

# DNA Methylation as Surrogate Marker For Gastric Cancer

REVIEW

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Stomach cancer remains, stubbornly, highly prevalent in East Asia. Still, stomach cancer has few biomarkers by which it can be predicted. *Helicobacter pylori* infection, a known carcinogen of stomach cancer, usually goes undetected prior to cancer diagnosis, due to the poor mucosal environments that its related gastric atrophy causes. We propose, herein, an endoscopic-biopsy-based cancer-predicting DNA methylation marker. We semi-quantitatively examined the methylation-variable sites near the CpG-island margins by radioisotope-labeling methylation-specific polymerase chain reaction in association with *H. pylori*, which increases age-related over-methylation in CpG islands of gastric mucosa. These age-related methylation patterns of the transitional-CpG sites are proposed as useful surrogate markers for stomach cancer. It would be helpful for setting the optimal screening interval for high-risk subjects as well as for estimating the prognosis and the predictability for recurrence of early gastric cancer in patients having undergone endoscopic submucosal dissection. New screening-interval guidelines for gastric cancer should be suggested considering individual risk based on age, severity of atrophy, *H. pylori* status, and DNA methylation pattern.

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## INTRODUCTION

Gastric cancer was the fifth most common malignancy and the third most common cause of cancer-related deaths worldwide in 2012.<sup>1</sup> In East Asia, it is the most prevalent cancer.<sup>2</sup> Both Japan and Korea, uniquely in the world, routinely screen for gastric cancer. The Japanese screening program recommends an upper-gastrointestinal series using barium.<sup>3</sup> The National Health Insurance Corporation of Korea, meanwhile, provides, for detection of early gastric cancer, biannual endoscopy for all adults over the age of 40 years. Early detection of cancer, which can be achieved by identification of high-risk individuals, is essential to the reduction of morbidity and mortality rates. In this effort, endoscopy plays a leading role. One recent gastric cancer study reported a higher mortality reduction rate for the endoscopic screening group relative to a radiographic screening group.<sup>4</sup>

However, no system of risk stratification assessment based on recommended screening intervals has yet been established.<sup>5</sup>

Even though a number of risk factors such as *Helicobacter pylori* infection, salt intake, smoking, alcohol, family history, atrophic gastritis (AG), and intestinal metaplasia (IM) are well known, they cannot account for all gastric cancers, since gastric carcinogenesis is a multifactorial process. Indeed, a very wide range of factors (genetic, epigenetic, and environmental) can contribute to gastric cancer carcinogenesis. The research into the epigenetic mechanisms of gastric cancer, in any case, has begun to make a mark. The aim of this review is to discuss DNA methylation changes according to *H. pylori* infection, especially focusing on the transitional-CpG site. Such changes in the transitional-CpG sites doubtless will become useful markers for detection of subjects at high risk of gastric cancer.

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## ENDOSCOPIC SCREENING

### 1. Atrophy/intestinal metaplasia

AG and the development of IM are considered to be the primary premalignant conditions leading to gastric cancer onset. However, the association of atrophy with gastric cancer and the link between its progression and gastric cancer development, in longitudinal cohort studies, have been tenuous. The rate of progression of AG to gastric cancer fluctuates from 0% to 10% with an annual person-per-year incidence lower than 1%.<sup>6</sup> The annual incidences of gastric cancer within five years of diagnosis were reported as 0.1% for patients with AG, 0.25% for IM, 0.6% for mild-to-moderate dysplasia, and 6% for severe dysplasia.<sup>7</sup>

For more rational classification of gastritis, the updated Sydney system was developed.<sup>8</sup> And to investigate the association between gastritis and gastric cancer risk, an international group of gastroenterologists and pathologists (the Operative Link for Gastritis Assessment [OLGA]) has suggested the OLGA staging system, which arranges the histological phenotypes of gastritis on a scale of gastric cancer risk progressively increasing from lowest (OLGA stage 0) to highest (OLGA stage IV).<sup>9</sup> Both systems, however, require five endoscopically obtained stomach biopsies for clarification of the distribution of atrophic change. This, unfortunately, renders problematic their application to all gastritis patients in actual clinical settings.

Correa proposed the precancerous cascade, which describes the process of active chronic inflammation's initiation of the development of gastric cancer from AG, IM, and dysplasia.<sup>10</sup> Fully one-third of gastric cancer patients in two studies, however, showed no precancerous lesions in the gastric mucosa.<sup>11,12</sup> Their cancers, moreover, were detected without signs of mucosal atrophy. Indeed, IM carries a low risk of progression to cancer, and such lesions are commonly found in everyday practice. Thus, surveillance programs based on the detection of precancerous lesions are of questionable utility. To overcome these problems, research into and implementation of promising biomarkers for early detection of gastric cancer should be vigorously pursued.

### 2. *Helicobacter pylori*

*H. pylori* is a known carcinogen that can form a developmental link between AG, IM, or dysplasia. It is related to both diffuse-type and intestinal-type cancer. *H. pylori* infection usually is acquired during childhood and persists for years, though it tends to disappear once AG and IM extend, which produces an unfavorable environment for its colonization.<sup>13</sup> It has been suggested that in order to prevent gastric cancer onset in Korea, *H.*

*pylori* should be eradicated before IM has a chance to develop.<sup>14</sup> According to a community-based study in Taiwan, mass eradication of *H. pylori* infection led to a substantial reduction in the incidence of AG; however, it did not reduce the incidence of IM.<sup>15</sup> It seems that the oncogenic potential of *H. pylori* infection persists after the infection itself is gone.<sup>16</sup> Indeed, whereas not all people infected with *H. pylori* develop gastric cancer (in fact, the majority do not), *H. pylori*-negative patients are among those afflicted with the disease.<sup>17</sup> The prevalence of *H. pylori*-negative gastric cancer, most of which cases might be expected to have involved previous *H. pylori* infection, reportedly ranges from 0.42% to 5.4% of all gastric cancers.<sup>18</sup> Therefore, *H. pylori* itself cannot be a useful marker for detection of gastric cancer.

## SERUM PEPSINOGEN AND *HELICOBACTER PYLORI* ANTIBODY

Serum pepsinogen (PG) was introduced as an objective method of atrophic-change detection that can overcome the problem of the interobserver variation in endoscopy-based AG diagnosis.<sup>19</sup> A PG I/II ratio < 3.0 has been accepted as the cut-off point for severity of AG. However, PG screening, due to its low predictive value, has not been adopted as a screening method. Instead, it has been developed for risk stratification of gastric cancer that entails assessment of two risk factors, namely serum immunoglobulin G anti-*H. pylori* antibody level and PG level.<sup>16</sup> To clarify the influence of the interaction of *H. pylori* infection with AG on gastric cancer risk, one study formed the following four subject groups: (1) *H. pylori* (-)/PG (-) (group A), (2) *H. pylori* (+)/PG (-) (group B), (3) *H. pylori* (+)/PG (+) (group C), and (4) *H. pylori* (-)/PG (+) (group D). It demonstrated that the risk of gastric cancer was higher in group C or group D than in group A or group B.<sup>16</sup> Notwithstanding the simplicity and non-invasive aspect of this methodology, it effectively raised the issue of the appropriateness of the PG cut-off level and showed that it could consistently distinguish gastric adenoma and gastric cancer. Subsequently, a new, modified ABCD method using two different PG cutoff values (I/II ratio 3.1 and 4.1) was proposed as a supplemental screening tool for both gastric adenoma and gastric cancer in Korea.<sup>20</sup>

## DNA METHYLATION AS NEW BIOMARKER

### 1. Role of DNA methylation

Epigenetics is a study that includes a number of modifications in the genomic structure that affect gene expression without

altering the primary DNA sequence. Epigenetic silencing of genes by aberrant DNA methylation is recognized as a crucial component of the mechanism underlying tumorigenesis.<sup>21</sup> DNA methylation refers to a methyl group's addition to or subtraction from cytosine residue in a sequence of DNA.<sup>22</sup> DNA methylation occurs almost exclusively at CpG dinucleotides. DNA methylation has been known to suppress repetitive DNA sequences initially such as retroviruses and retroelements that comprise one-fourth of the human genome.<sup>23</sup> Most CpG sites in the genome are thus highly methylated, though CpG islands, which are clusters of adjacent CpG sites, often are unmethylated.<sup>24</sup>

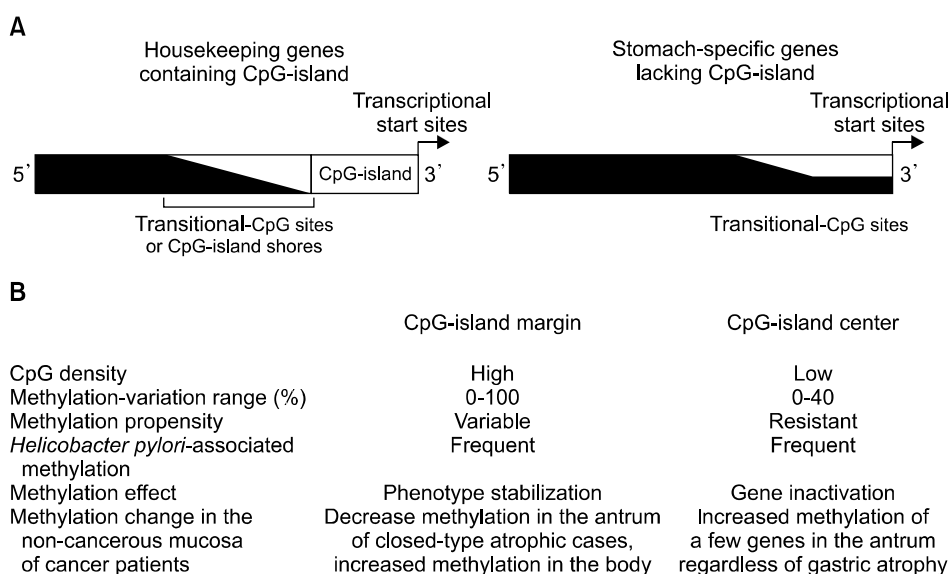
Studies have demonstrated that both hypermethylation of tumor-suppressor genes and hypomethylation of retroelements are frequently found in gastric cancer.<sup>25</sup> DNA hypermethylation of tumor-suppressor genes such as *CDHI*, *CDKN2A*, *MLH1*, and *RUNX3* was associated with early detection of gastric cancer,<sup>26</sup> while the methylation levels of retroelements are decreased in precancerous IM and dysplasia lesions as well as cancer lesions.<sup>27</sup> However, gene-specific hypermethylation and genome-wide hypomethylation are known to be independent events in carcinogenesis.<sup>28</sup> Obviously, the role of methylation in cancer development remains frustratingly murky.

## 2. CpG-island margins vs. CpG-island centers

There have been several studies on DNA methylation from endoscopy-obtained non-neoplastic gastric mucosa. The methylation level of *CDKN2A* was shown to be both high in patients with IM, and correlated with lower PG/I/I ratio.<sup>29</sup> Over-expression of *TP73* was suggested as a marker for assessment of high-risk chronic gastritis.<sup>30</sup> The methylation levels of *MOS* in non-neo-

plastic gastric mucosa were found to be increased in gastric cancer patients.<sup>31</sup> Meanwhile, in a comparison of in healthy *H. pylori*-infected subjects with cancer patients, it was determined that most of the CpG-island genes had been similarly methylated, whereas only a few were more frequently methylated in the cancer patients.<sup>32-34</sup> The genes displaying more frequent methylation in the cancer patients were frequently methylated in the antrum where *H. pylori* infection had initiated AG, but infrequently in the body.<sup>32</sup> Moreover, those genes' methylation was not associated with mucosal atrophy, IM, or degree of inflammation. Clearly, more precise *H. pylori*-associated methylation markers are needed for identification of high-risk individuals at early stages of gastric cancer.

We have suggested a new DNA methylation concept. CpG-island regions can be divided into two parts, the center and the margin. Contrary to most studies on DNA methylation focusing on the CpG-island center, we emphasized the CpG-island margin, or in other words the transitional-CpG sites, which are the methylation-variable sites between hypomethylated genes and hypermethylated retroelements (Fig. 1A).<sup>35</sup> The densely methylated retroelements that are distributed throughout the human genome might initiate the spread of DNA methylation to the adjacent genes in response to down-regulation of adjacent genes.<sup>36</sup> The retroelements are classified into two groups according to their ability to encode reverse transcriptase for self-replication: autonomous *LI* (a *LINE* element) and *LTR* (long terminal repeats) and non-autonomous *Alu* (a *SINE* element). Housekeeping genes contain rich CpG islands in promotor regions, where tissue-specific genes lack CpG islands.<sup>37</sup> The CpG-island centers of housekeeping genes are usually resistant to



**Figure 1.** Methylation-variable CpG sites around transcriptional start sites. (A) Schematics displaying locations of transitional-CpG sites in housekeeping genes and stomach-specific genes. The transitional-CpG sites are located between the highly methylated retroelements (closed box) and the weakly methylated genes (open box). (B) Comparison of methylation properties between CpG-island margins and CpG-island centers in housekeeping genes containing CpG islands.

DNA methylation (Fig. 1B). The methylation effect of CpG-island centers is the inactivation of individual genes. Compared with CpG-island centers, CpG-island margins are variably methylated in a tissue-type-dependent manner. In *H. pylori*-infected gastric mucosa, they display age-related methylation changes.<sup>38</sup> The term of CpG island shores, which is similar to the transitional CpG sites, was described in a sense that it was related to the induction of terminal cell differentiation.<sup>39</sup>

We have proposed that the role of methylation might be the stabilization of new stem cell phenotypes rather than gene inactivation.<sup>38</sup> Chronic *H. pylori* infection induces repopulation of the stomach with bone-marrow-derived cells, which suggests that epithelial cancers can arise from such cells.<sup>40,41</sup> New fixed stem cells in the *H. pylori*-infected mucosa can be methylated to preserve the re-established transcription profiles.<sup>36</sup> That is, the methylation of numerous housekeeping genes stabilizes newly established phenotypes of stem cells recruited into the *H. pylori*-infected stomach mucosa. The transitional-CpG sites are promptly methylated under the influence of adjacent retroelements, which can lock the re-established transcription profiles of new stem cells. Accordingly, the role of methylation is considered to be the stabilization of new stem cell phenotypes. Therefore, the transitional-CpG sites associated with *H. pylori* infection can be applied as potential markers for gastric stem cells.

### 3. *Helicobacter pylori*, DNA methylation, and gastric cancer

At the present time, there are no recognized suitable markers of gastric dysplasia or cancer other than atrophy, IM and *H. pylori* status.<sup>42</sup> According to the field cancerization theory, DNA methylation changes induced by *H. pylori* infection accumulate in the gastric mucosa before cancer cells are detected by endoscopy, since some new stem cells can possess both cancer-related methylation patterns and oncogenic mutations prior to the development of gastric cancer.<sup>43,44</sup> The prevalence of *H. pylori* in early gastric cancer has been found to be higher than that in advanced gastric cancer.<sup>45</sup> Whereas the histological characteristics of advanced cancer in the elderly typically show severe atrophic changes caused by the presence of long-standing *H. pylori* infection, by the time of cancer detection, the *H. pylori* infection has disappeared. This indicates that *H. pylori* infection potentially induces methylation, and that the methylation level decreases to a certain level after active *H. pylori* infection disappears. Specific CpG islands in non-cancerous gastric mucosa might be associated with gastric cancer risk in *H. pylori*-positive individuals who have higher methylation levels than those who

are *H. pylori*-negative.<sup>32,46</sup> Quantitative methylation levels have been found to be higher in GC patients than in healthy controls, irrespective of *H. pylori*.<sup>46</sup> They can be associated with gastric cancer in *H. pylori*-negative individuals who had past *H. pylori* infection.<sup>47</sup>

We have conducted several studies on transitional-CpG methylation as an epigenetic marker.<sup>35,36,38,48</sup> Most studies on DNA methylation of CpG-island centers have used DNA only from the antrum, not from the body of the stomach. However, we demonstrated that the methylation patterns of the transitional-CpG sites differed between the stomach antrum and body (Fig. 1B).<sup>38</sup> We semi-quantitatively analyzed, by radioisotope-labeling methylation-specific polymerase chain reaction, methylation-variable sites at the CpG-island margins and near the transcriptional start sites of tissue-specific genes lacking CpG islands. Specifically, we selected ten housekeeping genes adjacent to *Alu* (*CDHI*, *ARRDC4*, *PPARG*, *TRAPPC2L*, *MLH1*, and *SHH*) or *LTR* retroelements (*MMP2*, *CDKN2A*, *RUNX2*, and *RUNX3*) along with nine stomach-specific genes (*TFF2*, *PGC*, *ATP4B*, *TFF1*, *TFF3*, *GHRL*, *PGA*, *ATP4A*, and *APC*). Both the housekeeping genes and stomach-specific genes were rapidly methylated to a high-plateau frequency in the *H. pylori*-positive antrum, while most of the housekeeping genes slowly increased to a peak frequency in the body. High methylation levels of transitional-CpG sites in the body of non-cancerous gastric mucosa have been identified as potential cancer-risk-predicting markers.<sup>48</sup> The housekeeping genes according to our results tended to be frequently over-methylated in the body mucosa of the patients with gastric cancer compared with those of the *H. pylori*-positive controls. High-frequency over-methylation was defined as two or more over-methylated genes among the *LTR*-adjacent genes (*MMP2*, *CDKN2A*, *RUNX2*, and *RUNX3*) and one or more over-methylated genes in both the *Alu*-adjacent gene group (*CDHI*, *ARRDC4*, *PPARG*, and *TRAPPC2L*) and the stomach-specific gene group (*TFF2*, *PGC*, *ATP4B*, *TFF1*, *TFF3*, *GHRL*, *PGA*, and *ATP4A*). The body, not the antrum, was significantly associated with the risk of gastric cancer with open-type atrophy (OR, 13.4; 95% confidence interval, 3.8-46.8). Additionally, the OR value in early gastric cancer was much greater than in advanced gastric cancer (OR, 18.8 vs. 8.3).<sup>48</sup> That is, slowly over-methylated genes were frequent in the body of patients with gastric cancer with open-type atrophy. By contrast, the methylation of *Alu*-adjacent genes rapidly changed in the antrum, which was hardly increased in the antrum of patients with gastric cancer.<sup>48</sup> These markers therefore would be helpful for recognizing gastric cancer early, prior to detection of lesions by endoscopy.

#### 4. Clinical implications and future directions

DNA methylation is expected to be a good biomarker in clinical practice. First, it would prove helpful for setting the optimal screening interval for high-risk subjects, which at present remains controversial. A retrospective cohort study in Korea indicated that the optimal interval was 2 years, which finding is consistent with the nationwide gastric cancer screening program.<sup>49</sup> Another Korean study suggested that whereas endoscopy intervals of 3 years or less showed similar benefits, family members of gastric cancer patients should be screened more frequently than every three years.<sup>50</sup> Similarly, a European guideline recommended that patients with extensive AG and/or IM undergo an endoscopic check-up every three years.<sup>51</sup> But subjects showing low-risk results by biomarker would not need to be tested even every two years. This would improve, to a certain extent at least, cost effectiveness. Also, it would facilitate the formulation and adoption of new, other-than-mass-screening strategies for management of high-risk subjects. Secondly, DNA methylation would be helpful for establishing follow-up treatment plans for patients having undergone endoscopic submucosal dissection (an endoscopy-based therapeutic modality for treatment of gastric neoplasms), as it is highly possible that patients with gastric neoplasm will be found to have other synchronous and/or metachronous lesions as well. DNA methylation as a biomarker, again after completion of endoscopic submucosal dissection, would be useful as a means of estimating the prognosis and the predictability for recurrence of early gastric cancer.

It is well accepted that precancerous theory has explained the gastritis-metaplasia-carcinoma sequence.<sup>10</sup> Meanwhile, 'paracancerous' concept has been suggested, according to which most IMs, known as 'precancerous' lesions, are only 'paracancerous'.<sup>52</sup> In fact, about one-third of cancer patients have shown no precancerous lesions in the background mucosa of cancer tissues.<sup>11,13</sup> Thus, the paracancerous theory might complement the precancerous theory. In this light, new screening-interval guidelines for gastric cancer should be suggested considering individual risk based on age, severity of atrophy, *H. pylori* status, and DNA methylation pattern.

We classified atrophic severity into closed-type AG, in which the atrophic border remains on the lesser curvature of the corpus, and open-type AG, in which the atrophic border extends along the anterior and posterior walls of the stomach. This classification was based on the concept that, because *H. pylori*-induced damage progresses from the antrum into the gastric corpus, an atrophic

border can be visualized along the lesser curvature of the corpus followed by the greater curvature.<sup>53,54</sup> We have already suggested the high-risk methylation pattern in antrum and body.<sup>48</sup> Further studies on transitional-CpG methylation can accelerate the clinical application of epigenetic markers for *H. pylori* infection and cancer-risk prediction.

## CONCLUSIONS

*H. pylori* infection promotes the recruitment of bone-marrow stem cells that up-regulate stomach-specific genes for adaptation to the stomach-tissue environment. Methylation-variable sites around CpG islands, also known as transitional-CpG sites, are concurrently methylated in both *H. pylori*-positive subjects and *H. pylori*-negative subjects with a history of infection. The powerful methylation changes at transitional-CpG sites are likely to provide information on the process of glandular reorganization; as such, they might also be useful biomarkers for detection of high-risk factors related to gastric cancer. Certainly, they suggest powerful new guidelines for screening of high-risk subjects.

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## CONFLICTS OF INTEREST

No potential conflicts of interest were disclosed.

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