Examination of Whole Blood DNA Methylation as a Potential Risk Marker for Gastric Cancer

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Abstract

Whole blood DNA methylation analysis has been proposed to be a risk marker for cancer that can be used to target patients for preventive interventions. To test this, we examined whole blood DNA methylation of 16 CpG island promoters and LINE1 repetitive element in patients with gastric cancer and control subjects. Bisulfite pyrosequencing was used to quantify the methylation of 14 CpG island promoters (MINT25, RORA, GDNF, CDH1, RARB2, ER, CDH13, MYOD1, SFRP1, P2RX7, SLC16A12, IG2F, DPYS, and N33) and LINE1 from 72 patients with gastric cancer, 67 control, and 52 healthy young individuals. Quantitative methylation-specific real-time PCR was also conducted for 3 CpG island promoters (MINT25, MYO3A, and SOX11). Among all sites tested, only a marginal increase in the methylation of the SFRP1 promoter was observed in the blood of patients with gastric cancer when compared with the control group (11.3% vs 10.5%; age-adjusted P value: P = 0.009), and this association was also seen in a validation set of 91 patients with gastric cancer (11.5% vs 10.5%; age-adjusted P value: P = 0.001). The methylation of 9 sites (GDNF, CDH1, RARAB2, CDH13, MYOD1, SFRP1, SLC16A12, DPYS, N33, and LINE1) and their mean Z score was correlated with higher age (R = 0.41, P < 0.0001) and marginally with telomere shortening (R = −0.18, P = 0.01) but not with gastric cancer risk (other than SFRP1 methylation). Variability in whole blood DNA methylation of cancer markers is primarily associated with aging, reflecting turnover of white blood cells, and has no direct link to gastric cancer predisposition. SFRP1 methylation in whole blood may be associated with gastric cancer risk. Cancer Prev Res; 6(10); 1093–100. © 2013 AACR.

Introduction

Gastric cancer is a major cause of cancer-related death, worldwide. Helicobacter pylori (H. pylori) plays an important role in gastric carcinogenesis, although the majority of individuals with H. pylori infection do not develop gastric cancer (1). Surveillance of these high-risk patients using reliable and accurate predictive markers is important for reducing the incidence of cancer and its mortality. It is now widely accepted that changes in DNA methylation patterns, particularly promoter hypermethylation and global (genome-wide) hypomethylation, contribute to cancer development and tumor growth (2, 3). Neoplastic growth is frequently preceded by aberrant promoter methylation, which leads to a loss-of-function for the genes that promote cell proliferation (4). Hypomethylation is thought to contribute to carcinogenesis by inducing genomic instability (5, 6), leading to the formation of abnormal chromosomal structures (7, 8). Gene promoter hypermethylation and global hypomethylation in tumor tissues are common events in the development of many types of cancer (4), whereas gene promoter hypermethylation and global hypomethylation are also observed in aged or inflamed tissues and are associated with cancer occurrence in their targeted tissues (9–11).

The DNA methylation status of various tissues has been shown to be associated with aging and perhaps also exposures encountered throughout life (12, 13), and therefore is now increasingly seen as a mechanism of cancer predisposition (14–17). Evaluating whole blood DNA methylation as a risk marker for cancer is of particular interest because peripheral blood DNA is a convenient tissue to assay for constitutional methylation, as its collection is noninvasive. It is possible that the methylation status of cancer target tissues (i.e., neoplastic cells and the surrounding tissue/field defect) might reflect acquired or inherited somatic events that are detectable in nontargeted tissues (methylation memory of exposures/inheritance) and correlate with cancer susceptibility. Thus, epigenetic signatures in whole blood DNA could reflect the interaction of host genetic and environmental factors associated with cancer susceptibility.
In addition, rare cases of constitutional DNA methylation of tumor suppressor genes have been reported and proposed to be predisposing to cancer. To evaluate this concept in gastric cancer, we investigated the methylation status of 16 CpG island promoters selected on the basis of either cancer-associated or age-related methylation, and also studied the LINE1 repetitive element (representative of global methylation) in whole blood DNA in patients with gastric cancer and control subjects. We also investigated the association between DNA methylation status and telomere length shortening, which is an indicator for cell turnover and aging (18, 19).

Materials and Methods

Samples analyzed
For screening, we used DNA from 8 primary gastric cancer tissues and 6 non-neoplastic gastric mucosae from healthy subjects. For testing, we used two different gastric cancer cohorts (training set: n = 72; validation set: n = 91) and 67 cancer-free subjects (controls) who were attending the endoscopy center of Fujita Health University (Toyoake, Aichi, Japan) from January 2005 to March 2010. Five milliliters of whole blood DNA was collected from each participant in an EDTA tube and stored frozen until DNA extraction. Whole blood DNA extraction was carried out using a commercial kit (FlexiGene DNA Kit, Qiagen) and stored until processing for analysis. All patients with gastric cancer were admitted to Fujita Health University hospital for the treatment of gastric cancer. Noncancer patients underwent upper gastroscopy for various reasons, including yearly screening for gastric cancer, a secondary complete check-up after barium radiographic examination due to a suspicion of gastric cancer or peptic ulcer disease, and complaints of abdominal discomfort. They were finally diagnosed as not having gastric cancer. To avoid confounders, we excluded patients with chronic illness from the study. We also collected whole blood DNA from 52 healthy young individuals recruited from Japanese medical students and staff of the Fujita Health University School of Medicine from April 2006 to October 2007. The Ethics Committee of the Fujita Health University School of Medicine approved the protocol and written informed consent was obtained from all subjects.

Selection of candidate panels and CpG methylation analysis by bisulfite pyrosequencing and qMSP
The selection of genes is based on the hypothesis that the methylation status of targeted tissues (i.e., cancer and surrounding tissue) might reflect acquired or inherited somatic events that are detectable in nontargeted tissues (methylation memory of exposures/inheritance) and correlate with cancer susceptibility. We reasoned that there might be two approaches to selecting genes (all selected from the literature), one based on frequency of methylation in cancer (MINT25, RORA, GDNF, RARAB2, SLC16A12, SOX11, and MYO3A) and a separate one based on methylation in normal inflamed/aged tissues (CDH1, ER, CDH13, MYOD1, SFRP1, P2RX7, IGF2, and N33; refs. 9, 10, 20–26). Bisulfite-treated genomic DNA was used to evaluate the methylation status of these CpG island promoters by bisulfite pyrosequencing. We also evaluated the methylation status of the LINE1 repetitive element using bisulfite pyrosequencing. Bisulfite treatment of DNA was carried out with an EpiTect Bisulfite Kit (Qiagen) according to the manufacturer’s protocol. Pyrosequencing was carried out using a PyroGold Reagent Kit (Qiagen) and the results were analyzed using PyroMark Q96 ID software version 1.0 (Qiagen). The primers used for pyrosequencing are listed in Supplementary Table S1. All bisulfite pyrosequencing was conducted at least twice and averaged. To increase sensitivity, for 3 genes (MINT25, SOX11, and MYO3A), we conducted quantitative methylation-specific real-time PCR (qMSP). The cycle threshold (Ct) values for targeted genes were normalized in each reaction using a primer/probe set for the reference gene, mC-LESS, a unique sequence that does not contain cytosines (27). Supplementary Table S2 lists primers and TaqMan probes (Applied Biosystems) for mC-LESS and the 3 genes examined. Each qMSP reaction batch was controlled with one positive (M. SssI methylase–treated DNA) and multiple blanks with no DNA. For each plate, mC-LESS (the internal control) and the tested genes were determined together to avoid inter-assay variation. All qMSP reactions were conducted in triplicate and averaged.

Relative average telomere length measurement
Relative telomere length was measured as a comparative quantification, in particular as abundance of telomeric template versusa single-copy gene (T/S) by quantitative real-time PCR as described previously (28). For the quantitative real-time PCR, the iTaq SYBR Green Supermix (Bio-Rad) and StepOnePlus Real-Time PCR System (Applied Biosystems) were used. The primers for telomeres and single-copy genes (H-globin) are listed in Supplementary Table S3. All measurements were carried out in duplicate and averaged.

Statistical analysis
Methylation levels and telomere length in whole blood DNA between gastric cancer and control subjects were compared using the Student t test. When the P values were less than 0.05, the logistic regression model was used to adjust for age. The correlation between methylation levels, telomere length, and age was assessed using the Spearman correlation analysis. Two-sided P < 0.05 was considered statistically significant.

Results

Study populations
Table 1 describes the age and gender of the gastric cancer, control, and healthy young individuals groups. In the comparison of gastric cancer and control subjects, the age of the gastric cancer subjects was significantly higher than that of the controls (control vs gastric cancer training set: P = 0.01; control vs gastric cancer validation set: P < 0.0001).
Prevalence of male gender was also significantly higher in the gastric cancer validation set when compared with the healthy young individuals ($P = 0.006$).

### Methylation status of candidate genes in primary gastric cancer tissues and non-neoplastic gastric mucosa

Initially, we evaluated the methylation status of 16 CpG island promoters and LINE1 in 8 primary gastric cancer tissues and 6 non-neoplastic gastric mucosae from healthy subjects by bisulfite pyrosequencing. This comparison was carried out because we wished to include in our study genes that are methylated in the normal mucosa of patients with cancer but not in healthy mucosa (potential field defect). We reasoned that there might be two approaches to selecting genes (all selected from the literature), one based on frequency of methylation in cancer (MINT25, RORA, GDNF, RARAB2, SLC16A12, SOX11, and MYO3A) and a separate one based on methylation in normal inflamed/aged tissues (CDH1, ER, CDH13, MYOD1, SFRP1, P2RX7, IGF2, and N33). Considering the heterogeneity in methylation status in individual tumors, most of the genes (except for LINE1), showed higher methylation levels in gastric cancer tissues than in non-neoplastic gastric mucosa, as expected on the basis of the selection criteria. (Supplementary Fig. S1).

### Methylation status of candidate genes in whole blood DNA in patients with gastric cancer and control patients

Figure 1 shows the methylation status of 14 CpG island promoters (MINT25, RORA, GDNF, CDH1, RARAB2, ER, CDH13, MYOD1, SFRP1, P2RX7, SLC16A12, IGF2, DPYS, and N33) and LINE1 in the gastric cancer training set, control, and healthy young individuals groups examined by bisulfite pyrosequencing. We found a marginal increase in the methylation of the SFRP1 promoter in gastric cancer compared with the control group (11.3% vs 10.5%; age-adjusted $P$ value: $P = 0.009$), whereas the methylation of SFRP1 was not significantly different between controls and healthy young individuals (10.5 ± % vs 10.0%, $P = 0.25$). We did not find any association for the remaining 13 CpG island promoters and LINE1 among gastric cancer and control subjects (Fig. 1). We also examined the methylation status of whole blood DNA for 3 genes (MINT25, SOX11, and MYO3A) using qMSP. However, we did not observe a significant difference in the methylation status of these 3 genes between the gastric cancer and control groups (Fig. 2). Because $H. pylori$ infection is strong inducer of gastric mucosa methylation (10), we determined whether $H. pylori$ infection status would influence the methylation status of whole blood DNA. $H. pylori$ status was available in 69 cancer-free subjects (4 young individuals and 65 controls) and 71 patients with gastric cancer. As expected, $H. pylori$ infection was more prevalent in gastric cancer (61/70, 86%) than in cancer-free subjects (29/43, 62.3%, $P = 0.0006$). However, we did not find any significant association between DNA methylation and $H. pylori$ infection status (all $P$ values >0.1, data not shown).

### Methylation status of candidate genes in whole blood DNA in relation to aging

We investigated the association between whole blood DNA methylation and aging using the Spearman correlation analysis. We combined the training set, control, and healthy young individuals groups. One hundred and ninety-one subjects were included for this analysis. We found significant positive correlations between 8 CpG island promoters ($GDNF$, $CDH1$, $RARAB2$, $CDH13$, $MYOD1$, $SFRP1$, $SLC16A12$, $DPYS$, and $N33$) and LINE1 with aging. The methylation status of 2 genes ($DPYS$ and $N33$) showed a relatively strong correlation with higher age ($DPYS$: $R = 0.37$, $P < 0.0001$; $N33$: $R = 0.37$, $P < 0.0001$; Fig. 3A). Also, the $Z$ score of mean methylation for the nine genes was closely correlated with higher age ($R = 0.41$, $P < 0.0001$; Fig. 3B).

### Telomere length in gastric cancer and control subjects and its relationship to aging and gene methylation

We examined the relative telomere length of whole blood DNA with quantitative real-time PCR. Because of several gastric cancer subjects with short telomere length, the gastric cancer group showed shorter mean telomere length than the control group. However, this association was not significantly different (Fig. 4A). We investigated the clinicopathologic features of gastric cancer in 6 patients with gastric cancer with the lowest telomere length (histology, staging, location, etc.), but we did not find any significant associations. We also investigated the association of age and methylation status with telomere length.

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**Table 1. Study populations**

<table>
<thead>
<tr>
<th>Variables</th>
<th>Healthy young individuals</th>
<th>Control without gastric cancer</th>
<th>Gastric cancer training set</th>
<th>Gastric cancer validation set</th>
</tr>
</thead>
<tbody>
<tr>
<td>$N$</td>
<td>52</td>
<td>67</td>
<td>72</td>
<td>91</td>
</tr>
<tr>
<td>Age (mean ± SEM)$^a$</td>
<td>23.2 ± 0.3</td>
<td>57.4 ± 1.5</td>
<td>62.3 ± 1.1</td>
<td>71.3 ± 0.93</td>
</tr>
<tr>
<td>Male$^b$</td>
<td>26 (50%)</td>
<td>41 (61.2%)</td>
<td>46 (63.9%)</td>
<td>67 (73.6%)</td>
</tr>
</tbody>
</table>

$^a$Control without gastric cancer versus gastric cancer training set, $P = 0.01$, Control without gastric cancer versus gastric cancer validation set, $P < 0.0001$.

$^b$Healthy young individuals versus gastric cancer validation set, $P = 0.006$. 

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Blood Methylation in Gastric Cancer
Telomere length was significantly correlated with higher age ($R = -0.26$, $P = 0.0003$; Fig. 4B). We also found significant inverse correlation between shortened telomere length and methylation of 4 CpG island promoters ($DPYS$, $CDH13$, $MYOD1$, and $SLC16A12$; Supplementary Fig. S2). A marginal significant correlation was also found between shortened telomere length and $Z$ score of the mean methylation of nine age-related sites ($GDNF$, $CDH1$, $RARAB2$, $CDH13$, $MYOD1$, $SFRP1$, $SLC16A12$, $DPYS$, $N33$, and $LINE1$; $R = -0.18$, $P = 0.01$; Fig. 4C).

**Methylation status of the SFRP1 gene in the gastric cancer validation set**

The $SFRP1$ promoter displayed a significant increase in methylation in the gastric cancer training set when compared with the control group. To confirm these data, we

**Figure 1.** Methylation status of 14 promoter CpG islands and $LINE1$ in healthy young individuals (Young), cancer-free subjects (Control), and gastric cancer training set (gastric cancer) groups examined by bisulfite pyrosequencing. Horizontal bars represent mean methylation values. Methylation levels in whole blood DNA between gastric cancer and CONTROL were compared using the Student $t$ test and the logistic regression model was used to adjust for age when the $P$ values were less than 0.05.

**Figure 2.** Methylation status of 3 promoter CpG islands in healthy young individuals (Young), cancer-free subjects (Control), and gastric cancer training set (gastric cancer) groups examined by qMSP. Horizontal bars represent mean methylation values. The relative level of methylated DNA is depicted as 40-dCt ($C_t$ of specific gene$–C_t$ of mC-LESS (internal control)). A higher 40-dCt represents more methylation of the target biomarker. Methylation levels in whole blood DNA between gastric cancer and CONTROL were compared using the Student $t$ test.
evaluated its methylation status in a gastric cancer validation set (n = 91) and found a significant though marginal increase of SFRP1 methylation in the gastric cancer validation set in comparison with the control group (11.5% vs 10.5%; age adjusted P value: P = 0.001; Fig. 5). We also investigated whether higher methylation of the SFRP1 gene...
is associated with clinicopathologic characteristics of gastric cancer using the combined training and validation sets ($n = 158$); however, no significant association was found between SFRP1 methylation and any clinicopathologic features of gastric cancer (Table 2).

**Discussion**

In this study, we tested whether methylation analysis of selected loci in whole blood DNA could be useful as a biomarker of risk in gastric cancer. Among all the sites we tested, only a marginal increase in the methylation of the SFRP1 promoter was observed in both training and validation sets. The SFRP1 gene is located at 8p11.21 and its frequent methylation has been documented in colorectal cancer tissues as well as in aged non-neoplastic colon mucosae (25). Our results also suggest that the SFRP1 promoter is one of the regions where methylation in blood could reflect gastric cancer predisposition, but the differences between cases and controls are small and the data should be verified in other cohorts. Overall, our negative results stand in contrast to other studies. In recent studies on gastric cancer, the methylation levels of the IGF2 differentially methylated region 0 (DMR0) and the Alu and LINE1 repetitive elements tended to be lower in blood from patients with gastric cancer than in control groups, but the association in overall subjects was not significant ($P > 0.05$ for all; refs. 29, 30). Another study explored the association between methylation in prediagnostic blood leukocyte DNA and gastric cancer risk in the prospective cohort (31). The result showed Alu methylation was inversely associated with gastric cancer risk, mainly among cases diagnosed one or more years after. However, in overall subjects, methylation was not significantly different among the cases and controls (31). This issue has also been looked at in other tumors such as bladder cancer and others. Although several studies reported on promising results, the differences tended to be small and few of these have been validated by others. For example, white blood cells (WBC) DNA methylation of the LRE1 sequence and ATM intragenic loci (ATMmvp2a) were significantly associated with the risk of head and neck and breast cancers (15, 17). However, the difference in methylation levels for cases and controls in both studies was not large (0.753 vs 0.747 for breast cancer; refs. 15, 17). Wong and colleagues reported that methylation of the BRCA1 promoter in blood DNA was more frequent in early-onset breast cancer patients and correlated with higher BRCA1 methylation in tumors and BRCA1 mutation associated with pathologic features (16). It seems unlikely that any of the markers analyzed here fit this constitutional methylation paradigm. Taken together, these results suggest that methylation in whole blood might reflect cancer predisposition. However, these changes are relatively small, with a large overlap between cases and controls.
controls and the potential usefulness of blood DNA methylation as a screening/diagnostic biomarker for cancer may therefore be limited.

Our study had limitations worth discussing. The selection of genes was based on the hypothesis that the methylation status of targeted tissues (i.e., cancer and surrounding tissue) might be reflected in nontargeted tissues and correlate to cancer susceptibility. Therefore, we selected genes that have been reported to be hypermethylated in several cancers, including gastric cancer as well as in inflamed or aged tissues (9, 10, 20–26). It is possible that the methylation of other genes might serve as better markers of risk and be due to the tissue specificity of the link between aberrant methylation and disease, which may therefore not be detectable in whole blood. Alternatively, a link between methylation and disease is more likely if aberrant methylation is rate limiting. For cancer, it is possible that the rate-limiting step is acquisition of a mutation in a hypermethylated “field” (37, 38), a property that cannot be measured by DNA methylation alone. Although aging is the dominant factor in accounting for white blood cell methylation CpG island methylation, only a small fraction of the variation in blood remain unclear.

Table 2. SFRP1 methylation status and clinicopathologic subtypes of gastric cancer

<table>
<thead>
<tr>
<th>Variables (n)</th>
<th>SFRP1 methylation (mean ± SEM%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td></td>
</tr>
<tr>
<td>Male (110)</td>
<td>11.4 ± 0.26</td>
</tr>
<tr>
<td>Female (48)</td>
<td>11.6 ± 0.31</td>
</tr>
<tr>
<td><em>H. pylori</em> status</td>
<td></td>
</tr>
<tr>
<td>Negative (38)</td>
<td>11.2 ± 0.46</td>
</tr>
<tr>
<td>Positive (120)</td>
<td>11.5 ± 0.23</td>
</tr>
<tr>
<td>Histology</td>
<td></td>
</tr>
<tr>
<td>Intestinal (110)</td>
<td>11.5 ± 0.26</td>
</tr>
<tr>
<td>Diffuse (48)</td>
<td>11.3 ± 0.31</td>
</tr>
<tr>
<td>Location</td>
<td></td>
</tr>
<tr>
<td>Antrum (49)</td>
<td>11.9 ± 0.43</td>
</tr>
<tr>
<td>Body (81)</td>
<td>11.2 ± 0.25</td>
</tr>
<tr>
<td>Cardia (28)</td>
<td>11.4 ± 0.48</td>
</tr>
<tr>
<td>Staging</td>
<td></td>
</tr>
<tr>
<td>Early (82)</td>
<td>11.8 ± 0.33</td>
</tr>
<tr>
<td>Advanced (76)</td>
<td>11.2 ± 0.23</td>
</tr>
<tr>
<td>Morphology</td>
<td></td>
</tr>
<tr>
<td>Elevated or protruding (40)</td>
<td>11.8 ± 0.44</td>
</tr>
<tr>
<td>Depressed or with ulceration (105)</td>
<td>11.3 ± 0.25</td>
</tr>
<tr>
<td>Scirrhous (13)</td>
<td>11.1 ± 0.55</td>
</tr>
</tbody>
</table>

NOTE: Methylation status was not determined for 5 cases.

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Ehrlich M. DNA hypomethylation, cancer, the immunode

References

8. Ehrlich M. DNA hypomethylation, cancer, the immunodeficiency, cromeroic region instability, facial abnormalities and chromo-
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