Methylation status of blood leukocyte DNA and risk of gastric cancer in a high-risk Chinese population

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Key words: Methylation, blood leukocyte DNA, IGF2, N33, gastric cancer

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**Abbreviations:** IGF2, insulin-like growth factor 2; N33, tumor suppressor candidate 3; CAG, chronic atrophic gastritis; CI, confidence interval; DYS, dysplasia; GC, gastric cancer; *H. pylori*, Helicobacter pylori; IM, intestinal metaplasia; Ind DYS, indefinite dysplasia; OR, odds ratio; SG, superficial gastritis.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.
Abstract

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

**Methods:** Methylation levels of IGF2 and N33 were determined by quantitative methylation-specific PCR. The temporal trend of methylation levels during GC development was investigated in 133 GC cases from two cohorts with pre- and/or post-GC samples. As the references of pre-GCs, 204 intestinal metaplasia (IM) or dysplasia (DYS) subjects who did not progress to GC during the follow-up period were selected. Meanwhile, 285 subjects with superficial gastritis/chronic atrophic gastritis (SG/CAG) were also selected as controls.

**Results:** IGF2 median methylation level was significantly higher in GC cases than those with SG/CAG (61.47% vs 49.73%, p<0.001). IGF2 and N33 methylation levels were elevated at least 5 years ahead of clinical GC diagnosis comparing with SG/CAG (63.38% vs 49.73% for IGF2, 9.12% vs 5.70% for N33, all p<0.001). Furthermore, the frequency of hypermethylated IGF2 was markedly increased in IM or DYS subjects who progressed to GC in contrast to those remained with IM and DYS, and adjusted odds ratios (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 result was also found for N33 in subjects with IM (OR, 3.77, 95%CI: 1.20-11.86).

**Conclusions:** Our findings suggested that hypermethylated IGF2 and N33 in blood leukocyte DNA were associated with risk of GC in a Chinese population.

**Impact:** IGF2 and N33 methylation status may be related to gastric carcinogenesis.
Introduction

Gastric cancer (GC) is one of the most common cancers worldwide, including China [1, 2]. The 5-year relative survival rate of GC varies remarkably from 90% in stage I to less than 5% in stage IV [3]. Therefore, many studies have focused on identification of effective biomarkers for early detection of GC.

Epigenetic changes, which do not involve alternations in the DNA sequence but rather changes in DNA methylation, are found in almost every type of human tumor [4, 5, 6]. Studies have shown that aberrant methylation of some genes can be found in precancerous gastric lesions or GC [7, 8, 9], and environmental factors could be important to determine the methylation status of cancer-related genes [10, 11].

Insulin-like growth factor 2 (IGF2), as an important autocrine growth factor, binds to the specific receptor to initiate intracellular signalling cascades and leads to cell proliferation [12]. Increased IGF2 expression was found in different types of tumors, including colorectal cancer [13], prostate cancer [14] and Wilm’s tumor [15]. Loss of imprinting (LOI), an important cause of overexpression of IGF2, was reported to be a potential marker for cancer risk assessment [13]. The hypomethylation of IGF2 promoter may be one of the critical mechanisms in LOI mediated IGF2 overexpression [16]. Therefore, the methylation level of IGF2 in blood leukocyte DNA may serve as a surrogate marker for the risk and prognosis of GC [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 GC, 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 GC were based on case-control studies. To explore the dynamical changes of methylation level in the process of GC development, a cohort study is highly desirable. Since 1989, we conducted a series of epidemiological studies in Linqu County, a rural area in northeast of
China with one of the highest GC mortality rates in the world [20, 21, 22]. In these cohorts, blood samples collected from GC cases before and/or after the clinical diagnosis provided a unique opportunity to assess the potential DNA methylation biomarkers for GC risk.

In this study, we tested the hypothesis that the methylation levels of IGF2 and N33 in blood leukocyte DNA may be associated with the risk of GC and further evaluated the dynamic changes of methylation level during GC development.

**Materials and methods**

*Study population and gastric pathology*

Two cohort studies were conducted in Linqu County in 1989 (N=3433) and 2002 (N=2638) respectively [20, 22], and a total of 186 GC 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 GC. 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 GC cases from two cohorts were enrolled. According to the time of diagnosis, blood leukocyte samples collected from GC cases were defined into pre-GCs (before GC diagnosis ranging from 1 to 10 years) and post-GCs (the year of GC diagnosis or up to 10 years after). Among them, 43 pre-GC blood leukocyte samples from 38 GC cases (5 cases with two pre-GC samples with different time interval) and 64 post-GC
samples from 64 GC cases were collected. Additionally, 31 cases had both pre-GC and post-GC 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 IGF2 and N33 with the risk of GC, 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 GC during the follow-up period randomly from baseline as controls, because the corresponding gastric lesions for the pre-GC 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 samples 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 K$_2$ EDTA tubes (BD Vacutainer) and centrifuged at 3000 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, Madison, WI). Quantitative methylation-specific PCR was carried out for IGF2 and N33 using a 7500 fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) with the primers and probes as described previously [17]. The PCR amplification was performed with a final reaction mixture of 20μl consisting of 1x TaqMan Fast Universal PCR Master Mix (Applied Biosystems), 0.9 μM each of forward and reverse primers, 0.25 μM each of probes and 20 ng of DNA at the following conditions: 95°C for 20 sec, followed by 40 cycles at 95°C for 3 sec and 61°C for 1 min for IGF2 (60°C for 30 sec for N33). The efficiencies of PCR
amplification of all the genes were confirmed to be nearly 100%, and beta actin (ACTB) was used as a reference set to normalize for input DNA.

The methylation levels of IGF2 and N33 were expressed as percentage, calculated by dividing the IGF2 (or N33)/ACTB ratio of a sample by the IGF2 (or N33)/ACTB ratio of MNK74. The DNA of MKN74 cell line was kindly provided by Prof. Dajun Deng at Peking University Cancer Hospital & Institute [26], and was confirmed to be 100% methylated in the CpGs in IGF2 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 DNA were selected randomly for duplicate detection of IGF2 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-*H. pylori* IgG and IgA were measured separately in duplicate with enzyme-linked immunosorbent assay procedures. Quality control samples were assayed at Vanderbilt University, Nashville, TN. An individual was determined to be positive for *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’s $\chi^2$ test was used to examine the differences between SG/CAG and post-GC groups in age, gender, smoking, drinking and *H. pylori* infection status. Mann-Whitney test was used to examine the differences for the methylation levels between SG/CAG and post-GC groups. The differences among multiple groups were estimated by Kruskal Wallis Test for temporal trends of methylation levels. Odds ratios (ORs) with
corresponding 95% confidence intervals (CIs) for associations of methylation status in blood leukocyte DNA with potential risk factors or the risk of GC 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 GC diagnosis or last follow-up. The p values for the differences of methylation levels between pre-GC and post-GC 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 GC cases with both pre- and post-diagnosis 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, Cary, NC).

Results

Selected characteristics of the subjects in SG/CAG and post-GC groups

The distributions of age, gender, cigarette smoking, alcohol consumption and \textit{H. pylori} infection status in SG/CAG and post-GC groups were presented in Table 1. Compared with SG/CAG, GC group showed a higher frequency of \textit{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-GC groups.

Comparison of methylation levels between GC and SG/CAG groups

We first compared the methylation levels between SG/CAG and GC groups, and found the median methylation level of IGF2 was 49.73% in SG/CAG, but significantly increased to 61.47% in GC group (p<0.001). For N33, compared with SG/CAG, no significant difference was observed in GC group (5.74% vs. 5.70%, p=0.991). To further evaluate the relationship between methylation status and risk of GC, we set 50% and 5% as the cut-off values for IGF2 and N33 according to the median levels in SG/CAG, respectively. With SG/CAG as reference, unconditional logistic regression analysis adjusting for age, gender, \textit{H. pylori} infection, smoking and drinking revealed a close association between hypermethylated (≥50%) IGF2 and GC risk (OR, 2.46, 95% CI: 1.46-4.13), while no significant association
was found between hypermethylation status (≥5%) and GC risk for N33 (OR, 0.92, 95% CI: 0.55-1.54, p=0.751).

**Temporal trends of methylation levels in GC development**

We further evaluated the dynamic changes of methylation levels by using pre-GC and post-GC samples (Table 2). Compared to SG/CAG, the median methylation levels of IGF2 and N33 were both higher in pre-GC blood leukocyte samples (66.22% vs. 49.73%, p<0.01 for IGF2; 9.23% vs. 5.70%, p<0.01 for N33). While in the post-GC samples, the median methylation levels remained high for IGF2, but decreased significantly for N33 (p=0.001). Similar results were obtained in 31 GC cases with both pre-GC and post-GC samples (Table 2).

The temporal trends of methylation levels were further explored by dividing the pre-GC and post-GC samples into five groups according to the time interval between sample collection and GC diagnosis. As shown in Table 3, the median methylation level of IGF2 was significantly increased at least 5 years ahead of clinical GC diagnosis compared to SG/CAG (63.38% vs 49.73%, p<0.001). In 6-10 years pre-GC samples, IGF2 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% 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 GC diagnosis (p=0.004). A similar temporal trend was found by using 31 GC cases with both pre-GC and post-GC samples. IGF2 persisted in hypermethylation status from 6-10 years pre-GC (66.70%, n=7), 1-5 years pre-GC (61.39%, n=24), GC diagnosis year (68.09%, n=21) to 1-5 years post-GC (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 6-10 (9.29%) or 1-5 years (9.28%) pre-GC samples (p=0.003).

**Methylation levels of IGF2 and N33 in IM or DYS subjects with different outcomes**

Because the corresponding gastric lesions for the pre-GC diagnosis were mainly IM and DYS, we were very interested to compare the methylation levels in IM or DYS subjects who did not progress to GC 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 to the subjects remained with IM or DYS (Table 4), the frequency of hypermethylated IGF2 was markedly increased in IM or DYS subjects who progressed to GC during the follow-up period (OR, 12.52, 95% CI: 3.81-41.15 for IM and 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 epidemiological parameters**

We also evaluated the relationships between methylation status and age or other risk factors, and found lower frequency of hypermethylated IGF2 (≥50%) in older subjects in GC 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**

Based on the two long-term cohort studies in a high-risk population of GC, we found hypermethylated IGF2 and N33 in blood leukocyte DNA were associated with higher risk of GC. To our best knowledge, this is the first population-based cohort study to explore the aberrant methylation of blood leukocyte DNA dynamically in the process of GC development.

Until now, there is little study on methylation status of blood leukocyte DNA in various gastric lesions and GC. Our data revealed that IGF2 was half-methylated in SG/CAG (49.73%), which is consistent with the general knowledge that IGF2 is paternally expressed and maternally silenced as an imprinted gene [27]. A previous hospital-based case-control study in a Japanese population reported hypomethylation of IGF2 in blood leukocyte DNA was associated with high risk of GC [17]. In contradiction to their result, the median methylation level of IGF2 was much higher in GC (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 GC, in which some unknown environmental impact factor or special mechanisms may be involved in gastric carcinogenesis.

The functional regulation of IGF2 in the process of cancer development is intriguing. Studies showed biallelic expression of IGF2 resulting in overgrowth in mice [28, 29, 30], as well as in human tumor [31, 32, 33], was considered due to LOI and hypomethylation of a differentially methylated region (DMR) near promoter [16, 34, 35]. However, other studies suggested the remarkable overexpression of IGF2 (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 IGF2. 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 IGF2 in GC 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 GC 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 GC, a significant increase of N33 methylation (9.12%) was observed in pre-GC samples at least 5 years before the clinical diagnosis. Similar result was also found for IGF2 (63.38%). In addition, temporal trends were further explored by a self-control study using 31 cases with both pre-GC and post-GC blood leukocyte samples based on our long-term follow-up population. This increasing trend of methylation level during GC development suggested that IGF2 and N33 methylation status in blood leukocyte DNA may serve as biomarkers for GC 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 IGF2 imprinting in colorectal tissue may be a potential marker for colorectal cancer, and the LOI of IGF2 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 IGF2 or
N33 showed much higher risk of progression to GC, providing further evidence that IGF2 and N33 methylation may play roles in the process of GC 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, 40, 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 that N33 [19] and IGF2 [37] showed age-related methylation in colon, and eventually progressed to hypermethylation in cancers. We also found higher frequency of hypermethylated N33 in older subjects. While in case of IGF2, the methylation status of blood leukocyte DNA decreased with aging both in our study and Japanese study [17], which suggested the pattern of IGF2 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 IGF2 and N33 and *H. pylori* current infection in the subjects (n=329) with both results of ELISA and 13C-Urea Breath Test. There were no statistical associations between methylation levels of IGF2 and N33 and *H. pylori* current infection, and no significant differences of methylation levels of IGF2 and N33 between past and current infection (data not shown).

The major strength of our study lies in the prospective design, which provides pre-diagnostic blood leukocyte samples for the dynamic observation of methylation levels before and after the clinical diagnosis of GC and also for the comparison of methylation levels between subjects with different outcomes in long term follow-up (progressed and non-progressed to GC). A limitation of our study is that the number of GC cases, especially the GC cases with both pre-GC and post-GC 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 GC development are required in the future.

In conclusion, our population-based study provided evidence that the methylation levels of IGF2 and N33 in blood leukocyte DNA changed at least 5 years ahead of clinical diagnosis of GC, and may serve as early biomarkers for the risk of GC. Subjects with hypermethylated IGF2 or N33 in blood leukocyte DNA would need further examination to monitor the progression of gastric lesions.

Acknowledgements

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References


Table 1. Selected characteristics of the study participants in SG/CAG and post-GC groups

<table>
<thead>
<tr>
<th></th>
<th>SG/CAG (n=285)</th>
<th>Post-GC (n=95)</th>
<th>P&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;60</td>
<td>139 (48.8)</td>
<td>40 (42.1)</td>
<td>0.26&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>≥60</td>
<td>146 (51.2)</td>
<td>55 (57.9)</td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>215 (75.4)</td>
<td>69 (72.6)</td>
<td>0.59</td>
</tr>
<tr>
<td>Female</td>
<td>70 (24.6)</td>
<td>26 (27.4)</td>
<td></td>
</tr>
<tr>
<td>Smoking</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Yes</td>
<td>177 (62.1)</td>
<td>57 (60.0)</td>
<td>0.80</td>
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<td>No</td>
<td>108 (37.9)</td>
<td>37 (38.9)</td>
<td></td>
</tr>
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<td></td>
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<tr>
<td>Drinking</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td>160 (56.1)</td>
<td>48 (50.5)</td>
<td>0.72</td>
</tr>
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<td>No</td>
<td>122 (42.8)</td>
<td>40 (42.1)</td>
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</tr>
<tr>
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<td>3 (1.1)</td>
<td>7 (7.4)</td>
<td></td>
</tr>
<tr>
<td>H&lt;sub&gt;p&lt;/sub&gt;ylori</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>(+)</td>
<td>147 (51.6)</td>
<td>60 (63.2)</td>
<td>0.050</td>
</tr>
<tr>
<td>(-)</td>
<td>138 (48.4)</td>
<td>35 (36.8)</td>
<td></td>
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</table>

<sup>a</sup>χ<sup>2</sup> test

<sup>b</sup>The cut off value of age was set as 60 years, according to the median of age.
Table 2. The methylation levels in pre-GC and post-GC blood samples

<table>
<thead>
<tr>
<th>Methylation proportion median % (interquartile range)</th>
<th>IGF2</th>
<th>N33</th>
</tr>
</thead>
<tbody>
<tr>
<td>SG/CAG (n=285)</td>
<td>49.73</td>
<td>(41.04-57.82)</td>
</tr>
<tr>
<td></td>
<td>5.70</td>
<td>(3.79-9.25)</td>
</tr>
<tr>
<td>Total pre-GC and post-GC samples</td>
<td>66.22</td>
<td>9.23</td>
</tr>
<tr>
<td>Pre-GC (n=74)</td>
<td>(50.54-77.88)</td>
<td>(5.07-12.69)</td>
</tr>
<tr>
<td>Post-GC (n=95)</td>
<td>61.47</td>
<td>5.74</td>
</tr>
<tr>
<td></td>
<td>(46.88-75.43)</td>
<td>(3.13-9.79)</td>
</tr>
<tr>
<td>P&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&lt;0.001</td>
<td>0.007</td>
</tr>
<tr>
<td>P&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.959</td>
<td>0.001</td>
</tr>
<tr>
<td>Self-control study</td>
<td>63.96</td>
<td>9.29</td>
</tr>
<tr>
<td>Pre-GC (n=31)</td>
<td>(52.25-74.28)</td>
<td>(4.70-12.70)</td>
</tr>
<tr>
<td>Post-GC (n=31)</td>
<td>69.96</td>
<td>5.08</td>
</tr>
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<td></td>
<td>(49.49-77.96)</td>
<td>(2.84-7.23)</td>
</tr>
<tr>
<td>P&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.207</td>
<td>0.022</td>
</tr>
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</table>

<sup>a</sup> Unconditional logistic regression analysis between pre-GC 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 post-GC and pre-GC groups, adjusted for other characteristics (age, sex, smoking, drinking and *H. pylori* infection status)

<sup>c</sup> Conditional logistic regression analysis, adjusted for age
Table 3. The temporal trends of methylation levels in blood leukocyte DNA before and after GC diagnosis

<table>
<thead>
<tr>
<th>Methylation proportion median % (interquartile range)</th>
<th>6-10 years</th>
<th>1-5 years</th>
<th>GC diag. year</th>
<th>1-5 years</th>
<th>6-10 years</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>pre-GC</td>
<td>pre-GC</td>
<td>post-GC year</td>
<td>post-GC</td>
<td>post-GC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(n=25)</td>
<td>(n=49)</td>
<td>(n=46)</td>
<td>(n=36)</td>
<td>(n=13)</td>
<td></td>
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<tr>
<td>IGF2</td>
<td>69.28</td>
<td>63.38</td>
<td>60.29</td>
<td>60.16</td>
<td>63.72</td>
<td>0.74</td>
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<tr>
<td></td>
<td>(55.07-77.55)</td>
<td>(48.67-79.06)</td>
<td>(46.16-75.33)</td>
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<td>(53.97-74.97)</td>
<td></td>
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<tr>
<td>N33</td>
<td>9.29</td>
<td>9.12</td>
<td>4.88</td>
<td>6.09</td>
<td>9.73</td>
<td>0.004</td>
</tr>
</tbody>
</table>

* Kruskal Wallis Test
Table 4. Association between methylation levels and risk of progression to GC in IM and DYS

<table>
<thead>
<tr>
<th></th>
<th>IM (n=99)</th>
<th>IM (progress to GC, n=33)</th>
<th>OR(95%CI)(^a)</th>
<th>P</th>
<th>DYS (n=105)</th>
<th>DYS (progress to GC, n=35)</th>
<th>OR(95%CI)(^a)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGF2 (cut-off 50%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypo-methylated</td>
<td>79(79.8)</td>
<td>7(21.2)</td>
<td>1.00</td>
<td></td>
<td>77(73.3)</td>
<td>8(22.9)</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>Hyper-methylated</td>
<td>20(20.2)</td>
<td>26(78.8)</td>
<td><strong>12.52(3.81-41.15)</strong></td>
<td>&lt;0.001</td>
<td>28(26.7)</td>
<td>27(77.1)</td>
<td><strong>10.12(2.68-38.22)</strong></td>
<td>0.001</td>
</tr>
<tr>
<td>N33 (cut-off 5%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypo-methylated</td>
<td>45(50.6)</td>
<td>6(18.2)</td>
<td>1.00</td>
<td></td>
<td>49(46.7)</td>
<td>10(28.6)</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>Hyper-methylated</td>
<td>44(49.4)</td>
<td>27(81.8)</td>
<td><strong>3.77(1.20-11.86)</strong></td>
<td>0.023</td>
<td>56(53.3)</td>
<td>25(71.4)</td>
<td>1.40(0.44-4.50)</td>
<td>0.57</td>
</tr>
</tbody>
</table>

\(^a\) Unconditional logistic regression analysis, adjusted for age, sex, smoking, drinking, *H. pylori* infection status and time interval between blood collection and the dates of GC diagnosis or last follow-up.
Figure legend

Figure 1. Structure diagram for sample selection.
Figure 1

133 GC subjects

38 GC subjects with pre-GC blood leukocyte samples (including 5 cases with two samples)

43 pre-GC blood leukocyte samples

99 and 105 subjects with IM and DYS from baseline 3:1

74 pre-GC blood leukocyte samples (including 33 IM; 35 DYS)

31 GC subjects with both pre- and post-GC blood leukocyte samples

31 pre-GC blood leukocyte samples

95 post-GC blood leukocyte samples

64 GC subjects with post-GC blood leukocyte samples

64 post-GC blood leukocyte samples

285 subjects with SG/CAG from baseline 1:3
Methylation status of blood leukocyte DNA and risk of gastric cancer in a high-risk Chinese population


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