frequently in the cell lines (Figs. 1*b*–1*d*). *miR-193a* was partially methylated in one of the two normal gastric mucosae, and completely methylated frequently in gastric cancer cell lines. In contrast, *miR-127* was completely methylated in the normal gastric mucosae, but unmethylated in the gastric cancer cell lines.

We then examined the effect of methylation of the putative promoter regions on miRNA expression (*miR-124a* for *miR-124a-1*, *miR-124a-2* and *miR-124a-3* genes; *miR-137*; *miR-193a*; *miR-127*) in the 11 gastric cancer cell lines and gastric epithelial cells obtained by the gland isolation technique (Fig. 2). *miR-124a* was consistently unexpressed in six cell lines with simultaneous methylation of its three isoforms (*miR-124a-1*, *miR-124a-2* and *miR-124a-3*), but was expressed in the gastric epithelial cells. In contrast, *miR-137*, *miR-193a*, and *miR-127* were expressed even in cell lines with complete methylation. This showed that these three miRNA genes were not silenced by their "promoter" methylation, and indicated that, in contrast, *miR-124a* was silenced by promoter methylation of its three isoforms.

Methylation-silencing of *miR-124a* was further confirmed by analyzing its re-expression in association with its promoter demethylation after treatment with a demethylating agent, 5-aza-dC, in three cell lines (AGS, HSC57 and MKN28). Re-expression and appearance of unmethylated DNA molecules were observed in all the three cell lines, HSC57 being prominent. This further orted that *miR-124a* was methylation-silenced.

### *The presence of* miR-124a *methylation in primary gastric cancers*

Since methylation-silencing was identified only for *miR-124a*, we analyzed methylation levels of its three genes (*miR-124a-1*, *miR-124a-2* and *miR-124a-3*), along with a representative protein-coding gene (*LOX*), in 28 primary gastric cancer tissues (13 intestinal and 15 diffuse types) by qMSP. The fact that densely methylated DNA molecules were being measured was confirmed by bisulfite sequencing (Supp. Info. Fig. 1). *miR-124a-1* showed a distribution of methylation levels similar to *LOX*, some having no methylation and the others having various levels of methylation (Fig. 3*a*). This was consistent with our previous finding that cancer samples could be essentially classified into two groups (cancers with and without methylation), and that the various degrees of methylation levels in methylation-positive cancer samples were mainly due to contamination of normal cells.[18] On the other hand, *miR-124a-2* and *miR-124a-3* showed a unimodal distribution of methylation levels, suggesting that they are susceptible to methylation induction in cancer tissues. Using a cut-off value of 6%, as in previous reports,[31,32] *miR-124a-1*, *miR-124a-2* and *miR-124a-3* were methylated in 11, 23 and 26 of the 28 samples, respectively. Between the two histological types, the incidences of methylation were the same for *miR-124a-1*, *miR-124a-2* and *miR-124a-3* ($p$ = 0.95, 0.84 and 0.67) (Supp. Info. Fig. 2a).

We further analyzed an association between methylation and expression of *miR-124a* in an additional 19 gastric cancer samples. Using a cut-off value of 6%, eight samples had methylation of all the three *miR-124a* genes, and the other 11 samples had methylation of only one or two genes and retained at least one unmethylated gene. *miR-124a* was barely expressed in all the eight samples with methylation of the three genes whereas it was expressed in 5 of 11 cancer samples with at least one unmethylated gene (Fig. 3*b*).

### *Accumulation of methylation in* H. pylori *positive gastric mucosae, and its association with gastric cancer risk*

Methylation levels of *miR-124a-1*, *miR-124a-2* and *miR-124a-3*, again along with *LOX*, were analyzed by qMSP in gastric mucosae of 56 healthy volunteers (28 volunteers with *H. pylori* and 28 without) and noncancerous gastric mucosae of 45 gastric cancer patients (29 patients with *H. pylori* and 16 without) (Fig. 3*b*). Among the healthy volunteers, the mean methylation levels of *miR-124a-1*, *miR-124a-2*, *miR-124a-3* and *LOX* in the *H. pylori*-positive individuals were 13.1-, 7.8-, 8.9- and 46.7-fold, respectively, as high as those in *H. pylori*-negative individuals. This showed that *H. pylori* infection was associated with aberrant methylation of not only protein-coding genes but also miRNA genes.

Next, methylation levels in gastric mucosae of healthy volunteers were compared with those of noncancerous gastric mucosae of gastric cancer patients. Since potent methylation induction by *H. pylori* can mask a difference in *H. pylori*-positive individuals, the comparison was made among *H. pylori*-negative individuals only (28 healthy volunteers and 16 gastric cancer patients) (Table II; Fig. 3*c*). The mean methylation levels of the three miRNA genes and *LOX* were much higher in noncancerous gastric mucosae of gastric cancer patients than those of gastric mucosae of healthy volunteers (15.5-, 7.2-, 13.3- and 24.7-fold, respectively). Between the two histological types, the mean methylation levels were not different (Supp. Info. Fig. 2b).

Correlations among methylation levels of miRNA genes and *LOX* were examined by calculating correlation coefficients. Correlations among the three miRNA genes were very strong, but correlations between a miRNA gene and *LOX* were weak or absent (Table III; Supp. Info. Fig. 3).

### *No effect of age and sex on methylation levels on miRNA genes*

Methylation of various CGIs is reported to be correlated with age.[33,34] Also, males have twice as high an incidence of gastric cancers as females.[1] In *H. pylori*-negative healthy volunteers, methylation levels of *miR-124a-1*, *miR-124a-2 and miR-124a-3* were not correlated with age (Spearman correlation test: $r$ = 0.19, 0.01 and 0.29; $p$ = 0.35, 0.94 and 0.15), and not associated with sex ($p$ = 0.05, 0.68 and 0.19). Also, in *H. pylori*-positive healthy volunteers, methylation levels were not correlated with age ($r$ = 0.13, 0.18 and −0.1; $p$ = 0.51, 0.35 and 0.51), and not associated with sex ($p$ = 0.70, 0.20 and 0.67).

## Discussion

The present study showed that significantly higher methylation levels of three miRNA genes (*miR-124a-1*, *miR-124a-2* and *miR-124a-3*) were present in gastric mucosae of *H. pylori*-positive healthy volunteers, indicating that *H. pylori* infection can induce DNA methylation of miRNA genes, in addition to protein-coding genes. Moreover, it was also shown that methylation levels of the miRNA genes in noncancerous gastric mucosae of gastric cancer patients were higher than those in gastric mucosae of healthy volunteers among *H. pylori* negative individuals, indicating that miRNA silencing is involved in the formation of a field defect for gastric cancers. To our knowledge, the presence of miRNA silencing in a field for cancerization was shown here for the first time.

Recent studies demonstrated that expression of some miRNAs is regulated by epigenetic mechanisms.[24,35] From six miRNA genes that were reported to be silenced by promoter methylation and to have tumor-suppressor functions, we were able to confirm that three genes of *miR-124a* were methylation-silenced in gastric cancer cell lines. The other three genes, *miR-137*, *miR-193a* and *miR-127*, were expressed even in cell lines with complete methylation, and were unlikely to be silenced by promoter methylation in gastric cancers. Since methylation of putative promoter regions consistently represses transcription of their downstream genes,[29,30] the presence of the expression of the three genes in gastric cancer cell lines with complete methylation of their "promoter" CGI indicated that the three genes had additional or alternative promoters.

Lujambio *et al.*[22] discovered that *miR-124a* was silenced by promoter methylation after screening 320 miRNA genes. They also found that *miR-124a* down-regulates CDK6, a demonstrated oncogene involved in cell cycle progression and differentiation, and induces hypophosphorylation of RB.[22] Therefore, it is possible that *miR-124a* silencing is also involved in gastric carcinogenesis, and the presence of its silencing in noncancerous tissues

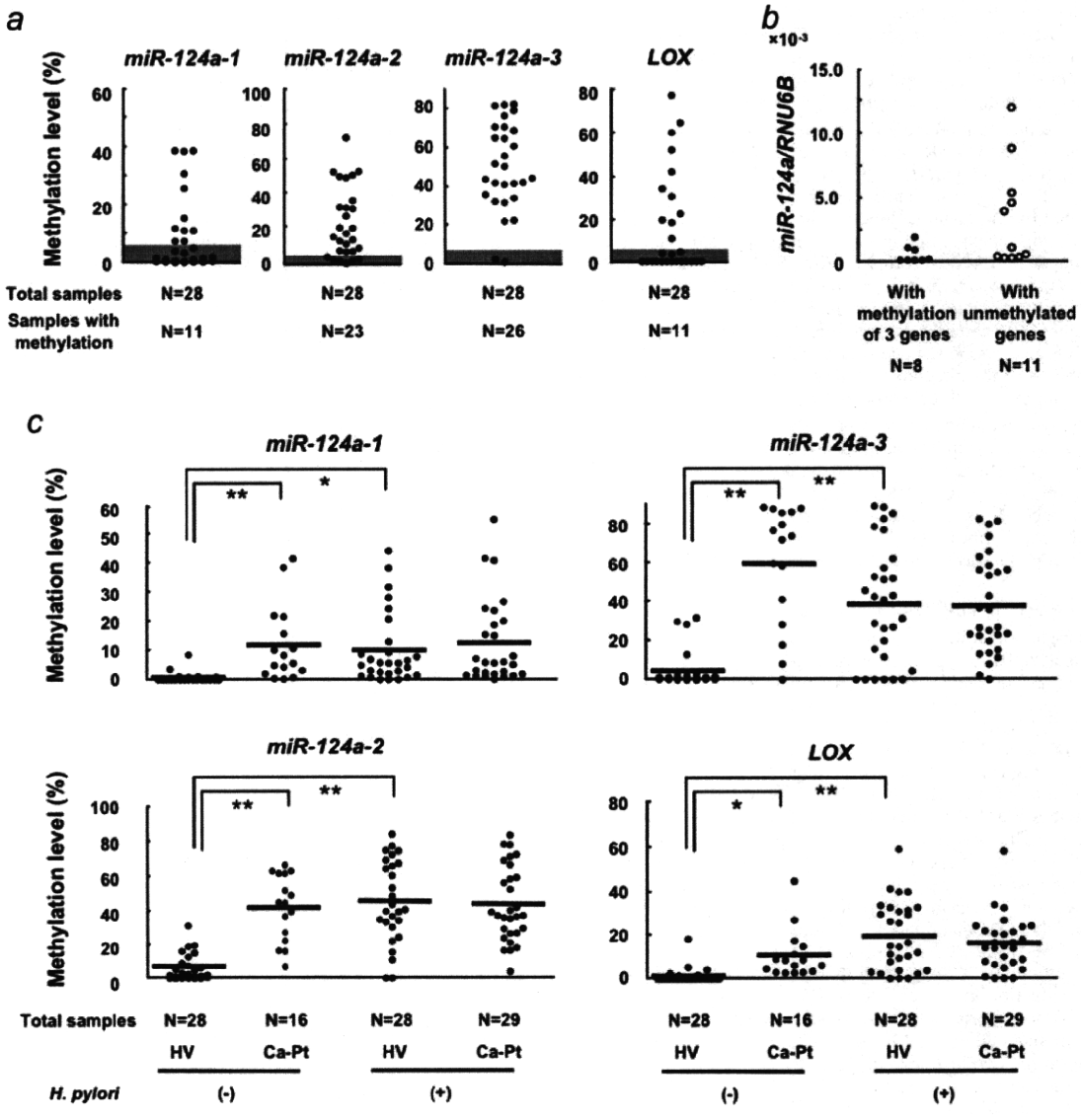


FIGURE 3 – Methylation levels of *miR-124a-1, miR-124a-2, miR-124a-3* and *LOX* in gastric mucosae of healthy volunteers, noncancerous mucosae of gastric cancer patients, and cancer tissues. (*a*) Distribution of methylation levels in gastric cancers. Gray areas show samples with methylation levels below the cut-off value of 6%. (*b*) Expression of *miR-124a* in eight cancer samples with methylation (three *miR-124a* genes, methylation positive) and 11 cancer samples with at least one unmethylated gene (*miR-124a-1* or *miR124a-3*). Five of the 11 cancers with unmethylated genes had high *miR-124a* expression levels. (*c*) Distribution of methylation levels in gastric mucosae of healthy volunteers (HV) and noncancerous mucosae of gastric cancer patients (Ca-Pt). A horizontal line represents a mean methylation level for each group. Among the healthy volunteers, *H. pylori*-positive individuals had 7.8–46.7-fold as high methylation levels as *H. pylori*-negative individuals (*$p < 0.005$; **$p < 0.001$). Among the *H. pylori*-negative individuals, noncancerous gastric mucosae of gastric cancer patients had 7.2–24.7-fold as high methylation levels as gastric mucosae of healthy volunteers (*$p < 0.005$; **$p < 0.001$).

TABLE II – MEAN METHYLATION LEVELS AND STANDARD DEVIATIONS OF THE FOUR GENES IN GASTRIC MUCOSAE OF HEALTHY VOLUNTEERS AND GASTRIC CANCER PATIENTS

| | | N | *miR-124a-1* | *miR-124a-2* | *miR-124a-3* | *LOX* |
|---|---|---|---|---|---|---|
| *H.pylori*(−) | (1) Healthy volunteers | 28 | 0.76 ± 1.70 | 5.75 ± 7.73 | 4.45 ± 9.29 | 0.43 ± 1.22 |
| | (2) Gastric cancer patients | 16 | 11.82 ± 12.94 | 41.66 ± 19.33 | 59.42 ± 30.71 | 10.64 ± 11.33 |
| *H.pylori*(+) | (3) Healthy volunteers | 28 | 9.96 ± 12.28 | 44.79 ± 23.96 | 39.66 ± 30.08 | 20.10 ± 15.72 |
| | (4) Gastric cancer patients | 29 | 12.28 ± 14.31 | 46.33 ± 28.36 | 37.48 ± 25.13 | 15.93 ± 12.58 |
| *p* value | (1) *vs.* (3) | | <0.001 | $<10^{-8}$ | $<10^{-5}$ | $<10^{-6}$ |
| | (1) *vs.* (2) | | 0.004 | $<10^{-5}$ | $<10^{-6}$ | 0.003 |
| | (3) *vs.* (4) | | 0.51 | 0.77 | 0.77 | 0.28 |

TABLE III – CORRELATION AMONG METHYLATION LEVEL OF *miR-124A-1, miR-124A-2, miR-124A-3* AND *LOX*

| | *miR-124a-1* | | *miR-124a-2* | | *miR-124a-3* | | *LOX* | |
|---|---|---|---|---|---|---|---|---|
| | *r* | *p* | *r* | *p* | *r* | *p* | *r* | *p* |
| *miR-124a-1* | – | – | 0.70 | $<10^{-15}$ | 0.77 | $<10^{-20}$ | 0.03 | 0.76 |
| *miR-124a-2* | 0.70 | $10^{-15}$ | – | – | 0.72 | $<10^{-16}$ | 0.37 | $<10^{-3}$ |
| *miR-124a-3* | 0.77 | $<10^{-20}$ | 0.72 | $<10^{-16}$ | – | – | 0.20 | 0.04 |

*r*, correlation coefficient.

could be directly associated with predisposition to developing gastric cancers.

As repeatedly shown by epidemiological studies, the majority of *H. pylori*-negative individuals with a gastric cancer are considered to have past exposure to *H. pylori*.[36] Methylation levels of protein-coding genes, including *LOX*, in the gastric mucosae of individuals with past infection (gastric cancer patients without *H. pylori*) were lower than those of individuals with current infection (both healthy volunteers and gastric cancer patients) in our previous study.[11] Actually, incidences of aberrant methylation and methylation levels of *CDH1* are reported to decrease after the eradication of *H. pylori*,[19,37] showing that DNA methylation in gastric mucosae decreases when *H. pylori* infection discontinues. Interestingly, methylation levels of the three miRNA genes in the gastric mucosae of individuals with past infection by *H. pylori* (gastric cancer patients without *H. pylori*) did not decrease compared with those of individuals with current infection (healthy volunteers and gastric cancer patients). Since aberrant methylation induced in stem cells is expected to persist even after *H. pylori* infection discontinues, DNA methylation of these miRNA genes might be relatively more easily induced in gastric stem cells than those of protein-coding genes.

In conclusion, our data indicated that DNA methylation of certain miRNA genes was associated with *H. pylori* infection, in addition to protein-coding genes, and involved in the formation of field defect for gastric cancers.

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# The presence of a methylation fingerprint of *Helicobacter pylori* infection in human gastric mucosae

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**Aberrant DNA methylation is deeply involved in human cancers, but its inducers and targets are still mostly unclear. *Helicobacter pylori* infection was recently shown to induce aberrant methylation in gastric mucosae, and produce a predisposed field for cancerization. Here, we analyzed the presence of target genes in methylation induction by *H. pylori* and the mechanism for the gene specificity. Noncancerous gastric mucosae were collected from 4 groups of individuals (with and without a gastric cancer, and with and without current *H. pylori* infection; $N = 11$ for each group), and methylation of promoter CpG islands of 48 genes that can be methylated in gastric cancer cell lines was analyzed by methylation-specific PCR. In total, 26 genes were consistently methylated in individuals with current or past infection by *H. pylori*, whereas 7 genes were not methylated at all. In addition, 14 genes were randomly or intermediately methylated in individuals with gastric cancers and the remaining 1 gene was methylated in all the cases. The methylation-susceptible genes had significantly lower mRNA expression levels than the methylation-resistant genes. *H. pylori* infection did not induce mRNA and protein expression of DNA methyltransferases; *DNMT1*, *DNMT3A* or *DNMT3B*. Gene specificity was present in the induction of aberrant DNA methylation by *H. pylori* infection, and low mRNA expression, which could precede methylation, was one of the mechanisms for the gene specificity. These findings open up the possibility that a methylation fingerprint can be used as a novel marker for past exposure to a specific carcinogenic factor.**


**Key words:** DNA methylation; epigenetic; fingerprint; *Helicobacter pylori*; molecular epidemiology

Aberrant DNA methylation is deeply involved in human cancer development and progression.[1] In some cancer types, such as gastric cancers, tumor-suppressor genes are more frequently inactivated by aberrant DNA methylation than by mutations.[2] Nevertheless, only limited information is available for inducers of aberrant DNA methylation, which include aging, viral infection and ulcerative colitis.[3,4] Also, almost no information is available for gene specificity in methylation induction by a specific factor. Using cancer tissues, it is very difficult to clarify an association between a specific inducer and methylation of a gene. Aberrant methylation of a gene can be present in cancer tissues because its methylation conferred a growth advantage although it was a rare and random event, or because its methylation was carried over from a precursor tissue to a cancer tissue since it was frequently induced in the precursor tissue. In contrast, using a noncancerous tissue, one can assess an effect of a methylation inducer by the fraction of cells with methylation in the polyclonal tissue.

Gastric mucosa infected by *Helicobacter pylori* is a useful model to examine the possible presence of gene specificity in methylation induction by a specific factor since *H. pylori* infection was recently shown to induce aberrant DNA methylation potently in gastric mucosae.[5] Moreover, the fraction of DNA molecules with aberrant methylation (methylation level) in gastric mucosae of individuals without current *H. pylori* infection was correlated with gastric cancer risk,[5,6] indicating that methylation in noncancerous tissues is related to gastric carcinogenesis. So far, 6 CpG islands in gene promoter regions methylated in gastric cancers[7] were analyzed, and all were methylated in gastric mucosae with current and past infection with *H. pylori*. However, it is unknown whether these 6 genes are preferentially methylated by *H. pylori* infection or *H. pylori* infection induces methylation of random genes.

In this study to analyze the presence of gene specificity for methylation induction, firstly we examined the methylation status of 48 promoter CpG islands in the noncancerous gastric mucosae of 4 groups of individuals (with and without a gastric cancer, and with and without current *H. pylori* infection). The 48 genes were selected as genes that can be methylation-silenced in gastric cancer cell lines[8] because the vast majority of CpG islands in gene promoter regions are not methylated at all in noncancerous tissues, and we had to newly select genes that have better chances to be methylated in noncancerous tissues. Secondly, we analyzed an association between susceptibility to methylation induction and mRNA expression levels in normal tissue without and with *H. pylori* infection.

## Material and methods

### *Tissue samples and DNA/RNA extraction*

For methylation analysis, (noncancerous) gastric mucosa samples were collected from 4 groups of individuals (with and without a gastric cancer, and with and without current *H. pylori* infection; $N = 11$ for each group, average age = 60.8 ± 13.8 years). For analysis of mRNA expression that determines gene specificity of methylation induction, we need to analyze the mRNA expression level in gastric mucosae free of methylation, which, once induced, will cause decreased gene transcription to avoid confusion between cause and consequence. Therefore, samples were collected from 11 healthy volunteers, who were considered to have less chance for methylation induction by *H. pylori* than elderly individuals (7 males and 4 females; 6 with *H. pylori* infection and 5 without; average age = 34.8 ± 3.1 years). Biopsy specimens were taken from one standard site of the stomach (antral regions in the lesser curvature) using sterilized biopsy forceps (Olympus, Tokyo, Japan). *H. pylori* infection status was analyzed by culture test (Eiken, Tokyo, Japan) and rapid urease test (Otsuka, Tokushima, Japan). All the materials were obtained with written informed consents, and the procedures were approved by the institutional review board. High molecular weight DNA was extracted by the standard phenol/chloroform method and total RNA was isolated using ISOGEN (Nippon Gene, Tokyo, Japan) and an RNeasy Mini kit (Qiagen, Valencia, CA).

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

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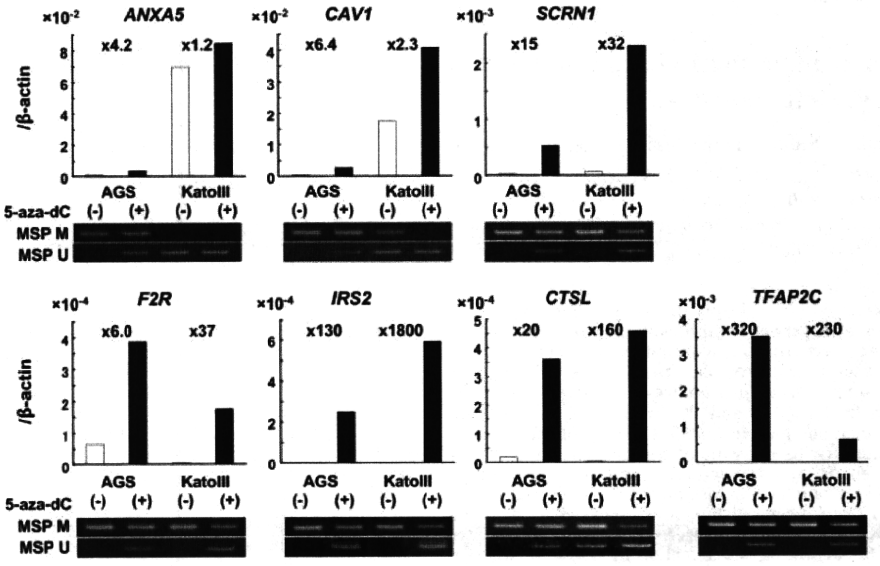


**FIGURE 1** – Gene silencing due to methylation of the regions analyzed. mRNA expression and methylation were analyzed by real-time RT-PCR and MSP, respectively, in gastric cancer cell lines (AGS and KATO-III) before and after 5-aza-dC treatment. The fold increases after 5-aza-dC treatment is shown for each cell line. No or little mRNA expression in a cell line(s) without unmethylated DNA molecules and upregulation by the 5-aza-dC treatment was confirmed for the 7 genes randomly selected from the 48 genes.

*Cell lines and 5-aza-dC treatment*

Gastric cancer cell lines, AGS and KATO-III, were obtained from the Japanese Collection of Research Bioresources (Tokyo, Japan) and the American Type Culture Collection (Manassas, VA). For treatment with a demethylating agent, 5-aza-2′-deoxycitidine (5-aza-dC, Sigma, St. Louis, MO), cells were seeded on day 0, media containing 0.3 μM 5-aza-dC was freshly added on days 1 and 3, and cells were harvested on day 5. Genomic DNA and total RNA were isolated in the same way as the primary samples.

*Bisulfite treatment and methylation-specific PCR*

Bisulfite treatment was performed as previously described.[9] 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-sec denaturation at 95°C and 15-min incubation at 50°C in 3.1 N sodium bisulfite (pH 5.0) and 0.5 mM hydroquinone. The samples were desalted with the Wizard DNA Clean-Up system (Promega, Madison, WI), and desulfonated in 0.3 N NaOH. DNA was ethanol precipitated and dissolved in 40 μL of TE buffer.

Methylation-specific PCR (MSP) was performed with a primer set specific to the methylated or unmethylated sequence (M or U set), respectively,[8] using 2 μL of the sodium bisulfite-treated DNA. A region upstream of a putative transcriptional start site (200 bp or less) was analyzed, and CpG maps of all the genes are shown in the Supporting Information Figure 1. DNA methylated with *Sss*I methylase was used to determine a specific condition of PCR for the M set, and DNA amplified by a GenomiPhi DNA amplification kit (GE Healthcare Bio-Sciences) was used for the U set. A number of PCR cycles that would yield a minimal visible band was determined using these fully methylated DNA (for M primers) and fully unmethylated DNA (for U primers), and a further 4 cycles were added for actual analysis of test samples. Methylation levels were classified as none (−), low (+), high (++) according to the intensity of the band for methylated DNA molecules compared with that for unmethylated DNA, respectively.

*Quantitative reverse transcription PCR*

cDNA was synthesized from 1 μg of total RNA using a Superscript II kit (Life Technologies, Rockville, MD) with a random primer. Real-time PCR was performed using an iCycler Thermal Cycler (Bio-Rad Laboratories, Hercules, CA) with SYBR Green I (BioWhittaker Molecular Applications, Rockland, ME). The number of molecules of a specific gene in a sample was measured by comparing its amplification with that of standard samples, which contained $10^1$–$10^7$ copies of the gene. The standard samples were produced by PCR amplification and purification using Zymo-Spin I™ Columns (Zymo Research, Orange, CA). The amount of the standard samples was measured by OD 260 nm and also by quantification of band intensities after electrophoresis. The mRNA quantity of each gene was normalized to that of β-actin. The primers and PCR conditions are shown in the Supporting Information Table 1. The difference of mRNA expression levels between 2 groups of genes was analyzed by the Welch *t*-test method (both sided).

*Western blot analysis*

Each 100 μg whole-cell lysate sample was subjected to SDS-PAGE (10% acrylamide gel) and blotted to PVDF membrane. DNMT1 and DNMT3A were detected using rabbit polyclonal antibody against human DNMT1 (NEB, Beverly, MA), human DNMT3A (Cell Signaling Technology, Danvers, MA), respectively at 1/1,000 dilution. DNMT3B was detected using goat polyclonal antibody against human DNMT3B (Santa Cruz Biotechnology, Santa Cruz, CA) at 1/500 dilution. Horse radish peroxidase-conjugated secondary antibody (antirabbit; Cell Signaling Technology, antigoat; Santa Cruz Biotechnology) was used at 1/5,000 dilution.

## Results

*Confirmation of gene silencing due to promoter CpG islands*

The 48 genes consisted of 32 randomly and 16 arbitrarily selected genes from 421 genes that had been identified as methylation-silenced genes in a gastric cancer cell line using microarray analysis of cells treated with 5-aza-dC, and MSP analysis.[8] First,

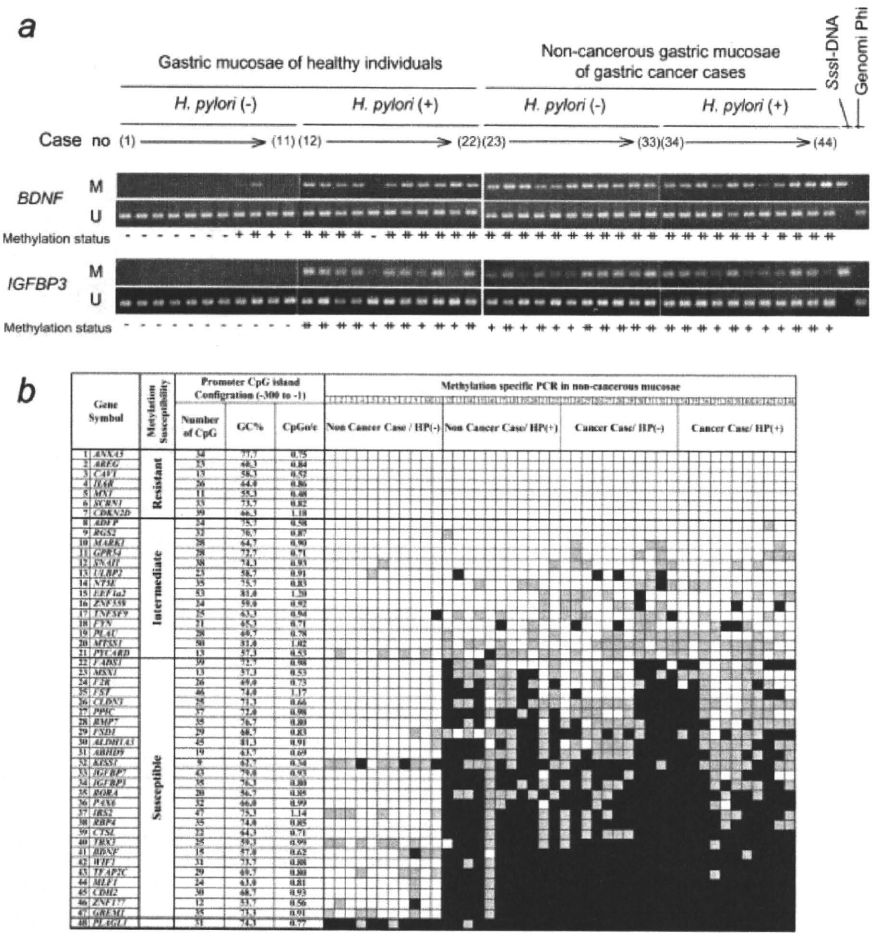


FIGURE 2 – Methylation profile of the 48 genes in noncancerous gastric mucosae. (*a*) Representative results of MSP. Samples 1–11, gastric mucosae of healthy individuals without *H. pylori* infection; 12–22, those with *H. pylori* infection; 23–33, noncancerous gastric mucosae of gastric cancer cases without *H. pylori* infection; and 34–44, those with *H. pylori* infection. Methylation levels were classified as none (−), low (+), high (++) according to the intensity of the band for methylated DNA molecules compared with that of fully methylated control DNA. (*b*) Overview of the results of all the 48 genes. The genes were aligned in the order of increasing numbers of individuals with methylation. Closed, hatched, and open boxes represent the methylation levels of high (++), low (+), and none (−), respectively. Rows 1–7, the 7 genes completely resistant to methylation induction in any cases; rows 8–21, genes methylated randomly or more frequently in individuals with cancers; and rows 22–47, genes susceptible to methylation induction by *H. pylori* infection. CpG island configuration (number of CpG sites, G+C content, and CpG score) in 300 bp upstream regions from transcription start sites is also shown. The presence of methylation-resistant and methylation-susceptible genes was clearly revealed. No clear difference in the CpG island configuration was observed between the 2 groups.

we analyzed mRNA expression of 7 of the 48 genes before and after 5-aza-dC treatment using real-time RT-PCR (Fig. 1). It was confirmed that no or little mRNA expression was present in cell lines without unmethylated DNA molecules and that mRNA expression was upregulated by the 5-aza-dC treatment.

### *Gene specificity in methylation induction by H. pylori infection in gastric mucosae*

We then analyzed the methylation status of the promoter CpG islands of the 48 genes in the (noncancerous) gastric mucosae of 4 groups of individuals; those with and without *H. pylori* infection and with and without a gastric cancer. Since MSP can produce inconsistent results if inappropriately performed, we carefully selected a PCR cycle for each primer set so that false positive and negative results were not produced. We scored the methylation status as negative, weakly positive or positive by comparing the band density with that of a fully methylated control (representative results in Fig. 2*a*).

When all the genes were aligned in the order of number of samples with methylation (Fig. 2*b*), the 48 genes were divided into 3 groups: (*i*) 7 genes that were completely unmethylated in any of the 4 groups (genes 1–7 in Fig. 2*b*; methylation-resistant genes), (*ii*) 14 genes that were methylated randomly or more frequently in individuals with cancers (genes 9–21; intermediate genes); and (*iii*) 26 genes that were consistently methylated in the individuals with *H. pylori* infection or with a gastric cancer (genes 22–47; methylation-susceptible genes). The remaining 1 gene, *PLAGL1*, was methylated in all the individuals. This demonstrated that some genes are resistant to methylation induction by *H. pylori* infection while others are susceptible, namely the presence of gene specificity in methylation induction.

### *Lack of association between CpG island configuration and methylation susceptibility*

The 48 genes analyzed here all had CpG islands in their promoter regions. However, based on recent reports,[10] there was a

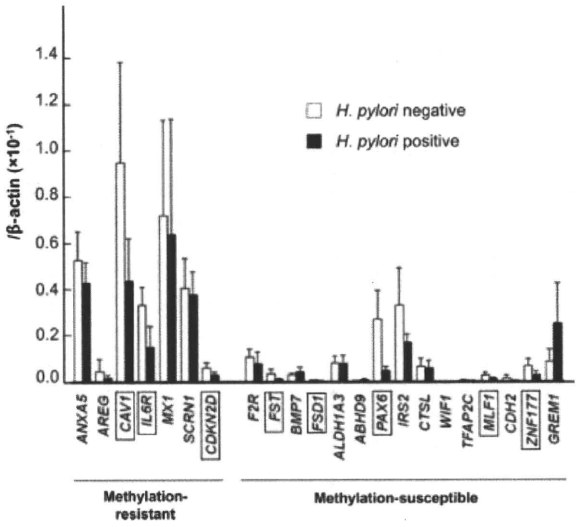


FIGURE 3 – The mRNA expression levels of genes resistant and susceptible to methylation induction. mRNA expression levels of 22 genes (7 resistant and 15 susceptible genes) in the noncancerous gastric mucosae of young healthy individuals with (closed columns) and without (open columns) *H. pylori* infection was analyzed by real-time RT-PCR. Error bar: standard deviation. The average mRNA expression level of methylation-resistant genes was much higher than that of methylation-susceptible genes among individuals without *H. pylori* infection ($4.3 \times 10^{-2}$ *vs.* $7.3 \times 10^{-3}$; $p = 0.0008$) and also among individuals with *H. pylori* infection ($2.9 \times 10^{-2}$ *vs.* $5.1 \times 10^{-3}$; $p = 0.0012$). The genes whose names are boxed showed a significant decrease in their mRNA expression levels by *H. pylori* infection ($p < 0.05$). Considering that all these 48 genes are those that can be methylated in gastric cancer cell lines, downregulation of mRNA expression could be involved in methylation induction.

possibility that, even among CpG islands, their configurations (number of CpG sites, G+C content, and CpG score) might influence the susceptibility of individual genes to methylation induction by *H. pylori*. Therefore, we examined their configurations in 300 bp upstream regions from transcription start sites (Fig. 2*b*), which corresponded to the nucleosome-free region and whose methylation is critical for gene silencing.[11,12]

The number of CpG sites in the region was 29.2 ± 10.4 (mean ± standard deviation) and 25.4 ± 9.3 for the susceptible and resistant genes, respectively ($p = 0.38$). The G + C content was 68.4 ± 7.4 and 66.4 ± 7.9% for the susceptible and resistant genes, respectively ($p = 0.52$). The CpG score was 0.82 ± 0.18 and 0.75 ± 0.21 for the susceptible and resistant genes, respectively ($p = 0.40$). In short, no significant difference was present between the 2 groups.

### *Involvement of low mRNA expression levels in gene specificity in methylation induction*

To investigate an association between the gene specificity in methylation induction and mRNA expression levels in gastric mucosae, we analyzed mRNA expression levels of all of the 7 methylation-resistant and 15 methylation-susceptible genes, which were randomly selected from the 26 methylation-susceptible genes. To compare mRNA expression levels among different genes, the numbers of cDNA molecules were measured by quantitative RT-PCR after accurate measurement of the weights (converted into the numbers of DNA molecules) of standard DNA samples of all the genes. mRNA expression levels were analyzed in the gastric mucosae of young healthy individuals with and without *H. pylori* infection, who were considered to have no or little methylation of the genes analyzed.

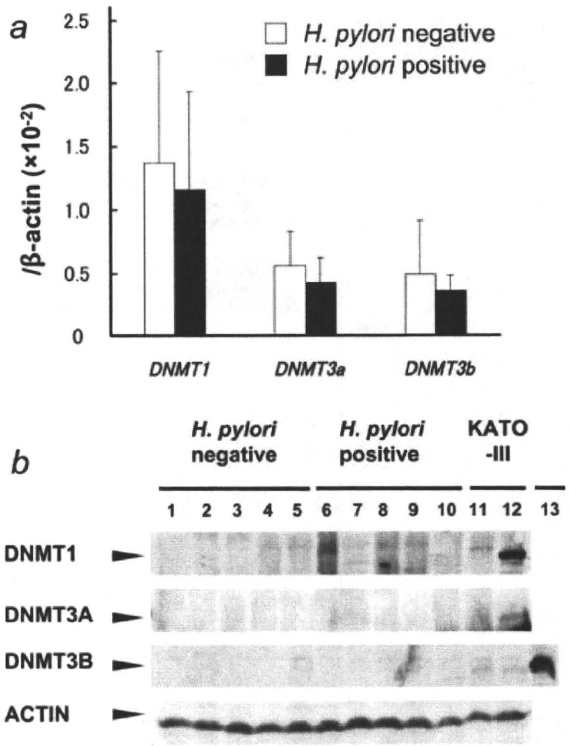


FIGURE 4 – The mRNA and protein expression levels of three DNA methyltransferases (*DNMT1, DNMT3A* and *DNMT3B*) in noncancerous gastric mucosae of young healthy individuals with and without *H. pylori* infection. (*a*) mRNA expression levels of *DMNTs*. Closed columns, individuals with *H. pylori* infection; open columns, those without. No significant increase was observed in the mRNA expression levels of these *DNMTs*. (*b*) Western blot analysis of DNMTs. For DNMT1 and DNMT3A, a stomach cancer cell line, KATO-III was used as a positive control (lane 12), and 5-aza-dC (1 μM)-treated KATO-III was used as a negative control (lane 11). ACTIN was used as a loading control. For DNMT3B, a commercially available positive control of DNMT3B (Santa Cruz, lane 13) was used. DNMT protein levels were below the detection limit in the noncancerous gastric mucosae of individuals without (lanes 1–5) and with (lanes 6–10) *H. pylori* infection, and no detectable increase was observed.

The average mRNA expression level of methylation-resistant genes was much higher than that of methylation-susceptible genes among individuals without *H. pylori* infection ($4.3 \times 10^{-2}$ *vs.* $7.3 \times 10^{-3}$; $p = 0.0008$) and also among individuals with *H. pylori* infection ($2.9 \times 10^{-2}$ *vs.* $5.1 \times 10^{-3}$; $p = 0.0012$) (Fig. 3). Three of the 7 resistant genes and 5 of the 15 susceptible genes showed a significant decrease of mRNA expression levels by *H. pylori* infection, but no genes showed significantly increased mRNA expression.

### *Expression levels of DNA methyltransferase*

To gain an insight into how *H. pylori* infection induces aberrant methylation, we analyzed mRNA expression levels of maintenance DNA methyltransferase, *DNMT1*, and *de novo* methyltransferases, *DNMT3A* and *DNMT3B*, in the gastric mucosae with and without *H. pylori* infection. However, no significant increase in their mRNA expression levels was observed (Fig. 4*a*). Further, at the protein level, expression levels of DNMT1, DNMT3A and DNMT3B were below the detection limit even in the gastric mucosae with *H. pylori* (Fig. 4*b*), indicating no increase was induced by *H. pylori* infection.

## Discussion

The presence of gene specificity for aberrant DNA methylation induction by a specific carcinogenic factor was demonstrated for the first time in this study. Also, genes susceptible to methylation had significantly lower mRNA expression levels than resistant genes. For clarification of the relationship between a methylation-inducing factor and gene specificity, use of noncancerous gastric tissue, which is polyclonal, was important because gene silencing due to promoter methylation can result in over- or under-presence of methylation in cancer tissues. Methylation in noncancerous tissues is also reported in the colonic mucosae of patients with ulcerative colitis[13,14] and liver tissues of patients with hepatocellular carcinomas,[15] but limited numbers of genes have been analyzed so far.

Methylation of specific genes can persist for a lifetime, and there is a possibility that the methylation profile can be used as a methylation fingerprint of *H. pylori* infection in the past, as specific *p53* and *APC* mutations are used to assess past exposure to specific carcinogens.[16,17] Use of DNA methylation has an advantage over mutations because methylation can be present in a significant fraction of cells in noncancerous tissues, and can be detected sensitively and reproducibly. The noncancerous gastric mucosae of cases with a gastric cancer without current *H. pylori* infection, most of which are considered to have had past exposure to *H. pylori*,[18] showed the same methylation profile as individuals with current *H. pylori* infection. This finding indicated that the methylation profile induced by *H. pylori* infection can persist even after discontinuation of *H. pylori* infection. Although eradication of *H. pylori* was reported to decrease incidences of individuals with methylation,[19,20] the decrease is only partial, not to zero, and highly variable among individuals (manuscript in preparation).

To establish a methylation profile as a fingerprint of *H. pylori* infection, the profile must be specific. Unfortunately, few gastric cancers can be considered as those induced solely by another carcinogenic factor, such as Epstein-Barr virus infection[21] or high salt intake,[22] and the specificity cannot be examined easily. However, since low mRNA expression levels are involved in gene specificity, there is a possibility that different carcinogenic factors induce different methylation profiles through induction of reduced mRNA expression of different genes. Once the specificity of a methylation profile is established, a methylation fingerprint will be very useful for clinicopathological analysis and epidemiology. Among the clinically used tests for *H. pylori* infection, the culture and rapid urease tests can detect only current *H. pylori* infection.[23,24] The serum antibody test remains positive in only half the patients as early as 1 year after successful eradication of *H. pylori*.[25,26]

The role of low mRNA expression in methylation induction has been reported.[4] De Smet *et al.* showed that weak transcriptional capacity leads to promoter remethylation by analysis of demethylation and mRNA expression of *MAGE-A1* in various cell lines.[27] Song *et al.* showed that decreased promoter activity leads to hypermethylation of a promoter CpG island of an exogenously introduced gene by disrupting its promoter activity.[28] We and others previously observed that most genes methylated in cancer tissues had no or little expression in cancer precursor cells.[29–32] This study showed that, in normal cells and *in vivo*, low mRNA expression is important for methylation induction. Also, it was suggested that downregulation by *H. pylori* infection precedes methylation since 8 of the 22 genes with expression analyses were downregulated by *H. pylori* infection, but none were upregulated. The 22 genes were selected from those that can be methylated in gastric cancer cell lines and even the resistant genes are considered to be relatively susceptible among the entire genes.

Even among the genes with similarly low mRNA expression levels, some genes were resistant and others were susceptible to methylation induction by *H. pylori*. As additional factors that determine the gene specificity of methylation induction, histone modification deregulation could be important. For example, a repressive histone modification, methylation at Lys27 of histone H3 (H3K27) induced by Polycomb group proteins, is associated with genes methylated in cancers.[33,34] Active chromatin marks, associated with active mRNA expression, could be important to protect DNA from methylation. At the same time, without *H. pylori* infection, even the susceptible genes were not methylated, indicating that abnormality in epigenetic regulation was induced by *H. pylori* infection. The final step of aberrant methylation must be mediated by DNA methyltransferases, and actually overexpression of *de novo* methyltransferases enhance methylation of specific genes in a mouse model.[35] Also, some inflammatory cytokines, such as IL-6, are reported to induce DNA methyltransferases.[36] However, contrary to initial expectations, *H. pylori* infection did not induce either mRNA or protein expression of DNMT1, DNMT3A and DNMT3B in gastric mucosae. Abnormalities in epigenetic regulation induced by *H. pylori* infection also need to be investigated.

In summary, methylation of specific genes was induced by *H. pylori* infection in noncancerous gastric membranes, and preceding low mRNA expression was suggested to be involved in the specificity. Use of the specific profile as a methylation fingerprint of past exposure to a specific carcinogenic factor was suggested.

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# Cancer High-Risk Subjects Identified by Serum Pepsinogen Tests: Outcomes after 10-Year Follow-up in Asymptomatic Middle-Aged Males

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## Abstract

Background: **Gastric cancer screening using the pepsinogen filter test is receiving wide recognition in Japan owing to convenience, freedom from discomfort or risk, efficiency, and economy. Because the long-term outcomes of cancer development in extensive atrophic gastritis detected by pepsinogen test are unclear, test-positive and test-negative subjects were investigated in a longitudinal cohort study.**

Methods: **Subjects comprised 5,209 middle-aged men with measured serum pepsinogen levels who were followed for 10 years. Cancer development based on "atrophy-positive" and "atrophy-negative" criteria used for cancer screening was investigated.**

Results: **During the study, 63 cases of cancer developed in the cohort, representing an incidence rate of 125 per 100,000 person-years. Pepsinogen test screening using the most widely used atrophy-positive criterion (pepsinogen I, ≤70 ng/mL; pepsinogen I/II ratio, ≤3.0) displayed 58.7% sensitivity, 73.4% specificity, and 2.6% positive predictive value. Cancer incidence rate was 276 per 100,000 person-years for the atrophy-positive group and 70 per 100,000 person-years for the atrophy-negative group. Incidence rate was higher in groups fulfilling stricter positive criteria detecting more extensive atrophy, reaching 424 per 100,000 person-years. In addition, 9.2% of atrophy-negative subjects with pepsinogen I of >70 ng/mL and pepsinogen I/II ratio of ≤3.0 (reflecting putative inflammation-based high pepsinogen II level) are at high risk for cancer, particularly diffuse-type cancer, with a cancer incidence rate comparable with atrophy-positive subjects (216 per 100,000 person-years).**

Conclusion: **Atrophy-positive subjects by pepsinogen filter test, particularly those fulfilling stricter criteria, and atrophy-negative subjects with low pepsinogen I/II ratio reflecting putative extensive active inflammation constitute populations at high risk for gastric cancer, requiring thorough endoscopic examination.** (Cancer Epidemiol Biomarkers Prev 2008;17(4):838–45)

## Introduction

Gastric cancer, despite a recent decline in incidence, is still one of the most common malignancies worldwide and remains a leading cause of cancer death not only in Japan but also in China, Korea, Central and Southern America, and some European countries (1-5). To reduce high mortality and morbidity rates in Japan, mass screening for gastric cancer has been conducted as a public health service since the mid-1960s. The screening program is currently done throughout the country, and ~6 million people annually undergo screening provided by either a community health service or the work place (6-8). Thousands of stomach cancer cases are detected each year, with 5,859,697 people undergoing screening and 5,529 cancers detected in 2004 (detection rate, 0.094%; ref. 8). Cancer screening has thus greatly contributed to reductions in cancer mortality rates in our country (9-13). Although nationwide stomach cancer screening has achieved unparalleled success, the number of people screened has not increased in recent years, with the same people appearing to receive gastric cancer screenings each year; in 2004, the cancer screening covered only 12.9% of the cancer-prone aged population throughout Japan. In addition, cancer screening programs have most commonly adopted gastrophotofluorography with an image intensifier as a filter test. The low resolution of this test is considered problematic because gastrophotofluorography is usually only indicative of abnormalities in the gastric mucosa. More than half of early cancer cases go undiagnosed; whereas sensitivity for advanced cancer is 92%, that for early-stage cancer is as low as 39% (14). Furthermore, gastrophotofluorography is expensive and requires

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technical skills on the part of the radiographic technicians and expert diagnostic abilities on the part of the radiologist. Although the incidence of leukemia has reportedly not increased among participants in the screening program, the risks of X-ray exposure are considered to represent another problem (15, 16). A more efficient screening system is thus sorely needed.

As a trial to improve the screening system, we recently introduced a serum pepsinogen test into gastric cancer screening as an alternative to gastrophotofluorography (17-19). This screening system is based on the hypothesis that chronic atrophic gastritis, including intestinal metaplasia, is a preneoplastic lesion of the stomach (20), together with the results of previous studies indicating that low serum pepsinogen levels reflect the extent of chronic atrophic gastritis (21, 22). In the screening system, individuals positive for extensive atrophic gastritis based on serum pepsinogen levels are further screened by endoscopy. Since 1992, when pepsinogen assay kits became commercially available, a number of screening services provided by work places or by community health services have adopted this serum test as a filter test (23-28). The results of the screening for the past 15 years have shown that addition of the serum test to the screening strategy has markedly increased the number of subjects undergoing screening and has also significantly improved detection rates of gastric cancer and early cancer, in particular, compared with conventional screening using gastrophotofluorography.

The effectiveness of the pepsinogen filter test is thus receiving wide recognition, and the observed high efficiency of the serum test in cancer detection strongly indicates that gastric cancer tends to develop from the atrophic stomach as detected by low serum pepsinogen. However, the long-term prognosis of subjects with extensive chronic atrophic gastritis identified by pepsinogen filter test is not fully known, including cancer incidence rates. In addition, various studies on cancer screening with the pepsinogen filter test have reported a nonnegligible number of test-negative cancers (17-19, 23-28), and the risk for cancer development among pepsinogen test-negative subjects has likewise not been fully elucidated. The present study investigated long-term outcomes of gastric cancer development among pepsinogen test-positive and test-negative subjects based on a 10-year follow-up study.

## Subjects and Methods

**Study Subjects.** Subjects comprised 5,706 male employees ages 40-60 years (mean ± SD, 49.2 ± 4.7 years) who participated in an annual multiphasic health screening program in Wakayama City, Japan, between April 1994 and the end of March 1995. This type of screening program is generally done by various work places throughout Japan to detect incident diseases in the early stages. Subjects with specific symptoms were thus guided to receive medical attention and were excluded from screening. Subjects who had previously undergone gastric resection were also excluded and were examined separately. Symptom-free subjects underwent a series of screening tests and procedures: an interview to ascertain general state of health, physical examination, chest radiography, electrocardiography, blood laboratory tests, urinalysis, and fecal occult blood test. Some of these subjects had been investigated in a previous cohort study (28).

All subjects were followed for the study period of 10 years, from April 1994 to the end of March 2004. Subjects underwent the aforementioned health screening program annually and were also screened to identify incident gastric cancer, as described in the following Gastric Cancer Screening section. The incident day of gastric cancer was defined as the day of the health checkup when the cancer was detected. Duration of the observation period was calculated for each subject from the time of the baseline survey to the diagnosis of gastric cancer.

**Analysis of Serum Pepsinogen Levels.** Aliquots of separated sera from fasting blood samples collected as routine laboratory tests for general health checkup were stored below −20°C until measurement of serum pepsinogen levels. Serum pepsinogen levels (pepsinogens I and II) were measured using a modification (RIAbeads kit, Dainabott) of our previously reported RIA (29). Subjects with renal failure were excluded from analyses of the results of serum pepsinogen levels. Subjects who had been prescribed medication that might affect gastrointestinal function, such as proton pump inhibitors or nonsteroidal anti-inflammatory drugs, before examination and subjects who had undergone eradication therapy for *Helicobacter pylori* were also excluded.

**Gastric Cancer Screening.** Cancer screening was done by double-contrast barium X-ray with digital radiography and by serum pepsinogen as filter tests. For upper gastrointestinal barium X-ray, a remote controlled X-ray fluoroscope (TU-230XB, Hitachi Medical Corp.) and real-time digital radiography (DR-2000H; Hitachi Medical) were used. The double-contrast upper gastrointestinal X-ray series used 150 mL of high-concentration barium at 200%, and 11 films were taken for each subject as described previously (28). Subjects were also screened using the serum pepsinogen filter test. Among several test-positive criteria of the pepsinogen filter test used for cancer screening in our country, the criterion of pepsinogen I of ≤70 ng/mL and pepsinogen I/II ratio of ≤3.0 is the most widely applied, with atrophy-positive defined when the criterion is fulfilled and atrophy-negative defined when the criterion is not fulfilled (19, 30-33). Additional and stricter criteria of pepsinogen I of ≤50 ng/mL and pepsinogen I/II ratio of ≤3.0 or pepsinogen I of ≤30 ng/mL and pepsinogen I/II ratio of ≤2.0 are also used to detect subjects with more extensive atrophy (18, 23, 24). The latter two criteria are used variously, independently or in combination with the aforementioned atrophy-positive criteria, depending on the purpose of screening. When used in combination, each criterion constitutes pepsinogen index 1+ to 3+ within the atrophy-positive group according to the first criterion of pepsinogen I of ≤70 ng/mL and pepsinogen I/II ratio of ≤3.0.

The third criterion, pepsinogen I of ≤30 ng/mL and pepsinogen I/II ratio of ≤2.0, is defined as pepsinogen index 3+. The second criterion, pepsinogen I of ≤50 ng/mL and pepsinogen I/II ratio of ≤3.0 but not meeting the criterion for pepsinogen index 3+, is defined

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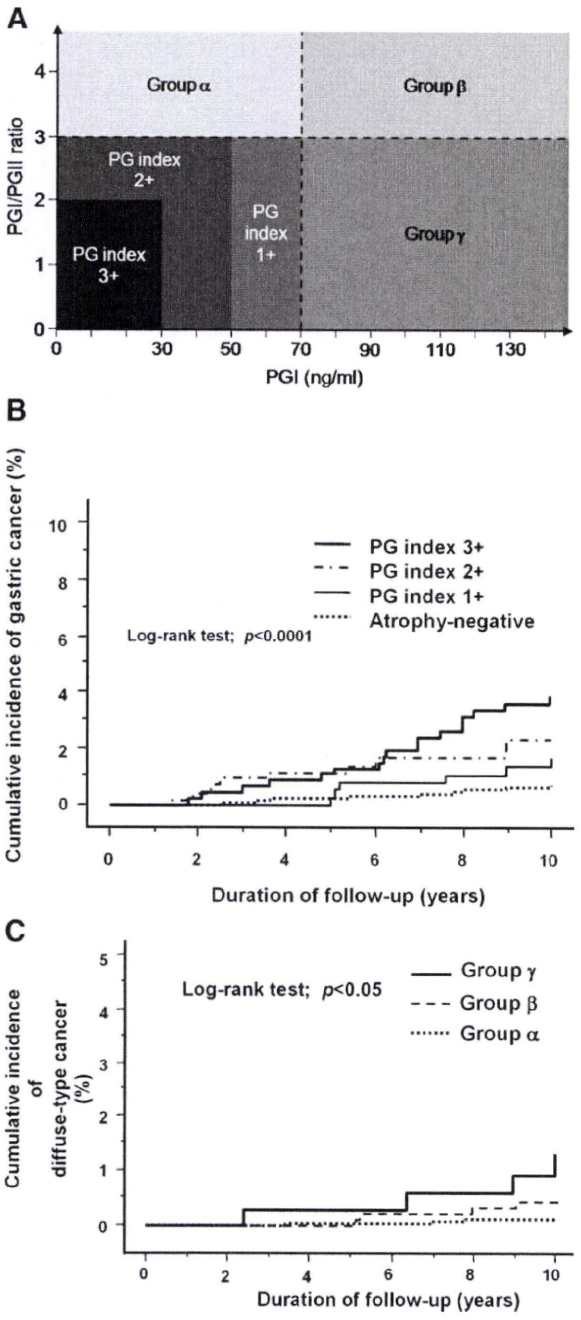
as pepsinogen index 2+. The first criterion, but not meeting the criteria for pepsinogen index 2+ or 3+, is defined as pepsinogen index 1+ (ref. 34; Fig. 1A). In the present study, data on serum pepsinogen levels of subjects were classified basically using the atrophy-positive criterion and pepsinogen index. If a subject was identified as test-positive by serum pepsinogen test, fulfilling the atrophy-positive criterion, that is, pepsinogen index 1+ to 3+, or by barium digital radiography, further examination was conducted by upper gastrointestinal endoscopy (XQ-200, Olympus).




Resected specimens of gastric cancer obtained by endoscopy or surgery were assessed histopathologically and classified according to the classification of Lauren (34) as intestinal or diffuse type. Location of the cancer in the stomach was classified as cardia or noncardia based on clinical or histopathologic records. The ethics committee of Wakayama Medical University approved the study protocols, and informed consent was obtained from all participating subjects.

**Statistical Analysis.** Data were analyzed using SPSS 11.0 (SPSS) and STATA (STATA Corp.). Differences were tested for significance using the $t$ test for comparisons between two groups, ANOVA for comparisons among multiple groups, and Scheffe's least significant difference test for comparisons of pairs of groups. The $\chi^2$ test was used to compare categorical variables. Long-term effects of pepsinogen test-positive or test-negative criteria on gastric cancer development were evaluated using Cox proportional hazards models.

## Results

A total of 5,706 eligible subjects were examined. Of these, 489 subjects who declined to participate in the study program or who met the exclusion criteria described were excluded from the study. In addition, eight cases of gastric cancer that developed within the 1st year of the study were also excluded. The remaining 5,209 subjects

**Figure 1.** Gastric cancer development based on criteria of pepsinogen filter test used for cancer screening in Japan. **A.** Schematic presentation of the atrophy-positive criterion and pepsinogen index, which are widely used for the pepsinogen filter test in Japan. Pepsinogen index is used to detect subjects with severe gastric atrophy by subdividing the atrophy-positive criterion identified by pepsinogen I of ≤70 ng/mL and pepsinogen I/II ratio of ≤3.0 into three groups (1+ to 3+), as described in the text. In addition, the figure illustrates three subgroups, groups α, β, and γ, in the atrophy-negative criterion, which is used in the present analysis. The atrophy-negative criterion was further classified into the three subgroups as follows: group α, pepsinogen I of ≤70 ng/mL and pepsinogen I/II ratio of >3.0; group β, pepsinogen I of >70 ng/mL and pepsinogen I/II ratio of >3.0; and group γ, pepsinogen I of >70 ng/mL and pepsinogen I/II ratio of ≤3.0. **B.** Kaplan-Meier analysis of gastric cancer development in subjects classified using the criteria of the pepsinogen filter test. Subjects were classified into four groups according to the atrophy-positive criterion and pepsinogen index and then followed for cancer development for 10 y. Cancer incidence rates for the atrophy-negative group and pepsinogen indices of 1+, 2+, and 3+ were 70 per 100,000, 166 per 100,000, 242 per 100,000, and 424 per 100,000 person-years, respectively. **C.** Kaplan-Meier analysis of diffuse-type gastric cancer development among subjects fulfilling the atrophy-negative criterion. Atrophy-negative subjects were classified into three subgroups, group α, β, and γ, according to the criteria described in the text and in **A**, and were followed for the study period of 10 y. Among these subgroups, incidence rate of diffuse-type cancer was significantly higher in group γ than in groups α and β, at 120 per 100,000 person-years ($P = 0.019$). *PG,* pepsinogen.

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**Table 1. Gastric cancer incidence rate by various criteria of pepsinogen filter test**

| Screening criteria | PG I ≤70 and PG I/II ≤3.0 | | PG I ≤50 and PG I/II ≤3.0 | | PG I ≤30 and PG I/II ≤2.0 | | Total |
|---|---|---|---|---|---|---|---|
| | Negative | Positive | Negative | Positive | Negative | Positive | Subjects |
| Subjects | 3,802 | 1,407 | 4,176 | 1,033 | 4,782 | 427 | 5,209 |
| Person-years | 37,010.0 | 13,416.0 | 40,626.5 | 9,799.5 | 46,419.0 | 4,007.0 | 50,426 |
| Age [mean (SD)] | 48.8 (4.8) | 50.3 (4.4)* | 48.9 (4.7) | 50.6 (4.3)* | 49.0 (4.7) | 51.3 (4.2)* | 49.2 (4.7) |
| Follow-up years [mean (SD)] | 9.7 (0.8) | 9.5 (1.1) | 9.7 (0.8) | 9.5 (1.2) | 9.7 (0.8) | 9.4 (1.2) | 9.7 (0.9) |
| Total gastric cancer | | | | | | | |
| Age [mean (SD)] | 50.7 (4.3) | 50.9 (3.4) | 50.3 (4.2) | 51.4 (3.2) | 50.5 (4.0) | 51.8 (2.9) | 50.8 (3.7) |
| Follow-up years [mean (SD)] | 6.3 (2.6) | 5.9 (2.6) | 6.5 (2.5) | 5.6 (2.6) | 6.0 (2.7) | 6.1 (2.4) | 6.0 (2.6) |
| Cases/incidence rate† | 26/70 | 37/276 | 32/79 | 31/316 | 46/99 | 17/424 | 63/125 |
| Intestinal gastric cancer | | | | | | | |
| Age [mean (SD)] | 51.1 (4.0) | 51.4 (3.4) | 50.6 (3.6) | 51.6 (3.4) | 50.6 (3.6) | 52.4 (3.0) | 51.1 (3.5) |
| Follow-up years [mean (SD)] | 6.0 (2.7) | 5.7 (2.5) | 6.2 (2.6) | 5.7 (2.4) | 5.8 (2.7) | 5.8 (2.2) | 5.8 (2.5) |
| Cases/incidence rate† | 16/41 | 26/194 | 18/44 | 24/245 | 30/65 | 12/299 | 42/83 |
| Diffuse gastric cancer | | | | | | | |
| Age [mean (SD)] | 50.2 (4.8) | 49.7 (3.2) | 50.0 (5.0) | 50.6 (2.0) | 50.3 (4.8) | 50.2 (1.9) | 50.3 (4.2) |
| Follow-up years [mean (SD)] | 6.8 (2.5) | 6.2 (2.8) | 6.7 (2.4) | 5.4 (3.3) | 6.4 (2.6) | 6.7 (2.9) | 6.5 (2.6) |
| Cases/incidence rate† | 10/30 | 11/82 | 14/35 | 7/71 | 16/34 | 5/125 | 21/42 |
| Accuracy of screening criteria | | | | | | | |
| Sensitivity (95% CI) | 58.7% (45.6-70.8) | | 49.2% (36.5-62.0) | | 27.0% (16.9-39.9) | | |
| Specificity (95% CI) | 73.4% (72.1-74.6) | | 80.5% (79.4-81.6) | | 92.0% (91.3-92.8) | | |
| Positive predictive value (95% CI) | 2.6% (1.9-3.6) | | 3.0% (2.1-4.3) | | 4.0% (2.4-6.4) | | |
| Negative predictive value (95% CI) | 99.3% (99.0-99.5) | | 99.2% (98.9-99.5) | | 99.0% (98.7-99.3) | | |
| Likelihood ratio of a positive test (95% CI) | 2.21% (1.78-2.73) | | 2.53% (1.95-3.27) | | 3.39% (2.23-5.14) | | |
| Likelihood ratio of a negative test (95% CI) | 0.56% (0.42-0.76) | | 0.63% (0.49-0.80) | | 0.79% (0.68-0.92) | | |

Abbreviation: PG, pepsinogen.
*Significantly different from each counterpart ($P < 0.05$).
† Per 100,000 person-years.

(mean age, 49.2 ± 4.7 years) were followed for 10 years and investigated for gastric cancer development. During 50,426 person-years of follow-up, 63 cases of cancer developed, representing an incidence rate of 125 of 100,000 person-years in the cohort (Table 1). Histopathologic features of the detected cancer revealed intestinal type in 42 cases (65%) and diffuse type in 21 cases (35%). Most cases (61 cases, 96.8%) were noncardia cancer, and only 2 cases (3.2%) were located in the gastric cardia. Fifty-four cases (86%) were in the early stage and confined to the mucosa or submucosa. The remaining nine cases were in the advanced stage. Mucosal cancers comprised 74% (40 of 54) of early cancers and were treated by endoscopic resection. The remaining 14 early cancers and 9 advanced cancers underwent surgical resection, and all cases were successfully treated.

Of 5,209 study subjects, 27.0% ($n$ = 1,407) were positive according to the first criterion, pepsinogen I of ≤70 ng/mL and pepsinogen I/II ratio of ≤3.0, that is atrophy positive. Likewise, 19.8% ($n$ = 1,033) and 8.2% ($n$ = 427) were positive by the second criterion, pepsinogen I of ≤50 ng/mL and pepsinogen I/II ratio of ≤3.0, and third criterion, pepsinogen I of ≤30 ng/mL and pepsinogen I/II ratio of ≤2.0, respectively (Table 1). Among the atrophy-positive group, 27.0% ($n$ = 374) were categorized as pepsinogen index 1+, 43.0% ($n$ = 606) as pepsinogen index 2+, and 30.0% ($n$ = 427) as pepsinogen index 3+ (Table 2). Mean age was significantly higher in test-positive subjects than in test-negative subjects in each of the three criteria and increased in a stepwise manner with increasing pepsinogen index, whereas no significant difference was seen in mean follow-up period among subjects, irrespective of test positivity or differences in pepsinogen index. Of 63 cases of gastric cancer that developed during the study period, 58.7% ($n$ = 37) developed in the atrophy-positive group and the remaining 41.3% ($n$ = 26) developed in the atrophy-negative group. With the second and third criteria, 49.2% ($n$ = 31) and 27.0% ($n$ = 17) of cancer cases were test-positive, respectively (Table 1). The accuracy of cancer screening by each of the three criteria was thus as follows: for pepsinogen I of ≤70 ng/mL and pepsinogen I/II ratio of ≤3.0, 58.7% sensitivity [95% confidence interval (95% CI), 45.6-70.8%], 73.4% specificity (95% CI, 72.1-74.6%), and 2.6% positive predictive value (95% CI, 1.9-3.6%); for pepsinogen I of ≤50 ng/mL and pepsinogen I/II ratio of ≤3.0, 49.2% sensitivity (95% CI, 36.5-62.0%), 80.5% specificity (95% CI, 79.4-81.6%), and 3.0% positive predictive value (95% CI, 2.1-4.3%); and for pepsinogen I of ≤30 ng/mL and pepsinogen I/II ratio of ≤2.0, 27.0% sensitivity (95% CI, 16.9-39.9%), 92.0% specificity (95% CI, 91.3-92.8%), and 4.0% positive predictive value (95% CI, 2.4-6.4%). The cancer incidence rate of the atrophy-negative group was 70 per 100,000 person-years compared with 276 per 100,000 person-years for the atrophy-positive group.

Among 37 cancers developed from atrophy-positive subjects, 16.2% ($n$ = 6) were from the pepsinogen index 1+ group, 37.8% ($n$ = 14) from the pepsinogen index 2+ group, and 46.0% ($n$ = 17) from the pepsinogen index 3+ group (Table 2). Kaplan-Meier analysis showed that after 6 years of follow-up, cancer development occurred in the order of pepsinogen index and was highest in the 3+ group, followed by the 2+, 1+, and atrophy-negative

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groups (Fig. 1B). A significant stepwise increase in cancer incidence rate was seen with increases in pepsinogen index among the atrophy-positive group from 166 to 424 per 100,000 person-years, and hazard ratio also increased significantly and in a stepwise manner from atrophy-negative to pepsinogen index 3+, reaching 5.16 (95% CI, 2.77-9.51; $P < 0.01$; Table 2). Of the two histologic types of cancer, a significant positive correlation between cancer incidence rate and pepsinogen index was observed only for intestinal-type cancer.

As described previously, 41.3% (26 of 63) of detected cancers developed from the atrophy-negative group. Of the two histopathologic types of cancer, 10 cancers (38.5%) derived from this group were diffuse-type, meaning that nearly half (47.6%) of the 21 diffuse-type cancers detected during the study period developed from nonatrophic stomachs. Whereas the atrophy-positive group comprised subjects with low values for both serum pepsinogen I and pepsinogen I/II ratio, the negative group was composed of not only subjects with high serum pepsinogen I and pepsinogen I/II ratio but also subjects with low serum pepsinogen I or low pepsinogen I/II ratio. The atrophy-negative group can thus be further classified into the following three groups: group α, with pepsinogen I of ≤70 ng/mL and pepsinogen I/II ratio of >3.0; group β, with pepsinogen I of >70 ng/mL and pepsinogen I/II ratio of >3.0; and group γ with pepsinogen I of >70 ng/mL and pepsinogen I/II ratio of ≤3.0 (Fig. 1A). Because previous studies (including our own) have suggested that a low serum pepsinogen I level or low pepsinogen I/II ratio is related to risk for gastric cancer (18, 35, 36), long-term outcomes of cancer development may differ among these three subgroups. Table 2 shows cancer development in the three groups during the study period. Mean age was significantly higher for group γ than for group α, but no difference in mean follow-up years was seen among the three groups. Mean serum levels of pepsinogens I and II increased significantly and in a stepwise manner from group α to γ, and the elevation was particularly marked for pepsinogen II. Pepsinogen I/II ratio was lowest in group γ. Groups α, β, and γ comprised 58.4% ($n$ = 2,219), 32.4% ($n$ = 1,234), and 9.2% ($n$ = 349) of atrophy-negative subjects, and proportions of cancers that developed in each group were 46.2% ($n$ = 12), 26.9% ($n$ = 7), and 26.9% ($n$ = 7) of proportions in the atrophy-negative group, respectively.

Cancer incidence rates for groups α and β were 55 and 58 per 100,000 person-years, respectively, lower than that in the atrophy-negative group overall (70 per 100,000 person-year), whereas group γ showed a high incidence rate of 211 per 100,000 person-years. A significant increase in hazard ratio was seen from group α to γ (hazard ratio, 3.49; 95% CI, 1.37-8.93), reflecting a significant increase in the hazard ratio of diffuse-type

**Table 2. Gastric cancer incidence rate in atrophy-negative and atrophy-positive groups**

| Group | Atrophy-negative group | | | | $P_{trend}$ | Atrophy-positive group | | | $P_{trend}$ |
|---|---|---|---|---|---|---|---|---|---|
| | Total | α | β | γ | | PG index 1+ | PG index 2+ | PG index 3+ | |
| Subjects | 3,802 | 2,219 | 1,234 | 349 | | 374 | 606 | 427 | |
| Person-years | 37,010.0 | 21,702.5 | 11,976.5 | 3,324.0 | | 3,616.5 | 5,792.5 | 4,007.0 | |
| Age [mean (SD)] | 48.8 (4.8) | 48.5 (4.8) | 49.2 (4.7) | 49.8 (4.6)* | | 49.5 (4.4) | 50.2 (4.5)† | 51.3 (4.2)† | |
| Follow-up years [mean (SD)] | 9.7 (0.8) | 9.8 (0.7) | 9.7 (0.8) | 9.5 (1.0) | | 9.7 (0.9) | 9.5 (1.2) | 9.4 (1.2) | |
| PG I [mean (SD)] | 69.3 (29.6) | 51.9 (10.1) | 93.1 (30.4)* | 96.2 (29.5)* | | 59.5 (5.8) | 38.1 (8.1)† | 16.7 (7.7)† | |
| PG II [mean (SD)] | 16.2 (11.4) | 10.4 (3.9) | 19.9 (8.4) | 40.7 (14.2)* | | 26.6 (6.8) | 18.5 (6.7)† | 14.0 (5.6)† | |
| PG I/II [mean (SD)] | 5.1 (1.8) | 5.4 (1.6) | 5.1 (1.7) | 2.4 (0.4)* | | 2.4 (0.5) | 2.2 (0.5) | 1.2 (0.4)† | |
| Total gastric cancer | | | | | | | | | |
| Age [mean (SD)] | 50.7 (4.3) | 51.5 (2.9) | 51.0 (5.5) | 49.1 (5.1) | | 48.5 (3.7) | 50.9 (3.6) | 51.8 (2.9) | |
| Follow-up years [mean (SD)] | 6.3 (2.6) | 5.9 (2.6) | 5.6 (2.2) | 7.8 (2.7) | | 7.0 (2.2) | 5.1 (2.8) | 6.1 (2.4) | |
| Cases/incidence rate‡ | 26/70 | 12/55 | 7/58 | 7/211 | | 6/166 | 14/242 | 17/424 | |
| HR (95% CI)§ | 1 | 1 | 0.99 (0.39-2.54) | 3.49 (1.37-8.93) | 0.019 | 3.60 (2.17-5.96) | 4.55 (2.62-7.43) | 5.16 (2.77-9.51) | <0.0001 |
| Intestinal gastric cancer | | | | | | | | | |
| Age [mean (SD)] | 51.1 (4.0) | 51.8 (2.6) | 51.7 (6.1) | 46.3 (1.5)* | | 48.5 (4.7) | 50.6 (3.7) | 52.4 (3.0) | |
| Follow-up years [mean (SD)] | 6.0 (2.7) | 5.8 (2.8) | 4.0 (1.3) | 9.0 (1.0) | | 7.0 (2.3) | 5.6 (2.6) | 5.8 (2.2) | |
| Cases/incidence rate‡ | 16/41 | 9/41 | 3/25 | 3/91 | | 2/55 | 12/207 | 12/299 | |
| HR (95% CI)§ | 1 | 1 | 0.57 (0.15-2.11) | 1.99 (0.53-7.39) | 0.31 | 4.47 (2.37-8.42) | 5.54 (2.91-10.55) | 6.62 (3.18-13.74) | <0.0001 |
| Diffuse gastric cancer | | | | | | | | | |
| Age [mean (SD)] | 50.2 (4.8) | 50.7 (4.0) | 50.5 (5.9) | 51.3 (6.1) | | 48.5 (0.7) | 53.0 (1.4) | 50.2 (1.9) | |
| Follow-up years [mean (SD)] | 6.8 (2.5) | 6.1 (2.3) | 6.8 (3.0) | 7.0 (3.4) | | 7.1 (2.8) | 1.8 (0.7) | 6.7 (2.9) | |
| Cases/incidence rate‡ | 10/29 | 3/14 | 4/33 | 4/120 | | 4/111 | 2/35 | 5/125 | |
| HR (95% CI)§ | 1 | 1 | 2.29 (0.51-10.24) | 8.04 (1.78-36.25) | 0.021 | 2.41 (1.02-5.71) | 1.96 (0.72-5.36) | 3.16 (0.99-10.06) | 0.23 |

Abbreviation: HR, hazard ratio.

* Significantly different from group α subjects ($P < 0.05$).

† Significantly different from pepsinogen index 1+ subjects ($P < 0.05$).

‡ Per 100,000 person-years.

§ In the atrophy-positive group, hazards ratio was calculated in comparison with cancer incidence rate of the atrophy -negative group (total) and, in the atrophy-negative group, was calculated in comparison with that of group α.

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cancer but not that of intestinal-type cancer (Table 2). Kaplan-Meier analysis revealed that after 5 years of follow-up, cumulative incidence of diffuse-type cancer was in the order of groups γ, β, and α (Fig. 1C), with an incidence rate of 120 per 100,000 person-years for group γ and significantly increased hazard ratio compared with that in group α (hazard ratio, 8.04; 95% CI, 1.78-36.25).

## Discussion

In the present study, 5,209 middle-aged male subjects with measured serum pepsinogen levels were followed for 10 years, and gastric cancer development was investigated based on the criteria of the pepsinogen filter test widely used for gastric cancer screening in Japan. For cancer screening in our country, the following pepsinogen test-positive criteria have been used depending on differences in target population and the purpose of screening: pepsinogen I of ≤70 ng/mL and pepsinogen I/II ratio of ≤3.0; pepsinogen I of ≤50 ng/mL and pepsinogen I/II ratio of ≤3.0; or pepsinogen I of ≤30 ng/mL and pepsinogen I/II ratio of ≤2.0.

Among these, the first criterion (pepsinogen I of ≤70 ng/mL and pepsinogen I/II ratio of ≤3.0) is considered both the most efficient for identifying extensive atrophic gastritis and the best cancer screening criterion based on the results of previous studies (19, 26, 30-33). In addition, the results of a recent meta-analysis of seven studies analyzing organized population-based cancer screening indicated that pooled sensitivity and specificity of the criterion were 77.3% and 73.2%, offering a better sensitivity/specificity balance than any other criteria and also providing strong support for the use of pepsinogen I of ≤70 ng/mL and pepsinogen I/II ratio of ≤3.0 (37). In our observation over 10 years, pepsinogen I of ≤70 ng/mL and pepsinogen I/II ratio of ≤3.0 was considered the best among the criteria currently used for cancer screening, showing 58.7% sensitivity (95% CI, 45.6-70.8%) and 73.4% specificity (95% CI, 72.1-74.6%).

As for the other two criteria, pepsinogen I of ≤50 ng/mL and pepsinogen I/II ratio of ≤3.0 or pepsinogen I of ≤30 ng/mL and pepsinogen I/II ratio of ≤2.0, the sensitivity of screening is <50%, at 49.2% (95% CI, 36.5-62.0%) and 27.0% (95% CI, 16.9-39.9%), respectively. Cancer screening using either of these criteria as an independent criterion for the filter test thus does not seem feasible. As a whole, the sensitivity of these criteria analyzed in the present study was considerably lower than that reported in other studies (37). This is probably due to the fact that previous studies analyzed an initial phase of screening when the pepsinogen filter test was newly applied, in cross-sectional evaluations, whereas the present study analyzed the subsequent phase of screening by prospective evaluation, with eight cases of cancer detected during the 1st year of investigation excluded from the analysis.

The present results strongly indicate that the sensitivity of pepsinogen filter test, which is high on initial prevalent screening, is not equally high for noninitial incident screenings in subsequent years. The reported high sensitivity in these studies was likely caused by overestimating the accuracy of the pepsinogen filter test. Nonetheless, the present results clearly show that the atrophy-positive criterion of pepsinogen I of ≤70 ng/mL and pepsinogen I/II ratio of ≤3.0 detects 58.7% of cancers developing over 10 years and also identifies a group of subjects susceptible for gastric cancer. The incidence rate of gastric cancer in the group defined by this criterion was 276 per 100,000 person-years and increased in a stepwise to a maximum level of 424 per 100,000 person-years with an increase in pepsinogen index from 1+ to 3+. The observed correlation between incidence rate of gastric cancer and grade of atrophy as indicated by pepsinogen index verifies the hypothesis that risk for cancer increases with progression of chronic atrophic gastritis (20) and that serum pepsinogen level offers a reliable marker for cancer development and coexisting atrophic gastritis.

Meanwhile, a nonnegligible proportion (41.3%) of gastric cancers developed in the atrophy-negative group during the study period. In this group, the percentage of diffuse-type cancers (38.5%) was significantly higher than that in the atrophy-positive group (29.7%), and nearly half of diffuse-type cancers (47.6%) developed in this group during the study period, in good accordance with the hypothesis that this type of cancer develops in the stomach following chronic inflammation without passing through an intermediate step of atrophic gastritis together with intestinal metaplasia (38-40).

Unlike the atrophy-positive group, this group displays heterogeneous serum pepsinogen levels, containing subjects with both high and low levels of serum pepsinogen I and pepsinogen I/II ratio, and can be classified into three subgroups: group α, with pepsinogen I of ≤70 ng/mL and pepsinogen I/II ratio of >3.0; group β, with pepsinogen I of >70 ng/mL and pepsinogen I/II ratio of >3.0; and group γ, with pepsinogen I of >70 ng/mL and pepsinogen I/II ratio of ≤3.0. Among these subgroups, cancer incidence rate was highest in group γ at 216 per 100,000 person-years, comparable with that in the atrophy-positive group and even higher than that for pepsinogen index 1+ (166 per 100,000 person-years). Establishment of *H. pylori* infection results in increased serum pepsinogen levels, and this elevated pepsinogen level (particularly pepsinogen II in the nonatrophic stomach) is considered to reflect the severity of gastritis (41, 42). Gastric inflammation is thus likely to be increasingly severe in groups α, β, and γ, in that order, as revealed by respective pepsinogen levels. In addition, the mean age in each of the three subgroups increased in the order of groups α, β, and γ, and the number of subjects in these subgroups decreased in the same order.

Furthermore, the proportion of *H. pylori*–negative subjects was larger in the order of group α (33%; 733 of 2,219), group β (19%; 229 of 1,234), and group γ (1%; 4 of 349). The process of gastritis thus seems to advance from group α thorough group β and finally to group γ after the establishment of *H. pylori* infection. These results strongly indicate that a group of subjects with putative extensive active gastritis in the nonatrophic stomach are at high risk for cancer comparable with that in subjects with extensive atrophy. These subjects comprised 9.2% (349 of 3,802) of the atrophy-negative group and can be identified using a criterion of pepsinogen I of >70 ng/mL and pepsinogen I/II ratio of ≤3.0.

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However, this criterion detects only about one quarter of cancers (26.7%) developing in the nonatrophic stomach. This is probably because serum pepsinogen level, especially pepsinogen II, does not show marked elevation in most *H. pylori*–related multifocal gastritis unless *H. pylori*–induced active inflammation becomes widespread, and we cannot detect subjects in whom the active inflammatory process is focally severe enough to commit epithelial cells to neoplastic transformation.

Taken together, the present results clearly indicate that the atrophy-metaplasia-dysplasia-cancer sequence described by Correa (20) represents the main route of stomach carcinogenesis in Japan, and the pepsinogen filter test using a criterion of pepsinogen I of ≤70 ng/mL and pepsinogen I/II ratio of ≤3.0 offers a reliable method for identifying individuals at high risk for the sequence-derived cancer, with an incidence rate of 276 per 100,000 person-years. In addition, we have revealed another group at high risk for cancer without gastric atrophy, identified by a criterion of pepsinogen I of >70 ng/mL and pepsinogen I/II ratio of ≤3.0. The characteristics of this minor group are that it comprises only 6.7% (349 of 5,209) of total cancer-prone aged subjects, shows a high cancer incidence rate comparable with that in subjects with extensive atrophy (216 per 100,000 person-years), and tends to develop diffuse-type cancers with higher malignant potential. Based on the present results, screening targeting both of these high-risk groups would provide 69.8% sensitivity (95% CI, 56.8-80.4%), 66.7% specificity (95% CI, 65.4-68.0%), and 2.5% positive predictive value (95% CI, 1.8-3.3%).

The major aim of cancer screening is to detect treatable early-stage cancer in asymptomatic individuals. For early detection of premalignant or malignant lesions, endoscopic visualization is considered the best method. Endoscopy is invasive, uncomfortable, and expensive and is thus only offered to subjects with positive results on a filter test in cancer screening. In Japan, mass screening for gastric cancer has primarily adopted gastrophotofluorography as a filter test. Compared with this traditional filter test, serum pepsinogen test is easier to perform, provides quicker results, and produces no patient discomfort (17, 19).

Furthermore, the test is inexpensive; the cost for detection of a single cancer was much less than that for conventional screening, comparable with that for surgical resection of a single cancer case (19, 28). Gastrophotofluorography reportedly offers 57% to 90% sensitivity, 77% to 91% specificity, and 0.9% to 2.0% positive predictive value (43). The present results indicate that specificity of the pepsinogen filter test using the criterion of pepsinogen I of ≤70 ng/mL and pepsinogen I/II ratio of ≤3.0 as a cutoff is slightly lower (73.4%), representing a higher false-positive rate than reported for gastrophotofluorography.

However, as clearly indicated in the present study, pepsinogen test-positive subjects are at high risk for cancer, and this low specificity (high false-positive rate) may be partly attributable to the relatively long latency between initiation of the carcinogenic step and clinically established cancer development. The incidence rate of cancer in pepsinogen test-positive subjects is 276 per 100,000 person-years, meaning that one cancer develops in about every 36 subjects during the 10-year period. Regular and strict long-term endoscopic surveillance of this group thus seems warranted. Whether eradication of *H. pylori* is effective for preventing cancer development in these pepsinogen test-positive subjects warrants further investigation.

In conclusion, atrophy-positive subjects and atrophy-negative subjects with putative extensive active inflammation as defined by pepsinogen test criteria constitute populations at high risk for gastric cancer and need thorough examination by endoscopy. Because the results of previous studies have clearly indicated that use of the same cutoff for pepsinogen test results in comparable outcomes in different sets of individuals and in different countries for the detection of preneoplastic or neoplastic lesions (37), the present data are probably useful not only for subjects in high-risk areas of gastric cancer outside Japan but also for subjects in low-risk areas. Mass cancer-screening programs may not be feasible, but strict follow-up of high-risk subjects will probably be effective even in Western countries (44). Further studies are necessary, particularly analyzing follow-up programs of subjects identified by pepsinogen tests and including examination of cost-effectiveness.

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創薬基盤推進研究事業

# 胃粘膜に蓄積したエピジェネティック異常の定量による多発胃がん発生予測に関する前向き研究

平成２０年度～２２年度　総合研究報告書

研究代表者　牛島　俊和

平成２３年（２０１１）年　４月
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