were collected on the day of endoscopy to measure serum pepsinogen I and II (SRL, Tokyo).

Bisulfite treatment and methylation-specific PCR

Bisulfite treatment was performed as previously described [14]. Briefly, DNA samples (1 μg each) digested by *Bam*HI

were denatured in 0.3 N NaOH at 37°C for 15 min. The samples underwent 15 cycles of 30-s denaturation at 95°C and 15 min incubation at 50°C in 3.6 N sodium bisulfite (pH 5.0) and 0.6 mM hydroquinone. The samples were desalted with a Zymo-Spin Column IC (Zymo Research, Orange, CA) and desulfonated in 0.3 N NaOH. DNA was ethanol-precipitated and dissolved in 40 μl of TE buffer.

For methylation analysis, two promoter CpG islands of the filamin C (*FLNc*) and thrombomodulin (*THBD*) genes were selected from eight CpG island regions previously analyzed because they were closely correlated with the risk of gastric cancer development among individuals without current *H. pylori* infection "epigenetic gastric cancer marker genes" [6, 7]. Methylation levels were quantified by real-time methylation-specific PCR (MSP) as in our previous report [6], and the standard DNA for real-time MSP is available upon request. The methylation level of a sample for a CpG island was calculated as the fraction of methylated molecules among the total DNA molecules (number of methylated molecules + number of unmethylated molecules). The methylation level of a sample was measured in triplicate, and standard deviation of the measurement was confirmed as less than 15% of a methylation level. A methylation level of an individual at a time point was obtained as an average of three samples from the three biopsy sites [7].

For bisulfite sequencing, 1 μl of the sodium bisulfite-treated DNA was used for PCR with the primers common to methylated and unmethylated DNA sequences. The sequences were: *THBD*-forward, 5′-ATTTTGTTGGGGTG TAAGAAGTAT-3′ and *THBD*-reverse, 5′-CTACCCCAT AACTAACCAAAAAC-3′. The PCR products were cloned into a pGEM-T Easy TA Vector (Promega, Madison, WI), and 20–22 clones were cycle-sequenced for each sample.

Histological analysis

Biopsy specimens for histological analysis were fixed in 10% buffered formalin and embedded in paraffin. All tissue sections were stained with hematoxylin-eosin for histological examination. Intensities of acute infiltrates (neutrophil), chronic infiltrates (mononuclear cells), gastric atrophy and intestinal metaplasia [15] were graded according to the updated Sydney System as follows: none (0), mild (1), moderate (2) or marked (3) [16]. Histological review was performed by a single experienced pathologist (YN) who had no prior knowledge of the clinical course of the patients.

Statistical analysis

Methylation levels, patient's age, histological grades and serum pepsinogen value were expressed as an average ± standard deviation. Methylation levels and the histological grades and serum pepsinogen value between two time points were compared by Welch's *t* test (paired samples, two sided).

Results

Effects of *H. pylori* eradication on *FLNc* and *THBD* methylation levels

Methylation levels were measured in patients with successful eradication ($n = 20$) and patients with persistent infection ($n = 15$) at three time points. They were also measured in nine healthy volunteers with successful eradication ($n = 9$) at two time points.

In the patients with successful eradication, the average *FLNc* methylation level decreased from 0.6 ± 0.5 to 0.4 ± 0.3% at 6 weeks after eradication ($P = 0.049$) (Fig. 1a), and it remained at a low level (0.4 ± 0.3%) ($P = 0.50$, compared with 6 weeks after eradication; $P = 0.022$, compared with the start point) at 1 year after eradication. When methylation levels were analyzed separately in three sites of the stomach (Supplemental Figure), those in the middle and upper gastric bodies showed significant decreases ($P = 0.020$ and 0.005, respectively), while that in the antrum did not change. The average *THBD* methylation level (30.1 ± 12.3% before eradication) did not show a significant decrease at 6 weeks after eradication (31.0 ± 9.7%) in the group of successful eradication ($P = 0.32$) (Fig. 1a). However, at 1 year after eradication, it showed a significant decrease to 19.0 ± 11.5% ($P = 0.0032$).

In the patients with persistent infection, the average *FLNc* methylation level (1.1 ± 0.9% before eradication) remained the same at 6 weeks (0.8 ± 1.0%, $P = 0.18$) and 1 year after eradication (0.8 ± 1.0%, $P = 0.27$), compared with the start point. The average *THBD* methylation level (31.2 ± 15.4% before eradication) did not show a significant decrease at 6 weeks after eradication (33.1 ± 14.3%, $P = 0.33$) or at 1 year after eradication (28.9 ± 15.4%, $P = 0.29$), compared with the start point. Patients with persistent infection included those with eradication failure and those who received no eradication therapy, and we analyzed methylation levels separately in these two subgroups. Even in the eradication failure subgroup, there was no significant decrease of the *FLNc* and *THBD* methylation level between the start point and 1 year after eradication therapy ($P = 0.068$ and 0.128, respectively).

In the nine healthy volunteers with successful eradication (Fig. 1b), the average *FLNc* methylation level decreased from 1.6 ± 1.1 to 1.2 ± 0.9%, and the average

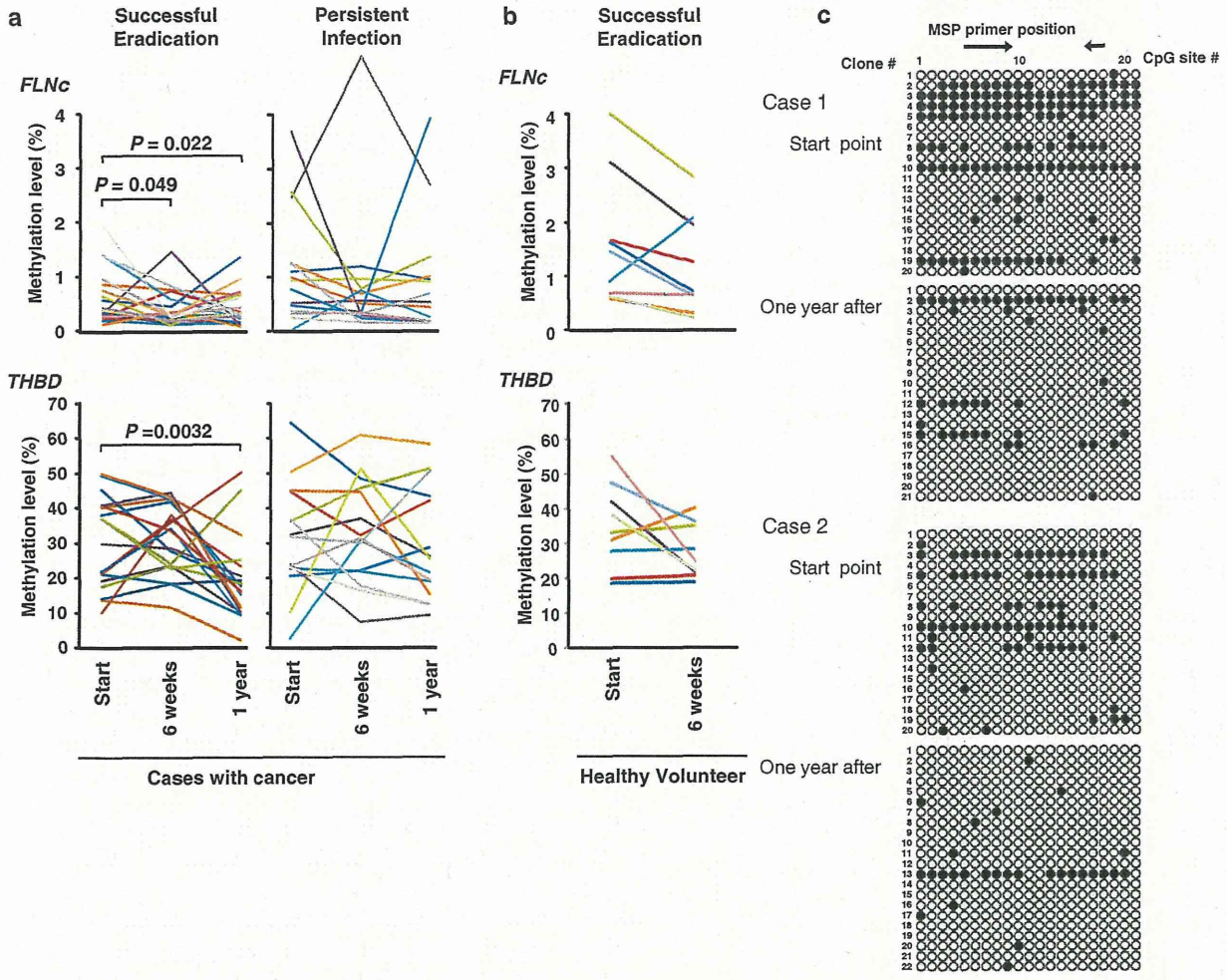

Fig. 1 **a** Effects of *H. pylori* eradication on *FLNc* and *THBD* methylation levels. A methylation level of a sample was measured in triplicate, and an average of three biopsy sites in the stomach was used as a methylation level of an individual. Methylation levels were measured in the group of successful eradication ($n = 20$) and that of persistent infection ($n = 15$) at three time points (before the eradication = start point; 6 weeks after eradication; and 1 year after eradication). Each *color* shows an individual patient. In the group of successful eradication, the *FLNc* methylation level decreased at 6 weeks after eradication ($P = 0.049$). The *THBD* methylation level showed a significant decrease at 1 year after eradication ($P = 0.0032$). In the group of persistent infection, neither the *FLNc* nor the *THBD* methylation level showed a significant decrease 1 year after eradication ($P = 0.27$ and 0.29, respectively). These findings demonstrate the presence of temporary components in DNA methylation. **b** Methylation changes in healthy volunteers. Eleven healthy volunteers received eradication therapy, and methylation levels were measured in nine volunteers with successful eradication before and 6 weeks after the eradication. The average *FLNc* methylation level decreased from 1.6 ± 1.1 to $1.2 \pm 0.9\%$, and the average *THBD* methylation level decreased from 34.7 ± 11.5 to $27.5 \pm 7.4\%$. Although there were tendencies, no significant difference was observed ($P = 0.054$ and 0.066 for *FLNc* and *THBD*, respectively). **c** Decrease of densely methylated DNA molecules shown by bisulfite sequencing. Twenty-one CpG sites of the *THBD* promoter region, covering the CpG sites used for its real-time MSP (shown by *arrows*) were analyzed in two pairs of samples. DNA molecules in which 9 or more of the 14 CpG sites between and on the MSP primers were considered to be densely methylated. Case 1 with a decrease from 54.8 to 6.0% by real-time MSP showed a decrease of densely methylated DNA molecules from 7/20 to 1/21 by bisulfite sequencing. Case 2 with a decrease from 33.7 to 5.1% showed a decrease from 3/20 to 1/22. The fraction of densely methylated DNA molecules by bisulfite sequencing was in accordance with the methylation level by real-time MSP. *Closed* and *open circles* show methylated and unmethylated CpG sites, respectively

THBD methylation level decreased from 34.7 ± 11.5 to $27.5 \pm 7.4\%$. Although both *FLNc* and *THBD* showed decreasing tendencies at 6 weeks, the decrease was not statistically significant ($P = 0.054$ and 0.066, respectively).

To confirm that we detected a decrease of densely methylated DNA molecules, not a decrease of methylated CpG sites, by real-time MSP we performed bisulfite sequencing of two pairs of samples (before and after eradication). The

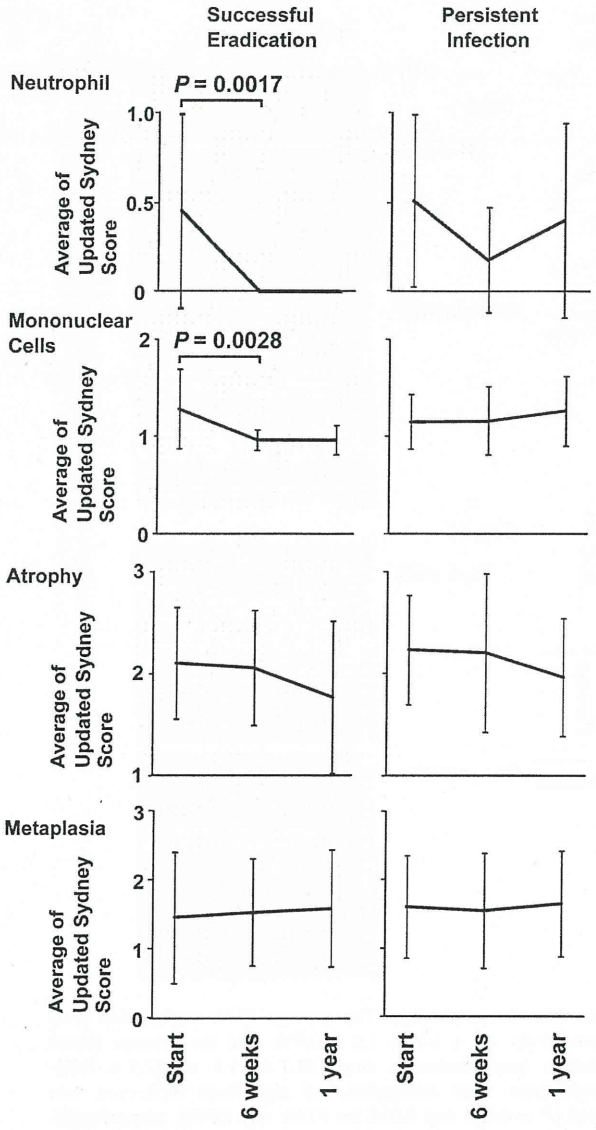

Fig. 2 Histological changes after *H. pylori* eradication. Average scores of histological gastritis of the three biopsy sites in the stomach were measured at three time points. Intensities of acute infiltrates (neutrophil), chronic infiltrates (mononuclear cells), gastric atrophy and intestinal metaplasia were graded according to the updated Sydney System as follows: none (0), mild (1), moderate (2) or marked (3). In the patients with successful eradication, neutrophil and mononuclear cell infiltration decreased at 6 weeks after eradication and remained at the decreased levels at 1 year. On the other hand, in the patients with persistent infection, neutrophil and mononuclear cell infiltration did not show any significant changes. The degree of gastric atrophy and intestinal metaplasia did not show any significant changes even in the group of successful eradication

number of densely methylated DNA molecules decreased in accordance with the decrease of the methylation level (Fig. 1c), confirming that methylation levels reflect the population of densely methylated DNA molecules, thus the population of cells with dense methylation.

Effects of *H. pylori* eradication on histological gastritis

Histological examination was performed on the infiltration of neutrophil and mononuclear cells, and the degree of atrophy and intestinal metaplasia (Fig. 2). In the patients with successful eradication, neutrophil infiltration (0.5 ± 0.5 before eradication) and mononuclear cell infiltration (1.3 ± 0.4) decreased at 6 weeks (zero, $P = 0.0017$ for neutrophil infiltration; 1.0 ± 0.1, $P = 0.0028$ for mononuclear cell infiltration), and remained at the decreased levels at 1 year (zero for neutrophil infiltration; 1.0 ± 0.1 for mononuclear cell infiltration). On the other hand, in the patients with persistent infection, the average score of neutrophils and mononuclear cells score did not show significant changes at 6 weeks and 1 year after eradication (from 0.5 ± 0.5 to 0.2 ± 0.3 and 0.4 ± 0.6, $P = 0.27$ for neutrophil infiltration; from 1.2 ± 0.3 to 1.2 ± 0.3 and 1.3 ± 0.3, $P = 0.068$ for mononuclear cell infiltration). The degree of gastric atrophy and intestinal metaplasia did not show any significant changes even in the group of successful eradication ($P = 0.062$ and 0.179, respectively). Pepsinogen II showed a clear decrease, leading to an increase of the pepsinogen I/II ratio at 6 weeks after eradication in the patients with successful eradication (Fig. 3), confirming previous reports [17, 18].

Association between the *THBD* methylation level before eradication and infiltration of inflammatory cells

To explore a mechanism for methylation induction, we analyzed the correlation between methylation levels and scores of histological analysis in all patients ($n = 35$). The *THBD* methylation level showed a weak correlation with scores of neutrophil and mononuclear cell infiltration (correlation coefficients = 0.40 and 0.45, $P = 0.017$ and 0.0067, respectively) (Table 1). On the other hand, the *FLNc* methylation level did not show any correlation with the scores of neutrophil or mononuclear cell infiltration. The degree of atrophic gastritis had no correlation with the *FLNc* and *THBD* methylation levels (Table 1), but that of intestinal metaplasia had an inverse correlation with the *THBD* methylation level.

Discussion

DNA methylation levels in non-cancerous gastric mucosae of gastric cancer patients were shown to decrease to certain levels after successful eradication of *H. pylori*, in association with the decrease of inflammatory cell infiltration. The same tendency was also observed in healthy volunteers with *H. pylori* infection. The decreased methylation levels after eradication were much higher than those of healthy

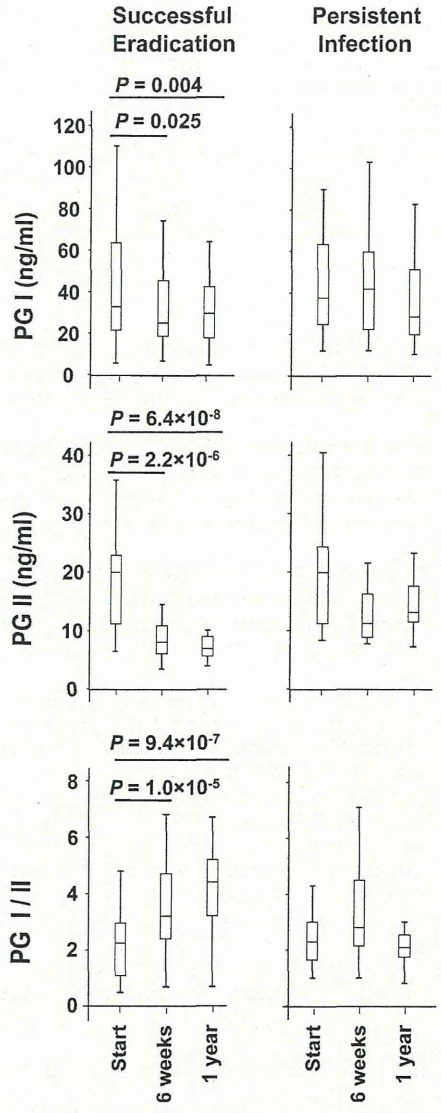

Fig. 3 Changes of serum pepsinogen I and II after *H. pylori* eradication. In the patients with successful eradication, a decrease of pepsinogen I, that of II, and increase of the I/II ratio were observed at 6 weeks after eradication ($P = 0.025$, 2.2×10^{-6}, and 1.0×10^{-5}, respectively) and 1 year ($P = 0.004$, 6.4×10^{-8}, and 9.4×10^{-7}, respectively). In the patients with persistent infection, pepsinogens I and II did not show any significant changes after eradication. The *boxes* represent the 75th and 25th percentiles, and the *line in the box* represents the 50th percentile (the median). *Whiskers* represent the maximum data within [75th percentile + 1.5 × (75th percentile − 25th percentile)] and the minimum data within [25th percentile − 1.5 × (75th percentile − 25th percentile)]

individuals without *H. pylori* infection (0.1% for *FLNc* and 0.8% for *THBD*) [6]. Our previous studies showed that methylation levels in individuals without active *H. pylori* infection correlate with gastric cancer risk [6, 7]. Taken together, methylation in gastric mucosae of individuals with active *H. pylori* infection consists of temporary and permanent components. The temporary component goes away when active *H. pylori* infection discontinues, and the remaining permanent component correlates with gastric cancer risk. The temporary component is likely to be attributed to turnover of cells with methylation, and the permanent component is likely to be methylation induced in long-living cells, possibly stem cells [19]. To use methylation levels in gastric mucosae as a risk marker, removal of the temporary component by *H. pylori* eradication will be important. This will become a fundamental basis for future studies that use DNA methylation levels in gastric mucosae as a risk marker.

FLNc and *THBD* methylation did not disappear in any of the 20 patients and 9 healthy volunteers with successful eradication. The most prominent decrease of the *THBD* methylation level was from 13.4 to 1.8%. Along with the finding by Miyazaki et al. [11], it was suggested that a decreased incidence ("disappearance" in some individuals) of *CDH1* methylation [8, 9] and that of multiple tumor-suppressor genes [10] was likely to be due to a decrease of their methylation levels below detection limits. However, there remains a possibility that eradication of *H. pylori* imposes negative selection pressure on cells with inactivation of tumor-suppressor genes.

We here analyzed methylation levels of *FLNc* and *THBD*. *FLNc* was selected because, in *H. pylori*-negative individuals, its methylation level had the strongest correlation with gastric cancer risk among the eight CGIs analyzed in previous studies [6, 7]. *THBD* was selected because its methylation level was relatively high among the eight CGIs. It was previously shown that methylation of the *FLNc* and *THBD* promoter CGIs can silence them [20]. As a result, the *FLNc* and *THBD* methylation levels showed different temporal profiles in this study. Recently, it was shown that the *miR-124a-1*, *-2*, and *-3* genes show methylation dynamics different from *FLNc* and *THBD* [21], and that *H. pylori* infection induces methylation of specific genes in gastric mucosae [22] with underlying mechanisms [23]. Taken together, it was suggested that target cells of methylation induction are different among various genes and that, even among the target genes in a specific type of cell, the susceptibility of individual genes are different. It was suggested that *FLNc* is methylated in close association with methylation of tumor-suppressor genes in cancer precursor cells although it is relatively resistant to DNA methylation.

A methylation level of an individual at a time point was obtained as an average of three samples from the three biopsy sites, because an average methylation level of the three sites reflects a risk of an individual [7]. When methylation levels were analyzed separately, those in the middle and upper gastric bodies showed significant

Table 1 Correlation between the methylation level and histological gastritis at the start point

	Neutrophil		Mononuclear cell		Atrophy		Metaplasia	
	Correlation coefficient	*P* value	Correlation coefficient	*P* value	Correlation coefficient	*P* value	Correlation coefficient	*P* value
FLNc	−0.02	0.91	0.04	0.82	0.04	0.84	0.01	0.93
THBD	0.40	0.017	0.45	0.0067	−0.30	0.084	−0.42[a]	0.010

[a] Inverse correlation

decreases while that in the antrum did not. This was considered to be because the permanent component consisted of the major part of methylation in the antrum while it consisted of a small part in the middle and upper gastric bodies.

Molecular mechanisms of how *H. pylori* infection induces aberrant DNA methylation in gastric mucosae are still unclear. In this study, we found that, before eradication, *THBD* methylation levels were weakly correlated with the scores of inflammatory cell infiltration. We also observed that, in the group of successful eradication, infiltration of inflammatory cells in gastric mucosae improved at 6 weeks after eradication and that the *FLNc* methylation level decreased significantly at this time point. In the subgroup of patients with eradication failure, there was no significant decrease of the methylation level even at 1 year after eradication therapy, and it was suggested that eradication therapy did not affect the methylation levels in gastric mucosae. These findings suggested that chronic inflammation induced by *H. pylori* infection is responsible for methylation induction.

In summary, a decrease, but not disappearance, of methylation in gastric mucosae after successful *H. pylori* eradication was confirmed, and a possible involvement of chronic inflammation in methylation induction was suggested.

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Epigenetic clustering of lung adenocarcinomas based on DNA methylation profiles in adjacent lung tissue: Its correlation with smoking history and chronic obstructive pulmonary disease

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The aim of this study was to clarify the significance of DNA methylation alterations during lung carcinogenesis. Infinium assay was performed using 139 paired samples of non-cancerous lung tissue (N) and tumorous tissue (T) from a learning cohort of patients with lung adenocarcinomas (LADCs). Fifty paired N and T samples from a validation cohort were also analyzed. DNA methylation alterations on 1,928 probes occurred in N samples relative to normal lung tissue from patients without primary lung tumors, and were inherited by, or strengthened in, T samples. Unsupervised hierarchical clustering using DNA methylation levels in N samples on all 26,447 probes subclustered patients into Cluster I (n = 32), Cluster II (n = 35) and Cluster III (n = 72). LADCs in Cluster I developed from the inflammatory background in chronic obstructive pulmonary disease (COPD) in heavy smokers and were locally invasive. Most patients in Cluster II were non-smokers and had a favorable outcome. LADCs in Cluster III developed in light smokers were most aggressive (frequently showing lymphatic and blood vessel invasion, lymph node metastasis and an advanced pathological stage), and had a poor outcome. DNA methylation levels of hallmark genes for each cluster, such as *IRX2, HOXD8, SPARCL1, RGS5* and *EI24*, were again correlated with clinicopathological characteristics in the validation cohort. DNA methylation profiles reflecting carcinogenetic factors such as smoking and COPD appear to be established in non-cancerous lung tissue from patients with LADCs and may determine the aggressiveness of tumors developing in individual patients, and thus patient outcome.

Lung cancer is the leading cause of cancer-related death worldwide,[1] and adenocarcinoma is the most common histological subtype, both in smokers and non-smokers. Differences in the genetic features of lung adenocarcinomas (LADCs) between smokers and non-smokers have been described.[2] LADCs arising in individuals who have never smoked, especially women and those of East Asian ethnicity, have been reported to have *EGFR* mutation and are thus responsive to tyrosine kinase inhibitors, whereas those arising in smokers frequently show oncogenic missense mutations in *KRAS*. *EGFR* and *KRAS* mutations in LADCs are almost entirely mutually exclusive. With regard to *TP53*

Key words: DNA methylation, infinium assay, lung adenocarcinoma, cigarette smoking, chronic obstructive pulmonary disease
Abbreviations: AAH: atypical adenomatous hyperplasia; C: normal lung tissue; COPD: chronic obstructive pulmonary disease; FDR: false discovery rate; LADC: lung adenocarcinomas; N: non-cancerous lung tissue; ROC: receiver operating characteristic curve; T: tumorous tissue; TNM: tumor-node-metastasis

Additional Supporting Information may be found in the online version of this article.
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