Title: Examination of whole blood DNA methylation as a potential risk marker for gastric cancer

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Running title: Blood methylation in gastric cancer

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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed

Key words: DNA methylation, whole blood DNA, gastric cancer, aging, telomere length
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 gastric cancer (GC) patients and control subjects. Bisulfite pyrosequencing was used to quantify the methylation of 14 CpG island promoters (MINT25, RORA, GDNF, CDH1, RARAB2, ER, CDH13, MYOD1, SFRP1, P2RX7, SLC16A12, IGF2, DPYS, and N33) and LINE1 from 72 GC, 67 control, and 52 healthy young individuals. Quantitative methylation-specific real-time PCR (qMSP) was also performed 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 GC patients when compared to 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 GC patients (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 GC predisposition. SFRP1 methylation in whole blood may be associated with gastric cancer risk.
Introduction

Gastric cancer (GC) 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) while 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 non-targeted 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 GC, we investigated the methylation status of 16 CpG island promoters selected based on either cancer-associated or age-related methylation, and also studied the LINE1 repetitive element (representative of global methylation) in whole blood DNA in gastric cancer (GC) patients 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 GC tissues and 6 non-neoplastic gastric mucosae from healthy subjects. For testing, we use two different GC 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 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 performed using a commercial kit (FlexiGene DNA Kit, QIAGEN, Hilden, Germany) and stored until processing for analysis. All GC patients were admitted to Fujita Health University hospital for the treatment of GC. Non-cancer patients underwent upper gastroscopy for various reasons, including yearly screening for GC, a secondary complete check-up after barium radiographic examination due to a suspicion of GC or peptic ulcer disease, and complaints of abdominal discomfort. They were finally diagnosed as not having GC. 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 non-targeted 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). (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 performed with an EpiTect bisulfite kit (Qiagen) according to the manufacturer’s protocol. Pyrosequencing was carried out using a PSQ96 system with a Pyro-Gold 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 1. All bisulfite pyrosequencing was conducted at least twice and averaged. To increase sensitivity, for 3 genes (MINT25, SOX11, and MYO3A), we performed quantitative methylation-specific real-time PCR (qMSP). The cycle threshold (Ct) values for targeted genes were normalized in each reaction by using a primer/probe set for the reference gene, mC-LESS, a unique sequence that does not contain cytosines. (27) Supplementary Table 2 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 done 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 vs. a 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 3. All measurements were performed in duplicate and averaged.

**Statistical analysis**

Methylation levels and telomere length in whole blood DNA between GC and control subjects were compared using the Student's 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 $p$ value $<$ 0.05 was considered statistically significant.
Results

Study populations

Table 1 describes the age and gender of the GC, control, and healthy young individuals groups. In the comparison of GC and control subjects, the age of the GC subjects was significantly higher than that of the controls (control vs. GC training set: \( p = 0.01 \); control vs. GC validation set: \( p < 0.0001 \)). Prevalence of male gender was also significantly higher in the GC validation set when compared to the healthy young individuals (\( p = 0.006 \)).

Methylation status of candidate genes in primary GC tissues and non-neoplastic gastric mucosae

Initially, we evaluated the methylation status of 16 CpG island promoters and \( LINE1 \) in 8 primary GC tissues and 6 non-neoplastic gastric mucosae from healthy subjects by bisulfite pyrosequencing. This comparison was done 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 based on the selection criteria. (Supplementary Fig. 1).

Methylation status of candidate genes in whole blood DNA in GC 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 GC 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 GC compared to the control group (11.3% vs. 10.5%; age-adjusted p value: p=0.009), while 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 GC 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 GC and control groups (Fig. 2). Since H. pylori infection is a 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 GC patients. As expected, H. pylori infection was more prevalent in GC (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. 191 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,
Telomere length in GC 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. Due to several GC subjects with short telomere length, the GC group showed shorter mean telomere length than the control group. However, this association was not significantly different (Fig. 4a). We investigated the clinicopathological features of GC in 6 GC patients 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. Shortened 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. 2). A marginal significant correlation was also found between shortened telomere length and Z score of the mean methylation of 9 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 GC validation set

The SFRP1 promoter displayed a significant increase in methylation in the GC training set when compared to the control group. To confirm these data, we evaluated its methylation status in a GC validation set (n=91), and found a significant though marginal increase of SFRP1 methylation in the GC validation set in comparison to 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 clinicopathological characteristics of GC using the combined
training and validation sets (n=158); however, no significant association was found between SFRP1 methylation and any clinicopathological features of GC (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 CRC tissues as well as in aged non-neoplastic colon mucosae. Our results also suggest that the SFRP1 promoter is one of the regions where methylation in blood could reflect GC 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 GC, 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 GC patients than in control groups, but the association in overall subjects was not significant ($p>0.05$ for all). Another study explored the association between methylation in pre-diagnostic blood leukocyte DNA and GC risk in the prospective cohort. The result demonstrated Alu methylation was inversely associated with GC risk, mainly among cases diagnosed one or more years after. However, in overall subjects, methylation was not significantly different among the cases and controls. This issue has also been looked at in other tumors such as bladder cancer and others. While several studies reported on promising results, the differences tended to be small and few of these have been validated by others. For example, 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. However, the difference in methylation levels for cases and controls in both studies was not large ($0.753$ vs. $0.747$ for LRE1 in the head and neck cancers; $76.8\%$ vs. $76.4\%$ and $76.9\%$ vs. $81.8\%$ for 2 independent breast cancer cohorts). There have also been several studies showing the possible role of constitutional methylation in blood DNA in cancer.
predisposition. 

Wong et al. 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 pathological features. 

(16) It appears 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 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 non-targeted tissues and correlate to cancer susceptibility. Therefore, we selected genes that have been reported to be hypermethylated in several cancers, including GC 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 this can be addressed in future studies using genome wide methylation analysis technologies. 

(32, 33) Our data suggest that true differences might be small however, and these studies will need careful attention to sample size, validation and quantitation to avoid the possibilities of false positive findings. Another issue is technical – pyrosequencing is quantitative but has a background of ~5% methylation and is therefore not adequate to detect methylation below that range. For 3 selected genes (MINT25, SOX11, and MYO3A), we examined the methylation status of whole blood DNA by qMSP. Since qMSP targets only methylated molecules relative to a reference gene, it would potentially be more sensitive to detecting methylated molecules, even those derived from circulating tumor DNA. All 3 genes showed higher methylation in GC tissues relative to healthy gastric mucosa (Supplementary Fig. 1). However, we did not find any difference in the methylation status of whole blood between GC and control subjects. It is also worth noting that whole blood DNA may
include circulating DNA derived from tumor cells; it is likely to be in small amounts (<1%)(34, 35), but it may be a confounder in evaluating the significance of marginally positive results (such as for SFRP1). Finally, it is important to note that a formal study of a risk marker requires follow-up of individuals positive for that marker rather than a case-control study (as done here), and this is most relevant to positive results.

Our study confirms a firm association between aging and promoter CpG island methylation; 9 sites (GDNF, CDH1, RARAB2, CDH13, MYOD1, SFRP1, SLC16A12, DPYS, N33, and LINE1) showed a significant increase in methylation throughout the aging process. The Z score of mean methylation for these 9 sites had a relatively good correlation with aging (R=0.41, p<0.0001). Moreover, methylation of the 9 sites was inversely associated with telomere length. Telomere length shortening has been observed in aged blood DNA and inflamed tissues and is considered to be associated with cell turnover.(18, 19) Therefore, our results indicate that DNA methylation in the blood increases at many sites throughout the lifespan, and this methylation increase is partly associated with the turnover of white blood cells. On the other hand, we did not find any difference in the mean methylation levels of these 9 age-related sites between GC and control groups (data not shown). Several other studies have examined age-related methylation in whole blood and found a number of sites that are hypermethylated with age(13,36), while very few were associated with age-related phenotypes.(31) This lack of association between the severity of age-related changes in DNA methylation and disease occurrence may 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. While aging is the dominant factor in accounting for white blood cell methylation CpG island methylation, only a small
fraction of the variation can be explained by age (R=0.41). We have previously reported that chronic inflammation and folate intake are associated with methylation change\((11, 39)\), but this information was not available for the current patients. *H. pylori* was previously associated with increased gastric mucosa methylation\((11)\), but this does not appear to extend to WBCs. Thus, many factors that contribute to methylation variation in blood remain unclear.
References


Table 1 Study populations

<table>
<thead>
<tr>
<th>Variables</th>
<th>Healthy young individuals</th>
<th>Control without GC</th>
<th>GC training set</th>
<th>GC 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)$^1$</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$^2$</td>
<td>26 (50%)</td>
<td>41 (61.2%)</td>
<td>46 (63.9%)</td>
<td>67 (73.6%)</td>
</tr>
</tbody>
</table>

$^1$: Control without GC vs. GC training set, p=0.01, Control without GC vs. GC validation set, p<0.0001.

$^2$: Healthy young individuals vs. GC validation set, p=0.006.
<table>
<thead>
<tr>
<th>Variables (n)</th>
<th>SFRP1 methylation (mean+/-SEM%)</th>
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</thead>
<tbody>
<tr>
<td><strong>Gender</strong></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>
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<tr>
<td><strong>H. pylori status</strong></td>
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</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><strong>Histology</strong></td>
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<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><strong>Location</strong></td>
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</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>
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<tr>
<td><strong>Staging</strong></td>
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<tr>
<td>Early (82)</td>
<td>11.8+/-0.33</td>
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<tr>
<td>Advanced (76)</td>
<td>11.2+/-0.23</td>
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<tr>
<td><strong>Morphology</strong></td>
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<tr>
<td>Elevated or protruding (40)</td>
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<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.
Figure legends

Fig. 1 Methylation status of 14 promoter CpG islands and LINE1 in healthy young individuals (YOUNG), cancer-free subjects (CONTROL), and GC training set (GC) groups examined by bisulfite-pyrosequencing. Horizontal bars represent mean methylation values. Methylation levels in whole blood DNA between GC and CONTROL were compared using the Student's t-Test and the Logistic Regression model was used to adjust for age when the p values were less than 0.05.

Fig. 2 Methylation status of 3 promoter CpG islands in healthy young individuals (YOUNG), cancer-free subjects (CONTROL), and GC training set (GC) groups examined by qMSP. Horizontal bars represent mean methylation values. The relative level of methylated DNA is depicted as 40-dCt [Ct of specific gene—Ct of mC-LESS (internal control)]. A higher 40-dCt represents more methylation of the target biomarker. Methylation levels in whole blood DNA between GC and CONTROL were compared using the Student's t-Test.

Fig. 3 Age-related methylation. (a) Methylation status of 8 promoter CpG islands and LINE1 in relation to aging. The healthy young individuals, control, and GC training set groups were all included in this analysis. Statistical analyses were performed using the Spearman correlation analysis. (b) Mean Z score of the methylation levels of 9 age-related loci in relation and aging. The healthy young individuals (Young subjects), control, and GC training set groups (control and GC patients) were all included in this analysis. Statistical analyses were performed using the Spearman correlation analysis. Left is a scatter plot of all the data by age, while right is a bar graph of all the patients divided by study group.

Fig. 4 Relative telomere length examined by quantitative real-time PCR. (a) Telomere length in healthy young individuals (YOUNG), cancer-free subjects (CONTROL), and GC training set (GC) groups. Horizontal bars represent mean telomere length. (b) Telomere length, in relation to
age. (c) Telomere length in relation to the mean Z score of the methylation levels of 9 age-related loci. Statistical analyses were performed using the Student's t-Test (a) and Spearman correlation analysis (b,c).

**Fig. 5** Methylation status of *SFRP1* in cancer-free subjects (CONTROL) and the GC validation set. Horizontal bars represent mean methylation values. Methylation levels in whole blood DNA between GC and CONTROL were compared using the Student's t-Test and the Logistic Regression model was used to adjust for age.
Figure 1
Figure 2

- **MINT25 qMSP**
- **SOX11 qMSP**
- **MYO3A qMSP**

The graphs show methylation levels for different genes under various conditions (Young, Control, GC).
Figure 3
Figure 4

(a) 

(b) 

(c) 

Relative telomere length

R = -0.26, p = 0.0003

Age (y)

Mean Z score of methylation of age related genes

R = -0.18, p = 0.01
Figure 5

\[ p = 0.001 \]
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