Short Communication

Higher Methylation Levels in Gastric Mucosae Significantly Correlate with Higher Risk of Gastric Cancers

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Abstract

Background: ***Helicobacter pylori* infection potently induces methylation of CpG islands in gastric mucosae, which is considered to decrease to a certain level after active *H. pylori* infection discontinues. Noncancerous gastric mucosae of *H. pylori*–negative cases with a gastric cancer had higher methylation levels than those of *H. pylori*–negative healthy individuals. Here, using cases with multiple gastric cancers, we analyzed whether the higher methylation levels correlated with the higher risk of gastric cancers.**

Methods: **Twenty-six healthy volunteers (HV), 30 cases with a single well-differentiated gastric cancer (S cases), and 32 cases with multiple well-differentiated gastric cancers (M cases) were recruited. *H. pylori* infection status was analyzed by the culture method. Methylation levels were quantified by real-time methylation-specific PCR of seven CpG islands.**

Results: **In *H. pylori*–negative individuals, significant increasing trends were present in the order of HV, S cases, and M cases for *FLNc* and *HAND1* methylation levels ($P < 0.01$, Spearman's rank-order test). Furthermore, the *FLNc* methylation level of M cases was significantly higher than that of S cases ($P < 0.01$, t test). Even adjusted by the extent of gastric atrophy, the *FLNc* methylation level retained a significant increasing trend ($P = 0.03$). In contrast, methylation levels in *H. pylori*–positive individuals were increased to various degrees in all the three groups.**

Conclusions: **In *H. pylori*–negative individuals, methylation levels in gastric mucosae significantly increased in cases with a single gastric cancer and more in cases with multiple gastric cancers. Quantitative analysis of methylation levels is a promising risk marker for gastric cancers.** (Cancer Epidemiol Biomarkers Prev 2006;15(11):2317–21)

Introduction

Gastric cancer is one of the most common malignancies worldwide (1). As a novel therapeutic procedure, endoscopic resection (ER), including both endoscopic mucosal resection and endoscopic submucosal dissection, is becoming common not only in Japan but also in western countries (2, 3). ER preserves a larger portion of the stomach than partial gastrectomy, and cumulative incidence of metachronous gastric cancers reaches as high as 8.5% to 14.0% (4, 5), in contrast with the incidence of 1.8% to 2.4% after partial gastrectomy (6, 7). Therefore, it is important to clarify whether specific individuals with a very high risk of gastric cancers develop multiple gastric cancers and, if so, to develop a marker to estimate the risk of individual cases.

The presence of individuals with a very high risk is indicated by the fact that cases with multiple gastric cancers have a much higher chance of developing another gastric cancer than those of cases with a single gastric cancer (8, 9). To identify these people, markers for multiple gastric cancers, such as gastric atrophy (10), microsatellite instability in cancers (11), and expression of brain-type glycogen phosphorylase in noncancerous gastric mucosae (12), have been developed. However, their sensitivity and specificity are not satisfactory, and a more sensitive marker whose value correlates with the degree of an individual's risk of cancer is awaited.

We showed recently that *Helicobacter pylori* infection induces methylation of various but preferential CpG islands (CGI) in gastric mucosae (13). Among *H. pylori*–negative individuals, methylation levels in noncancerous gastric mucosae were higher in gastric cancer cases than in healthy volunteers (HV). *H. pylori*–positive individuals, those who are with current, or active, *H. pylori* infection, had higher methylation levels than *H. pylori*–negative gastric cancer cases, most of whom are expected to have had past *H. pylori* infection (14). Therefore, it was indicated that current *H. pylori* infection potently induces methylation, that the high methylation level decreases to a certain level after active *H. pylori* infection discontinues, and that the final methylation level is associated with the risk of gastric cancers (15).

In this study, to develop a novel risk marker for multiple gastric cancers, we aimed to clarify that higher methylation levels in noncancerous gastric mucosae correlate with higher risks of developing gastric cancers. Risk levels in HV, cases with a single gastric cancer (S cases) with follow-up longer than 1 year, and cases with multiple gastric cancers (M cases) can be considered to be low, high, and very high, respectively (5, 8, 9), and their methylation levels were quantitatively analyzed.

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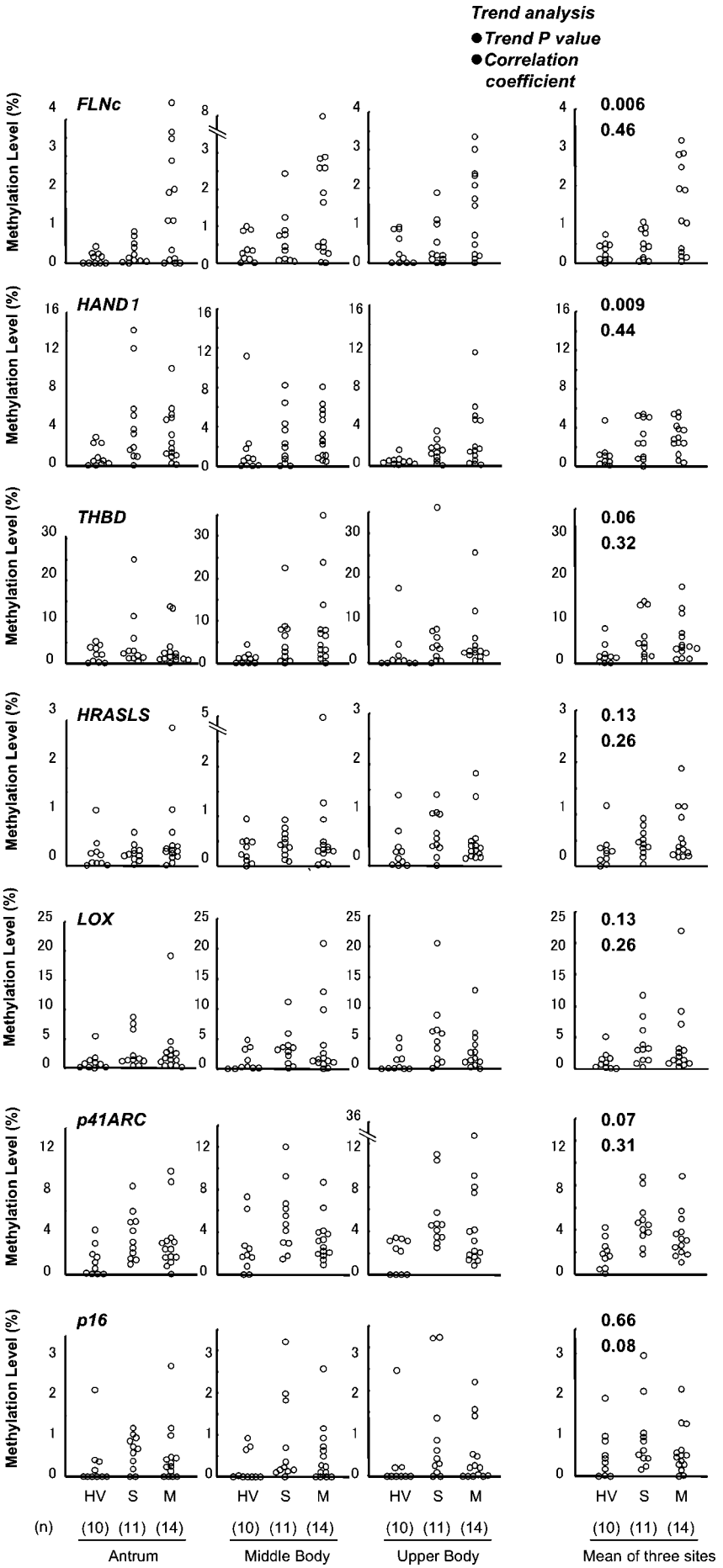

Figure 1. Methylation levels of the seven CGIs in *H. pylori*–negative HV, S cases, and M cases. Methylation levels in three sites within the stomach and their mean values. Trend *P* values and correlation coefficients are shown for the mean values, which were most closely associated with gastric cancer risk levels. Significant increasing trends were observed for *FLNc* and *HAND1* by the Spearman's rank-order correlation test ($P = 0.006$ and 0.009). Tendency of increasing trends was observed for *THBD* and *p41ARC*. Even when S and M cases are compared, *FLNc* methylation levels showed a significant increase in M cases ($P = 0.009$, *t* test).

Materials and Methods

Cases and Biopsy Materials. Twenty-six HV (male/female, 18/8), 30 S cases (23/7), and 32 M cases (23/9) were recruited from June 2004 to March 2005 at the National Cancer Center Hospital (Tokyo, Japan) under approval of the institutional review board and with written informed consents. The S and M cases previously underwent ER for well-differentiated early gastric adenocarcinoma according to the pre-ER indications, and curative resection was confirmed using expanded histologic criteria (16). M cases were defined according to the criteria of Moertel et al. (17): (*a*) each lesion is histopathologically malignant, (*b*) each lesion is separated from another, and (*c*) each lesion is not the result of a local extension or metastasis of another lesion. Because distinction between ''real'' metachronous gastric cancer and ''missed'' synchronous gastric cancer is difficult and has little meaning from the viewpoint of their risk levels, both of them were classified as M cases. S cases were defined as those who underwent ER and were followed up without a new gastric cancer for at least 1 year (median, 2.4 ± 1.9 years; range, 1.0-7.3 years, respectively). HV were recruited so that their mean age (66.0 years; range, 56-76 years) would match that of M cases (69.5 years; range, 55-77 years) and S cases (69.0 years; range, 55-76 years). Cases with hereditary cancer syndromes (hereditary nonpolyposis colorectal cancer, familial adenomatous polyposis, hereditary diffuse gastric cancers, and Li-Fraumeni syndrome), those with malignancy of other organs, and those who received chemotherapy or radiation therapy were excluded based on clinical history.

By endoscopic biopsy using sterilized biopsy forceps (Boston Scientific, Natick, MA), gastric mucosa were obtained from three standard sites: (*a*) the lesser curvature at 2 to 3 cm from the pyloric ring (antrum), (*b*) the lesser curvature at 2 to 3 cm proximal from incisura angularis (middle body), and (*c*) the lesser curvature at 2 to 3 cm distal to the cardia (upper body). Cases with any endoscopic abnormal appearance at biopsy points, such as ulceration, reddish or discolored areas, deformity, or elevation, were excluded. The samples were snap frozen immediately and stored at −80°C. *H. pylori* infection status was analyzed by culture of two sites, which are known to have a sensitivity of 94% and a specificity of 100% for current infection status (18-20). The endoscopic extent of gastric atrophy was assessed using the atrophic pattern system proposed by Kimura et al. (21), which well correlates with the histologic degree of atrophic gastritis.

CGIs Analyzed. Seven CGIs were selected for methylation analysis from the eight CGI regions previously analyzed (13). These included CGIs of *FLNc, HAND1, THBD, HRASLS, LOX*

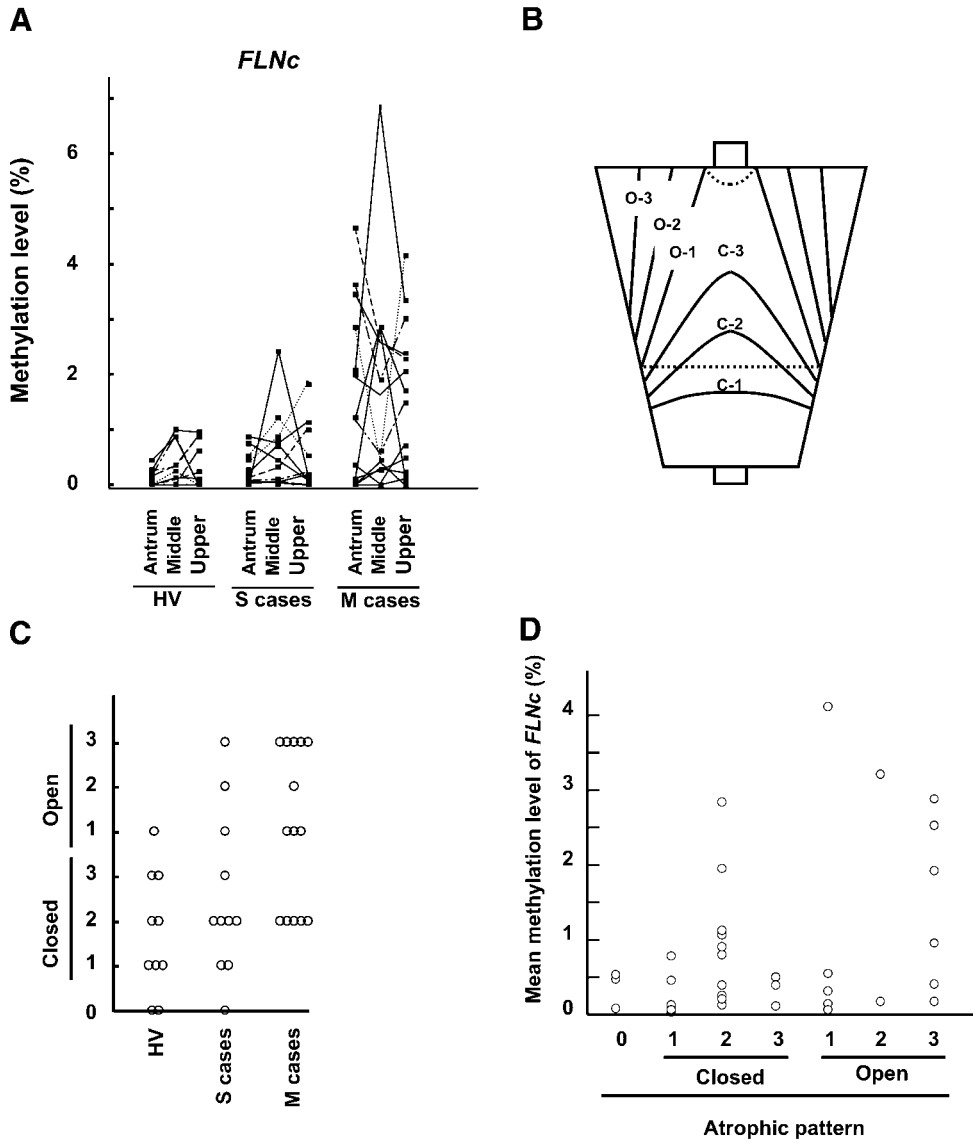

Figure 2. Methylation distribution and effect of atrophy. **A.** methylation levels in the three sites of the same *H. pylori*–negative individuals ($n = 35$). The *FLNc* methylation levels are shown for the three sites. Some individuals had relatively homogeneous methylation levels, but others, especially M cases, had divergent methylation levels. The occasional presence of cases with divergent methylation levels also supported that the mean methylation level best reflects the risk of gastric cancer of a specific individual. **B.** schematic representation of the spread of gastric atrophy through the stomach (21). Each line is a border between an endoscopically atrophic area and nonatrophic area. C and O are closed-type and open-type gastritis, respectively. In C-1, the atrophic area is localized in the antral region, and the area expands in C-2, C-3, O-1, O-2, and O-3. In O-3, almost the entire stomach has atrophy. **C.** correlation between the extents of atrophy and gastric cancer risk levels in *H. pylori*–negative individuals ($r = 0.52$; $P = 0.001$). **D.** correlation between the extent of atrophy and mean *FLNc* methylation level in *H. pylori*–negative individuals. A weak correlation was observed ($r = 0.36$; $P = 0.035$).

Table 1. Correlations between the methylation level and atrophy and between the methylation level and risk levels after adjustment by atrophy

	Extent of gastric atrophy		Gastric cancer risk levels after adjustment by the extent of gastric atrophy	
	Correlation coefficient	P_{trend}	Correlation coefficient*	P_{trend}
FLNc	0.36	0.035	0.37	0.029
HAND1	0.34	0.043	0.32	0.06
THBD	0.18	0.31	0.22	0.21
HRASLS	0.46	0.006	0.04	0.79
LOX	0.08	0.63	0.1	0.57
p41ARC	0.28	0.10	0.22	0.19
p16	0.09	0.61	−0.003	0.99

*Spearman's partial correlation coefficients adjusted by atrophy levels.

(up to here is in promoter regions), *p41ARC* (exon 8), and *p16* (exon 1). The *p16* promoter region was hardly methylated in our previous study and thus was excluded in this study.

Quantitative Methylation-Specific PCR. Methylation levels were quantified by real-time methylation-specific PCR as in our previous report (13). The methylation level of a sample for a CGI was calculated as the fraction of methylated molecules in the total DNA molecules (number of M molecules + number of U molecules). The standard DNA for real-time methylation-specific PCR is available on request.

Statistical Analysis. Methylation levels between S and M cases were compared by Welch's *t* test (two sided). Correlation between methylation levels and risk levels was analyzed by the Spearman's rank-order correlation coefficient (*r*), and the effect of gastric atrophy was adjusted by calculating Spearman's partial correlation coefficients.

Results

Correlation between Methylation Levels and Gastric Cancer Risk Levels in *H. pylori*–Negative Individuals. Methylation levels of the seven CGIs were analyzed for the three biopsy sites of each case. Because increased methylation levels in *H. pylori*–positive individuals are composed of both permanent and temporary components and do not necessarily reflect gastric cancer risk (13), we first focused on correlation between methylation levels and risk levels in *H. pylori*–negative individuals (Fig. 1). Methylation levels of *FLNc* and *HAND1* showed significant increasing trends ($r = 0.47$; $P_{trend} = 0.006$ for *FLNc*; $P_{trend} = 0.44$ and 0.009 for *HAND1*). The *THBD* and *p41ARC* methylation levels also showed increasing tendencies ($r = 0.32$; $P_{trend} = 0.06$ for *THBD*; $P_{trend} = 0.31$ and 0.07 for *p41ARC*). Even between S and M cases, the *FLNc* methylation level was significantly higher in M cases than in S cases ($P = 0.009$, Welch's *t* test). Interestingly, the methylation level of exon 1 of *p16* had no correlation with risk levels of gastric cancer. These data showed that the methylation levels of specific CGIs correlate with the risk levels of gastric cancers.

Comparison of the Three Biopsy Sites and Their Mean Value. Distribution of methylation levels in three biopsy sites was scrutinized using *FLNc* (Fig. 2A), which had the largest correlation coefficient and the smallest trend *P* value with gastric cancer risk levels. Some individuals had relatively similar methylation levels among the three sites, but others, especially M cases, had divergent methylation levels. This suggested that different sites of gastric mucosae have different methylation levels and possibly cancer risks, and gastric cancer risk of an individual was considered to be most accurately estimated by the mean values of these sites. This explained the finding that smaller trend *P* values were obtained using the mean methylation levels than those for individual sites.

Association between the Methylation Level and Endoscopic Extent of Gastric Atrophy and Their Independent Predictive Powers. The extent of gastric atrophy (Fig. 2B) is known to have a correlation with gastric cancer risk (10). Also in this study, the correlation between the extent of gastric atrophy and the gastric cancer risk level was confirmed ($r = 0.52$; $P = 0.001$; Fig. 2C).

The *FLNc* methylation level had a weak correlation with the extent of atrophy ($r = 0.36$; $P = 0.035$; Fig. 2D; Table 1). To examine the independent predictive power of the methylation level for the gastric cancer risk, we calculated Spearman's partial correlation coefficients between the methylation level and gastric cancer risk levels after adjustment by the extent of atrophy. The methylation level retained its correlation with the gastric cancer risk level ($r = 0.37$; $P = 0.029$; Table 1). The *HAND1* methylation level showed a similar tendency. This finding indicated that the methylation level of *FLNc*, and possibly *HAND1*, is a promising risk marker for gastric cancers, independent of gastric atrophy.

Effect of Active *H. pylori* Infection. In *H. pylori*–positive individuals, methylation levels in HV, S cases, and M cases were increased to various degrees, as in our previous study (13). The *FLNc* methylation level in *H. pylori*–positive HV individuals (1.9 ± 0.6 %) was almost comparable with those in *H. pylori*–negative M cases (1.6 ± 0.4). In contrast, *HAND1* (4.6 ± 0.9), *THBD* (21.1 ± 3.7), *HRASLS* (2.3 ± 0.5), *LOX* (13.2 ± 2.3), *p41ARC* (13.5 ± 2.0), and *p16* (3.6 ± 0.8) in *H. pylori*–positive HV individuals had much higher levels than those in *H. pylori*–negative M and S cases. This finding supported that current, or active, *H. pylori* infection potently induces methylation with both temporary and permanent components and that methylation levels in *H. pylori*–positive individuals do not necessarily correlate with gastric cancer risk levels.

Discussion

Significant increasing trends of *FLNc* and *HAND1* methylation levels in noncancerous gastric mucosae were observed in the order of HV, S cases, and M cases in *H. pylori*–negative individuals, those who are without current, or active, *H. pylori* infection. Even between the S and M cases, the *FLNc* methylation level was significantly higher in the M cases. In addition, when adjusted by the extent of gastric atrophy, *FLNc* methylation levels retained their increasing trend in the S and M cases. These findings showed that DNA methylation levels in the gastric mucosae correlate with gastric cancer risk levels and that they are good candidates for a risk marker of gastric cancers. In other words, the so-called ''field defect'' produced by past exposure to carcinogens could be detected and measured using DNA methylation levels as a marker. Now, a prospective study in gastric cancers is warranted, and application of the concept to other cancers seems promising.

Noncancerous gastric mucosae of individuals with active or current *H. pylori* infection, whether they had cancers or not, showed higher methylation levels than those of *H. pylori*–negative S and M cases, most of whom were considered to have had past exposure to *H. pylori* (14). This indicated that high methylation levels induced by *H. pylori* infection would decrease after the *H. pylori* infection discontinues, as in our previous study (13). The most likely mechanism was a decrease due to turnover of gastric epithelial cells with methylation. Methylation in the infiltrating lymphocytes was unlikely because peripheral lymphocytes did not have methylation of the CGI analyzed (data not shown). It is known that cells in a gastric gland are renewed every 3 days by cells supplied from progenitor cells, and progenitor cells themselves are replaced by those supplied from a single stem cell with a much longer cycle (22). If we assume that active *H. pylori* infection continually induces methylation at the level of progenitor cells, their replacement by fresh progenitor cells without methylation after discontinuation of *H. pylori* infection explains the decrease of methylation levels. The methylation level in *H. pylori*–negative individuals is considered to reflect the fraction of stem cells with methylation and to correlate with gastric cancer risk (15).

The seven CGIs analyzed showed different levels of methylation induced by *H. pylori* infection and association with risk levels in the S and M cases. The effect of *H. pylori* was prominent in *THBD, LOX*, and *p41ARC* but not clear in *FLNc*. In contrast, association between methylation levels and risk levels was clear in *FLNc* and *HAND1* followed by *THBD* and *p41ARC*. Especially, the difference between S and M cases was significant only for *FLNc*. This suggested that *H. pylori* infection induces methylation preferentially in some CGIs and that the degree of methylation induction in nonstem cells is also different among CGIs. Low levels of gene transcription are an important factor that promote methylation of promoter CGIs (23), and transcription levels are different among various genes and between stem cells and nonstem cells. Methylation of genes useful as a risk marker, such as *FLNc*, is considered to occur in association with that of genes critical for cancer development but to have a higher rate. If there is a CGI that is methylated preferentially in stem cells and rarely in progenitor cells due to low transcription in stem cells, such a CGI would be useful as a risk marker.

In summary, the higher DNA methylation levels in gastric mucosae correlated with the higher risk levels of gastric cancers, and the DNA methylation levels are a promising risk marker.

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