Methylation Status of Blood Leukocyte DNA and Risk of Gastric Cancer in a High-Risk Chinese Population

Yang Zhang¹, Hui-juan Su¹, Kai-feng Pan¹, Lian Zhang¹, Jun-ling Ma¹, Lin Shen², Ji-you Li³, Wei-dong Liu⁴, Isao Oze⁵, Keitaro Matsuo⁵, Yasuhito Yuasa⁶, and Wei-cheng You¹

Abstract

**Background:** To evaluate the relationship between methylation status of blood leukocyte DNA and risk of gastric cancer, a population-based study was conducted in Linqu County.

**Methods:** Methylation levels of IGFII and N33 were determined by quantitative methylation-specific PCR. The temporal trend of methylation levels during gastric cancer development was investigated in 133 gastric cancer cases from two cohorts with pre– and/or post–gastric cancer samples. As the references of pre-GCs, 204 intestinal metaplasia (IM) or dysplasia (DYS) subjects who did not progress to gastric cancer during the follow-up period were selected. Meanwhile, 285 subjects with superficial gastritis/chronic atrophic gastritis (SG/CAG) were also selected as controls.

**Results:** IGFII median methylation level was significantly higher in gastric cancer cases than those with SG/CAG (61.47% vs. 49.73%; \( P < 0.001 \)). IGFII and N33 methylation levels were elevated at least 5 years ahead of clinical gastric cancer diagnosis comparing with SG/CAG (63.38% vs. 49.73% for IGFII, 9.12% vs. 5.70% for N33; all \( P < 0.001 \)). Furthermore, the frequency of hypermethylated IGFII was markedly increased in IM or DYS subjects who progressed to gastric cancer in contrast to those who remained with IM and DYS, and adjusted ORs were 12.52 [95% confidence interval (CI), 3.81–41.15] for IM and 10.12 (95% CI, 2.68–38.22) for DYS. Similar results were also found for N33 in subjects with IM (OR, 3.77; 95% CI, 1.20–11.86).

**Conclusions:** Our findings suggested that hypermethylated IGFII and N33 in blood leukocyte DNA were associated with risk of gastric cancer in a Chinese population.

**Impact:** IGFII and N33 methylation status may be related to gastric carcinogenesis.

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level of IGFII in blood leukocyte DNA may serve as a surrogate marker for the risk and prognosis of gastric cancer (17).

Tumor suppressor candidate 3 (TUSC3), also called N33, was identified from an area of frequent allelic losses in prostate cancer (18). As a putative tumor suppressor gene, hypermethylated N33 was found in colorectal cancer, and was age-related in normal colorectal mucosa (19). The presence of Helicobacter pylori (H. pylori), an important risk factor of gastric cancer, showed a significant association with a higher methylation level of N33 in blood leukocyte DNA (17).

Most of the previous conclusions on the association between methylation biomarkers and risk of gastric cancer were based on case–control studies. To explore the dynamical changes of methylation level in the process of gastric cancer development, a cohort study is highly desirable. Since 1989, we conducted a series of epidemiologic studies in Linqu County, a rural area in northeast of China with one of the highest gastric cancer mortality rates in the world (20–22). In these cohorts, blood samples collected from gastric cancer cases before and/or after the clinical diagnosis provided a unique opportunity to assess the potential DNA methylation biomarkers for gastric cancer risk.

In this study, we tested the hypothesis that the methylation levels of IGFII and N33 in blood leukocyte DNA may be associated with the risk of gastric cancer and further evaluated the dynamic changes of methylation level during gastric cancer development.

Materials and Methods

Study population and gastric pathology

Two cohort studies were conducted in Linqu County in 1989 (N = 3,433) and 2002 (N = 2,638), respectively (20, 22), and a total of 186 gastric cancer cases were identified until 2009. Endoscopic screening was performed at baseline of each cohort and followed by a repeated endoscopic examination using the same procedures in 1999, 2003, and 2009, respectively. For each subject, the biopsy specimens were taken from 5 to 7 standard sites of the stomach, and given its corresponding histopathologic diagnosis by three senior pathologists independently from Peking University Cancer Hospital according to the Updated Sydney System (23) and Padova International Classification (24). The detailed information of the study population, endoscopic procedures, and criteria of gastric pathology had been described elsewhere (25). Each biopsy was classified according to the presence or absence of superficial gastritis (SG), chronic atrophic gastritis (CAG), intestinal metaplasia (IM), dysplasia (DYS), or gastric cancer. Each biopsy was given a diagnosis based on the most severe histology, and each subject was assigned a “global” diagnosis based upon the most severe diagnosis among any of the biopsies.

For the current study, a total of 133 gastric cancer cases from two cohorts were enrolled. According to the time of diagnosis, blood leukocyte samples collected from gastric cancer cases were defined into pre-GCs (before gastric cancer diagnosis ranging from 1 to 10 years) and post-GCs (the year of gastric cancer diagnosis or up to 10 years after). Among them, 43 pre–gastric cancer blood leukocyte samples from 38 gastric cancer cases (5 cases with two pre–gastric cancer samples with different time interval) and 64 post–gastric cancer samples from 64 gastric cancer cases were collected. In addition, 31 cases had both pre–gastric cancer and post–gastric cancer samples were also selected as self-control to measure the methylation levels in the two time intervals (Fig. 1).

To test the methylation levels of IGFII and N33 with the risk of gastric cancer, we selected 285 SG or mild CAG subjects with a ratio of 1:3 at random from the baseline of the two cohorts as the control. We further selected 99 IM and 105 DYS subjects who did not progress to gastric cancer during the follow-up period randomly from baseline as controls, because the corresponding gastric lesions for the pre–gastric cancer diagnosis were mainly IM (n = 33) and DYS (n = 35).

All the blood leukocyte samples were collected before the endoscopic process. Information on gender, date of birth, cigarette smoking, and alcohol drinking was obtained from the questionnaires at the baseline of the two cohorts. Age was calculated according to the year when blood leukocyte sample was collected. Because of repeated endoscopic examinations, more than one blood leukocyte sample might be collected from the same person; consequently, different ages were calculated correspondingly in data analysis.

DNA preparation and methylation measurement

Peripheral blood samples were collected with K2 EDTA tubes (BD Vacutainer) and centrifuged at 3,000 rpm for 10 minutes for separation from plasma. The leukocyte fractions were washed by Tris-EDTA for 3 times and high molecular weight genomic DNA was isolated by standard proteinase K digestion and phenol-chloroform extraction, and then modified with sodium bisulfite to convert the unmethylated cytosines to uridines. Bisulfite-treated DNA was then purified with a genomic DNA purification kit (Promega). Quantitative methylation-specific PCR was carried out for IGFII and N33 using a 7500 fast Real-Time PCR System (Applied Biosystems) with the primers and probes as described previously (17). The PCR amplification was performed with a final reaction mixture of 20 μL consisting of 1 × TaqMan Fast Universal PCR Master Mix (Applied Biosystems), 0.9 μmol/L each of forward and reverse primers, 0.25 μmol/L each of probes, and 20 ng of DNA at the following conditions: 95°C for 20 seconds, followed by 40 cycles at 95°C for 3 seconds, and 61°C for 1 minute for IGFII (60°C for 30 seconds for N33). The efficiencies of PCR amplification of all the genes were confirmed to be nearly 100%, and β-actin (ACTB) was used as a reference set to normalize for input DNA.

The methylation levels of IGFII and N33 were expressed as percentage, calculated by dividing the IGFII (or
N33)/ACTB ratio of a sample by the IGFII (or N33)/ACTB ratio of MNK74. The DNA of MNK74 cell line was kindly provided by Prof. Dajun Deng at Peking University Cancer Hospital and Institute (Beijing, China; ref. 26), and was confirmed to be 100% methylated in the CpGs in IGFII and N33 probes (17). The analysis was performed blind by one technician, and various lesion groups were randomly mixed for bisulfite treatment and real-time PCR. As a quality control, about 40 blood leukocyte DNAs were selected randomly for duplicate detection of IGFII and N33 methylation levels, respectively.

**H. pylori antibody assays**

H. pylori antibody assays were used for determination of H. pylori infection status with the serum separated from blood samples collected. Details of serologic assay were described previously (25). In brief, serum levels of anti-\textit{H. pylori} IgG and IgA were measured separately in duplicate with ELISA procedures. Quality control samples were assayed at Vanderbilt University (Nashville, TN). An individual was determined to be positive for \textit{H. pylori} infection if the mean optical density for either the IgG or the IgA was >1.0.

**Statistical analysis**

According to the median age of total participants, subjects were classified into two categories: <60 and ≥60 years old. The Pearson $\chi^2$ test was used to examine the differences between the SG/CAG and post–gastric cancer groups in age, gender, smoking, drinking, and \textit{H. pylori} infection status. Mann–Whitney test was used to examine the differences for the methylation levels between the SG/CAG and post–gastric cancer groups. The differences among multiple groups were estimated by Kruskal–Wallis Test for temporal trends of methylation levels. ORs with corresponding 95% confidence intervals (CI) for associations of methylation status in blood leukocyte DNA with potential risk factors or the risk of gastric cancer were computed by unconditional logistic regression, adjusting for age, gender, smoking, drinking, \textit{H. pylori} infection, or time interval between the dates of blood collection and gastric cancer diagnosis or last follow-up. The $P$ values for the differences of methylation levels between the pre– and post–gastric cancer groups were calculated by unconditional logistic regression, adjusting for age, gender, smoking, drinking, and \textit{H. pylori} infection. To compare the methylation levels in the 31 gastric cancer cases with both pre- and postdiagnosis blood leukocyte samples, conditional logistic regression was applied adjusting for age. All statistical analyses were carried out using Statistical Analysis System software (version 9.0; SAS Institute).

**Results**

**Selected characteristics of the subjects in SG/CAG and post–gastric cancer groups**

The distributions of age, gender, cigarette smoking, alcohol consumption, and \textit{H. pylori} infection status in the SG/CAG and post–gastric cancer groups were presented in Table 1. Compared with SG/CAG, the gastric cancer
A group showed a higher frequency of *H. pylori* infection \( (P = 0.05) \), and two years older in median age \( (P = 0.05) \). There were no significant differences in other characteristics between SG/CAG and post–gastric cancer groups.

### Comparison of methylation levels between gastric cancer and SG/CAG groups

We first compared the methylation levels between the SG/CAG and gastric cancer groups, and found the median methylation level of *IGFII* was 49.73% in SG/CAG, but significantly increased to 61.47% in the gastric cancer group \( (P < 0.001) \). For N33, compared with SG/CAG, no significant difference was observed in the gastric cancer group \( (5.70\% \text{ vs. } 5.74\%; \ P = 0.991) \). To further evaluate the relationship between methylation status and risk of gastric cancer, we set 50% and 5% as the cutoff values for *IGFII* and N33 according to the median levels in SG/CAG, respectively. With SG/CAG as reference, unconditional logistic regression analysis adjusting for age, gender, *H. pylori* infection, smoking, and drinking revealed a close association between hypermethylation \( (\geq 50\%) \) *IGFII* and gastric cancer risk \( \text{OR, 2.46; 95\% CI, 1.46–4.13} \), whereas no significant association was found between hypermethylation status \( (\geq 5\%) \) and gastric cancer risk for N33 \( \text{OR, 0.92; 95\% CI, 0.55–1.54; } P = 0.751 \).

### Temporal trends of methylation levels in gastric cancer development

We further evaluated the dynamic changes of methylation levels by using pre–gastric cancer and post–gastric cancer samples (Table 2). Compared with SG/CAG, the median methylation levels of *IGFII* and N33 were both higher in pre–gastric cancer blood leukocyte samples \( (66.22\% \text{ vs. } 49.73\%; \ P < 0.01 \text{ for } *IGFII*; 9.23\% \text{ vs. } 5.70\%; \ P < 0.01 \text{ for N33}) \). In the post–gastric cancer samples, the median methylation levels remained high for *IGFII*, but decreased significantly for N33 \( (P = 0.001) \). Similar results were obtained in 31 gastric cancer cases with both pre– and post–gastric cancer samples (Table 2).

The temporal trends of methylation levels were further explored by dividing the pre– and post–gastric cancer samples into five groups according to the time interval between sample collection and gastric cancer diagnosis. As shown in Table 3, the median methylation level of *IGFII* was significantly increased at least 5 years ahead of clinical gastric cancer diagnosis compared with SG/CAG \( (63.38\% \text{ vs. } 49.73\%; \ P < 0.001) \). In 6– to 10-year pre–gastric cancer samples, *IGFII* methylation was also found to be increased, but the case number was only 25. For N33, the median methylation level also increased significantly at least 5 years ahead of diagnosis with SG/CAG as reference \( (9.12\% \text{ vs. } 5.70\%; \ P < 0.001) \), while significantly decreased in the year of diagnosis or 5 years after the diagnosis compared with the hypermethylated status before the gastric cancer diagnosis \( (P = 0.004) \). A similar temporal trend was found by using 31 gastric cancer cases with both pre– and post–gastric cancer samples. *IGFII* persisted in hypermethylation status from 6 to 10 years pre–gastric cancer \( (66.70\%, \ n = 7) \), 5 years pre–gastric cancer \( (61.39\%, \ n = 24) \), and post–gastric cancer \( (68.09\%, \ n = 21) \) to 1 to 5 years post–gastric cancer \( (72.25\%, \ n = 10) \). For N33, median methylation level also decreased to 4.15% or 5.75% in the year of diagnosis or 5 years after diagnosis compared with 6 to 10 \( (9.29\%) \) or 1 to 5 \( (9.28\%) \) years post–gastric cancer \( (P = 0.003) \).

### Methylation levels of *IGFII* and N33 in IM or DYS subjects with different outcomes

Because the corresponding gastric lesions for the pre–gastric cancer diagnosis were mainly IM and DYS, we were very interested to compare the methylation levels in

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**Table 1.** Selected characteristics of the study participants in the SG/CAG and post–gastric cancer groups

<table>
<thead>
<tr>
<th>SG/CAG ( \text{( n = 285 )} )</th>
<th>Post–gastric cancer ( \text{( n = 95 )} )</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age, y</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;60</td>
<td>139 (48.8)</td>
<td>40 (42.1)</td>
</tr>
<tr>
<td>≥60</td>
<td>146 (51.2)</td>
<td>55 (57.9)</td>
</tr>
<tr>
<td><strong>Gender</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>215 (75.4)</td>
<td>69 (72.6)</td>
</tr>
<tr>
<td>Female</td>
<td>70 (24.6)</td>
<td>26 (27.4)</td>
</tr>
<tr>
<td><strong>Smoking</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>177 (62.1)</td>
<td>57 (60.0)</td>
</tr>
<tr>
<td>No</td>
<td>108 (37.9)</td>
<td>37 (38.9)</td>
</tr>
<tr>
<td>Unknown</td>
<td>1 (1.1)</td>
<td>1 (1.1)</td>
</tr>
<tr>
<td><strong>Drinking</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>160 (56.1)</td>
<td>48 (50.5)</td>
</tr>
<tr>
<td>No</td>
<td>122 (42.8)</td>
<td>40 (42.1)</td>
</tr>
<tr>
<td>Unknown</td>
<td>3 (1.1)</td>
<td>7 (7.4)</td>
</tr>
<tr>
<td><strong>H. pylori</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(+)</td>
<td>147 (51.6)</td>
<td>60 (63.2)</td>
</tr>
<tr>
<td>(–)</td>
<td>138 (48.4)</td>
<td>35 (36.8)</td>
</tr>
</tbody>
</table>

\( ^a \) \( \chi^2 \) test.

\( ^b \) The cutoff value of age was set as 60 years, according to the median of age.
IM or DYS subjects who did not progress to gastric cancer during the follow-up period. We first compared the distributions of age, gender, cigarette smoking, alcohol consumption, and H. pylori infection status between two groups, and no significant differences were found. Compared with the subjects remained with IM or DYS (Table 4), the frequency of hypermethylated IGFII was markedly increased in IM or DYS subjects who progressed to gastric cancer during the follow-up period (OR, 12.52; 95% CI, 3.81–41.15 for IM; OR, 10.12; 95% CI, 2.68–38.22 for DYS). Similar result was also observed for N33 in subjects with IM (OR, 3.77; 95% CI, 1.20–11.86), but no significant difference was found for DYS (OR, 1.40; 95% CI, 0.44–4.50).

**Relationship between methylation status and epidemiologic parameters**

We also evaluated the relationships between methylation status and age or other risk factors, and found lower frequency of hypermethylated IGFII (≥50%) in older subjects in the gastric cancer group (OR, 0.33; 95% CI, 0.11–0.98) and ever smokers in total participants (OR, 0.54; 95% CI, 0.30-0.95). For N33, the frequency of hypermethylation (≥5%) was significantly higher in older subjects in SG/CAG (OR, 3.61; 95% CI, 2.16–6.02) and total subjects (OR, 3.26; 95% CI, 2.10–5.04). However, no significant associations were found between methylation levels and gender, H. pylori infection or drinking.

**Discussion**

On the basis of the two long-term cohort studies in a high-risk population of gastric cancer, we found hypermethylated IGFII and N33 in blood leukocyte DNA were associated with higher risk of gastric cancer. To our best knowledge, this is the first population-based cohort study

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**Table 2. The methylation levels in pre–gastric cancer and post–gastric cancer blood samples**

<table>
<thead>
<tr>
<th>Methylation proportion median % (interquartile range)</th>
<th>IGFII</th>
<th>N33</th>
</tr>
</thead>
<tbody>
<tr>
<td>SG/CAG (n = 285)</td>
<td>49.73 (41.04–57.82)</td>
<td>5.70 (3.79–9.25)</td>
</tr>
<tr>
<td>Total pre– and post–gastric cancer samples</td>
<td>66.22 (50.54–77.88)</td>
<td>9.23 (6.07–12.69)</td>
</tr>
<tr>
<td>Pre–gastric cancer (n = 74)</td>
<td>61.47 (46.88–75.43)</td>
<td>5.74 (3.13–9.79)</td>
</tr>
<tr>
<td>Post–gastric cancer (n = 95)</td>
<td>&lt;0.001</td>
<td>0.007</td>
</tr>
<tr>
<td>P&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.959</td>
<td>0.001</td>
</tr>
<tr>
<td>Self-control study</td>
<td>69.96 (49.49–77.96)</td>
<td>5.08 (2.84–7.23)</td>
</tr>
<tr>
<td>Pre–gastric cancer (n = 31)</td>
<td>63.96 (52.25–74.28)</td>
<td>9.29 (4.70–12.70)</td>
</tr>
<tr>
<td>Post–gastric cancer (n = 31)</td>
<td>0.207</td>
<td>0.022</td>
</tr>
</tbody>
</table>

<sup>a</sup>Unconditional logistic regression analysis between the pre–gastric cancer and SG/CAG groups, adjusted for other characteristics (age, sex, smoking, drinking, and H. pylori infection status).

<sup>b</sup>Unconditional logistic regression analysis between the post– and pre–gastric cancer groups, adjusted for other characteristics (age, sex, smoking, drinking, and H. pylori infection status).

<sup>c</sup>Conditional logistic regression analysis, adjusted for age.

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**Table 3. The temporal trends of methylation levels in blood leukocyte DNA before and after gastric cancer diagnosis**

<table>
<thead>
<tr>
<th>Methylation proportion median % (interquartile range)</th>
<th>6–10 years pre-GC (n = 25)</th>
<th>1–5 years pre-GC (n = 49)</th>
<th>GC diag. year (n = 46)</th>
<th>1–5 years post-GC (n = 36)</th>
<th>6–10 years post-GC (n = 13)</th>
<th>P&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGFII</td>
<td>69.28 (55.07–77.55)</td>
<td>63.38 (48.67–79.06)</td>
<td>60.29 (46.16–75.33)</td>
<td>60.16 (47.53–75.87)</td>
<td>63.72 (53.97–74.97)</td>
<td>0.738</td>
</tr>
<tr>
<td>N33</td>
<td>9.29 (4.56–12.22)</td>
<td>9.12 (5.28–13.13)</td>
<td>4.88 (2.85–9.28)</td>
<td>6.09 (3.38–9.42)</td>
<td>9.73 (3.52–13.96)</td>
<td>0.004</td>
</tr>
</tbody>
</table>

Abbreviation: GC, gastric cancer.

<sup>a</sup>Kruskal–Wallis test.
IGFII gene (27). A previous hospital-based case–control study expressed and maternally silenced as an imprinted gene (27). A previous hospital-based case–control study in a Japanese population reported hypomethylation of IGFII in blood leukocyte DNA was associated with high risk of gastric cancer (17). In contradiction to their result, the median methylation level of IGFII was much higher in gastric cancer (61.47%) than in SG/CAG in our current study. Considering the same detection method in the two studies, one may speculate that the difference might come from the source of the study population. In this study, all of the subjects were selected from a high-risk area of gastric cancer, in which some unknown environmental impact factor or special mechanisms may be involved in gastric carcinogenesis.

The functional regulation of IGFII in the process of cancer development is intriguing. Studies showed biallelic expression of IGFII, resulting in overgrowth in mice (28–30), as well as in human tumor (31–33), was considered due to LOI and hypomethylation of a differentially methylated region near promoter (16, 34, 35). However, other studies suggested the remarkable over-expression of IGFII (usually more than 10 times in tumor) might be a result of mechanisms unrelated to LOI or aberrant methylation (36). Four distinct promoters (P1–P4) were found to be responsible for the transcription of IGFII. Hypermethylation of P2–P4 promoter (CpG island rich) would not affect the upstream non-imprinted P1 promoter, which may result in a higher expression (37). While the mechanisms that may underlie the hypermethylated IGFII in gastric cancer cases in our study still need further study.

N33, which was reported to be hypermethylated in colorectal cancer as a tumor suppressor gene (19), showed no difference between the gastric cancer and SG/CAG groups in neither our study nor a Japanese study (17). However, by comparing blood leukocyte samples collected before and after the diagnosis of gastric cancer, a significant increase of N33 methylation (9.12%) was observed in pre–gastric cancer samples at least 5 years before the clinical diagnosis. Similar result was also found for IGFII (63.38%). In addition, temporal trends were further explored by a self-control study using 31 cases with both pre–gastric cancer and post–gastric cancer blood leukocyte samples based on our long-term follow-up population. This increasing trend of methylation level during gastric cancer development suggested that IGFII and N33 methylation status in blood leukocyte DNA may serve as biomarkers for gastric cancer early diagnosis.

Blood leukocyte is one of the most accessible cells in human body. The epigenetic status of blood leukocyte DNA may be variable corresponding to the alternations of tumor-related environmental exposure factors. A previous study (13) showed loss of IGFII imprinting in colorectal tissue may be a potential marker for colorectal cancer, and the LOI of IGFII in peripheral blood leukocyte DNA was closely correlated with that in biopsies. However, their results were not confirmed by a latter prospective study in a Northern Swedish population (38). Our current study revealed that subjects with IM or DYS and hypermethylated IGFII or N33 showed much higher risk of progression to gastric cancer, providing further evidence that IGFII and N33 methylation may play roles in the process of gastric cancer development. Further studies with a larger sample size are warranted to confirm these findings.

Growing evidences suggested epigenetic status can be affected by aging and environmental factors (39–41). For example, aging, an important risk factor for most cancers, may result in cumulative exposure to carcinogens and multiple hits for the onset of neoplasia. Studies illustrated...
Methylation of Blood Leukocyte DNA and Gastric Cancer

that N33 (19) and IGFII (37) showed age-related methylations in colon, and eventually progressed to hypermethylation in cancers. We also found higher frequency of hypermethylated N33 in older subjects. While in case of IGFII, the methylation status of blood leukocyte DNA decreased with aging both in our study and Japanese study (17), which suggested the pattern of IGFII methylation may be tissue-specific.

H. pylori infection shows strong associations with aberrant methylation of many tumor-related genes in gastric mucosa, such as p16 (10), COX-2 (42) and RUNX3 (11) by releasing reactive oxygen species and nitric oxide (43, 44). Eradication of H. pylori infection was found to reduce the methylation levels of many genes, such as p16 and COX-2 (45, 46). Although hypermethylated N33 was found in blood leukocyte with positive H. pylori antibodies by the previous Japanese study (17), no similar relationship was observed in our study, perhaps due to the less case number in each group and the higher prevalence of H. pylori infection in advanced gastric lesions. Because the antibody assay used in this study can only show the past H. pylori infection, we also evaluated the associations between methylation levels of IGFII and N33 and H. pylori current infection in the subjects (n = 329) with both results of ELISA and U-13C-Urea Breath Test. There were no statistical associations between methylation levels of IGFII and N33 and H. pylori current infection, and no significant differences of methylation levels of IGFII and N33 between past and current infection (data not shown).

The major strength of our study lies in the prospective design, which provides prediagnostic blood leukocyte samples for the dynamic observation of methylation levels before and after the clinical diagnosis of gastric cancer and also for the comparison of methylation levels between subjects with different outcomes in long-term follow-up (progressed and nonprogressed to gastric cancer). A limitation of our study is that the number of gastric cancer cases, especially the gastric cancer cases with both pre-gastric cancer and post-gastric cancer diagnostic blood leukocyte samples, is still not large enough. In addition, studies on the mechanisms involving in the association between methylation levels of blood leukocyte DNA and gastric cancer development are required in the future.

In conclusion, our population-based study provided evidence that the methylation levels of IGFII and N33 in blood leukocyte DNA changed at least 5 years ahead of clinical diagnosis of gastric cancer, and may serve as early biomarkers for the risk of gastric cancer. Subjects with hypermethylated IGFII or N33 in blood leukocyte DNA would need further examination to monitor the progression of gastric lesions.

Disclosure of Potential Conflicts of Interest

L. Shen reports receiving a commercial research grant from Roche and Pfizer (not related to the current study), has received speakers' bureau honoraria from Roche, Pfizer, Amgen, Novartis, and Sanofi, and is a consultant/advisory board member for Roche, Merck, Amgen, and Pfizer. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

Conception and design: K.-F. Pan, Y. Yuasa, W.-C. You

Development of methodology: Y. Zhang, H.-J. Su, W.-C. You

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Y. Zhang, H.-J. Su, J.-L. Ma, L. Shen, J.-Y. Li, W.-D. Liu, W.-C. You

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Y. Zhang, L. Zhang, K. Matsuo, W.-C. You

Writing, review, and/or revision of the manuscript: Y. Zhang, K.-F. Pan, L. Shen, Y.-Y. Li, L. Oue, Y. Yuasa, W.-C. You

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): H.-J. Su, L. Zhang

Study supervision: K.-F. Pan, W.-C. You

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