Research Article

Association between Genetic Instability and Helicobacter pylori Infection in Gastric Epithelial Dysplasia

Jin Su Kim, Woo Chul Chung, Kang-Moon Lee, Chang Nyol Paik, Kyeong Soo Lee, Hye Ji Kim, Young Wook Kim, Ji Han Jung, Seung June Noh, and Yun Kyung Lee

1 Department of Internal Medicine, College of Medicine, The Catholic University of Korea, 93 Jungbu-daero, Paldal-gu, Suwon 442-723, Republic of Korea
2 Department of Pathology, College of Medicine, The Catholic University of Korea, 93 Jungbu-daero, Paldal-gu, Suwon 442-723, Republic of Korea
3 The Research Institute of St. Vincent Hospital, 93 Jungbu-daero, Paldal-gu, Suwon 442-723, Republic of Korea
4 Department of Pathology, Samsung Medical Center of Korea, 50 Ilwon-dong, Gangnam-gu, Seoul 135-710, Republic of Korea

Correspondence should be addressed to Woo Chul Chung, jwchulkr@catholic.ac.kr

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Background. In gastric carcinogenesis, changes of DNA methylation appear to be an early molecular event, and the genome-wide methylation state is closely correlated with the level of long interspersed nucleotide element-1 (LINE-1) methylation. In this study, we measured LINE-1 methylation level according to genetic instability and evaluated the effect of Helicobacter pylori infection on genetic instability in gastric epithelial dysplasia.

Methods. Total 100 tissue samples of gastric epithelial dysplasia were analyzed. Seven loci that linked to tumor suppressor genes were used to identify significant structural chromosomal aberrations. Microsatellite status was investigated for two different microsatellite marker loci (BAT25 and BAT26). Also, we measured LINE-1 methylation level by combined bisulfite restriction analysis (COBRA-LINE-1) method.

Results. There were no significant differences of LINE-1 methylation level according to chromosomal/microsatellite instability and H. pylori state. In the dysplastic lesions with H. pylori infection, LINE-1 methylation level of MSI lesion was significantly lower than that of microsatellite stable (MSS) lesion (40.23 ± 4.47 versus 43.90 ± 4.81%, P < 0.01).

Conclusions. In gastric epithelial dysplasia with H. pylori infection, MSI is correlated with reduced LINE-1 methylation level. Coexistence of H. pylori infection and MSI might be a driving force of gastric carcinogenesis.

1. Introduction

Epidemiological studies in the last decade have established a strong causal relationship between Helicobacter pylori (H. pylori) infection and gastric cancer, and this bacteria has been classified as a Group I carcinogen by the World Health Organization (WHO) [1–3]. Previously, Correa suggested a human model of gastric carcinogenesis, and he postulated that the development of gastric cancer starts from chronic gastritis to gastric atrophy, intestinal metaplasia, dysplasia, and finally invasive cancer [4]. H. pylori infection stimulates cell proliferation in the gastric epithelium and induces apoptosis. It results in imbalance between apoptosis and proliferation and produces alterations or mutations of genes [5, 6]. Eventually, it increases the risk of developing gastric cancer. In the view of this point, the eradication therapy of H. pylori would be an attractive therapeutic modality, but it does not prevent the development of gastric cancer in all patients [7, 8]. Researchers are needed to further elucidate how H. pylori infection increases the risk of gastric cancer.

In cancer cell, abnormal DNA methylation is characterized by bidirectional changes—regional CpG island hypermethylation and generalized genomic hypomethylation. Both kinds of changes are observed simultaneously, but these two changes are not reciprocal. They might be independent events [9]. Several studies suggest that genome-wide hypomethylation generally arises earlier, whereas hypermethylation occurs in promoters and is usually
2.1. DNA Extraction and Assessment of Loss of Heterozygosity for evaluation of both from antrum and corpus after 4 weeks of the endoscopic or CLO test. In the present study, two biopsies were taken evaluated according to the histological results (silver stain ≥ tumor sites were checked for the tumor cell contents diagnosis of the tissue samples was confirmed by two glass slide and stained with hematoxylin and eosin. The dysplasia/cancer and normal tissues were placed on a lesion; they were obtained by gastric biopsy just after an intact mucosa and were at least 1 cm from the mucosal was excluded from the study. All normal tissues had grossly surgical blade, the pathologist performed the microdissection according to chromosomal/microsatellite instability and H. pylori status was measured by stereomicroscope under a xylenes and alcohol. Using a 30-gauge needle and a pointed depara

2. Materials and Methods

All tissues were excised by therapeutic endoscopic mucosal resection. The diagnosis of tissue sample was confirmed by two different histopathologists according to the revised Vienna classification; when they disagreed, the tissue sample was excluded from the study. All normal tissues had grossly intact mucosa and were at least 1 cm from the mucosal lesion; they were obtained by gastric biopsy just after an endoscopic mucosal resection. The H. pylori status was evaluated according to the histological results (silver stain or CLO test). In the present study, two biopsies were taken both from antrum and corpus after 4 weeks of the endoscopic resection for evaluation of H. pylori infection.

2.2. Assessment of Microsatellite Instability (MSI). DNA samples were amplified using two different oligonucleotide pairs specific for the recommended microsatellite loci BAT25 and BAT26. Primer sequences (Integrated DNA Technologies, Iowa, USA) were: BAT25 (forward 59-TGGCTCCTCAAGAATGTAAGTGTAACAAATATAAA, reverse 59-ATGTAA GT-39, and reverse 59-TCTGCATTTTAACTA-TCGCCCTCCAAGAATGTAAGTGTAACAAATATAAA, at an annealing temperature of 50°C. The PCR products were digested with the T aqI restriction enzyme, which recognizes sequences containing CpG. The extracted DNA was treated with sodium bisulfite and isolated using the EZ DNA methylation kit (Zymo Research, Orange, CA, USA). Bisulfite-treated DNA was amplified by 40 cycles of PCR with two primers, LINE 3 (5V-GYGTAAGGGGTAGGAGTTTTTT) and LINE 4 (5V-AACRTAAAAACCTCCRAACCAATAAAA), at an annealing temperature of 50°C. The PCR products were digested with the TaqI restriction enzyme, which recognizes TCGA, for 1 hour at 65°C, and then were separated by electrophoresis on 2% agarose gels. The densities of the

2.3. Assessment of LINE-1 Methylation Status. A modified long interspersed nucleotide elements-combined bisulfite restriction analysis (COBRA LINE-1) method was used to analyze LINE-1 methylation status of the cancers [17, 21, 22]. This method is based on the principle that cytosine in DNA is converted to uracil when DNA is treated with sodium bisulfite, whereas methylated cytosine is protected from the conversion. Thus, the methylated and unmethylated cytosine could be distinguished by digestion with a restriction enzyme that recognizes sequences containing CpG. The extracted DNA was treated with sodium bisulfite and isolated using the EZ DNA methylation kit (Zymo Research, Orange, CA, USA). Bisulfite-treated DNA was amplified by 40 cycles of PCR with two primers, LINE 3 (5V-GYGTAAGGGGTAGGAGTTTTTT) and LINE 4 (5V-AACRTAAAAACCTCCRAACCAATAAAA), at an annealing temperature of 50°C. The PCR products were digested with the TaqI restriction enzyme, which recognizes TCGA, for 1 hour at 65°C, and then were separated by electrophoresis on 2% agarose gels. The densities of the
digested and undigested bands were obtained by scanning with Gel Doc XR (Bio-Rad, Philadelphia, USA) and scoring with Quantity One Software (Bio-Rad, Philadelphia, USA). The ratio of the digested fragments (80 bp) derived from the methylated DNA divided by the sum of the digested fragments and the undigested fragments (160 bp) derived from the unmethylated DNA represents the fractional methylation (expressed as a percentage) at the LINE TaqI site (Figure 2).

2.4. Statistical Analysis. For the quantitative variables, the mean and its standard deviation were calculated. For the qualitative variables, the percent and its 95% confidence interval (95% CI) were calculated. We used the $\chi^2$ test to analyze the association between the $H. pylori$ status and other baseline characteristics. For comparison of age and the level of LINE-1 methylation we used the unpaired $t$ test. We used the SPSS statistical package (version 12.0.1) for all analyses.

3. Results

Total 100 tissue samples (from 61 men, mean age 62.57 ± 6.76 years; 39 women, mean age 63.97 ± 6.34 years) were examined and analyzed. When the gastric epithelial dysplasia (GED) was divided according to the revised Vienna classification, 50 tissues were low-grade (category 3) and 50 were high-grade dysplasia or intramucosal cancer (category 4). Total 54 tissue samples of GED had associated $H. pylori$ infection.

3.1. LOH in Gastric Epithelial Dysplasia. The incidence of LOH was 83% (83/100) in GED, and the frequencies of
LOH were 34% on APC (DSS505), 40% on 10p (D10S501), 48% on 10p (D10S602), 14% on p53 (TP 53), 40% on BRCA1 (D17S855), 51% on DCC (D18S58), and 45% on DCC (D18S61), respectively. According to the classification of chromosomal loss described previously, GED was divided into negative (LOH-negative), low-level (LOH-L; 3 or fewer losses), and high level (LOH-H; 4 or more losses). The incidence of LOH-L was 47% (47/100), whereas LOH-H was 36% (36/100). The frequencies of LOH with \( H. pylori \) infection were 76.5% (13/17), 48.9% (23/47), and 50.0% (18/36) in LOH-negative, LOH-L, and LOH-H lesion, respectively. There was no significant difference between \( H. pylori \) infection and LOH state \( (P = 0.06) \). LINE-1 methylation level of lesions with LOH-negative was not significantly different from that of LOH-positive. Also, LINE-1 methylation level of lesions with LOH-L was not significantly different from that of LOH-H \( (P = 0.06) \). LINE-1 methylation level of lesions with LOH-H was not significantly different from that of LOH-positve \( (P = 0.06) \).

3.2. MSI in Gastric Epithelial Dysplasia. The frequency of microsatellite instability (MSI) was 36% (36/100), and instability rates for the BAT25 and BAT26 were 19% and 29%, respectively. The frequency of BAT25 (+) with \( H. pylori \) infection was 11%, and BAT26 (+) with \( H. pylori \) infection was 22%.

LINE-1 methylation level of MSI was not significantly different from that of microsatellite stable (MSS) lesions \( (P = 0.06) \). The frequency of MSI with \( H. pylori \) infection was 61.1% (22/36), and it was not different from that of MSS lesion \( (50.0\%, 32/64) \) \( (P = 0.28) \). In the GED with \( H. pylori \) infection, LINE-1 methylation level of MSI lesion was significantly lower than that of microsatellite stable (MSS) lesion \( (40.23 \pm 4.47, 43.90 \pm 4.81, P < 0.01) \) \( (P = 0.06) \).

3.3. Gastric Epithelial Dysplasia Subgrouped by the Revised Vienna Classification. The tissue samples were divided into two groups according to the revised Vienna classification: low-grade dysplasia (category 3) and high-grade dysplasia/intramucosal cancer (category 4). Twenty-six patients with category 3 GED and 28 patients with category 4 GED had associated \( H. pylori \) infection \( (P = 0.68) \). The category 4 lesion had lower level of LINE-1 methylation than the category 3 lesion \( (38.95 \pm 4.28 \text{ versus } 44.93 \pm 4.29\% , P < 0.01) \). For categories 3 and 4, the difference in the frequency of LOH-H was not significant \( (30.0\%, 15/50 \text{ versus } 42.0\%, 21/50, P = 0.21) \). The frequencies of MSI positive were 30% and 42.0% \( (22/51) \) in categories 3 and 4 \( (P = 0.05) \). In category 3 lesion, LINE-1 methylation level of MSI was significantly lower than that of MSS \( (43.18 \pm 3.66 \text{ versus } 45.68 \pm 4.41\% , P = 0.05) \).

4. Discussion

It is widely accepted that gastric cancer develops through the accumulation of genetic or epigenetic alterations affecting oncogenes and tumor suppressor genes. These alterations involve the mechanisms that control genetic instability. Genetic instability is divided into two categories, chromosomal instability (CIN) and microsatellite instability (MSI) and whether the instability is at the chromosomal or nucleotide level in a lesion \[23, 24\]. CIN has been recognized as the most common feature of sporadic gastric cancers and CIN phenotype has been reported in up to 84% of gastrointestinal tumors \[25\], which is compatible with our result. The consequence of CIN is an imbalance in the chromosome number and an increased rate of loss of heterozygosity (LOH). An increased rate of LOH is an important property of CIN, because it accelerates the inactivation of the tumor suppressor genes \[26\]. In colon cancer model, CIN is an important event in the tumor initiation and progression, and LOH and MSI are inversely correlated \[27\]. However, in gastric cancer, these are not mutual. In present study, CIN and MSI coincided in 24% of gastric epithelial dysplasia, whereas evidence of both CIN and MSI was lacking in 4%. In the latter cases, it may be associated with the transcriptional silencing of genes by epigenetic alterations.

It is postulated that persistent infection with \( H. pylori \) initiates chronic inflammation, which induces increased...
Figure 3: Level of LINE-1 hypomethylation of gastric epithelial dysplasia according to H. pylori state and genetic instability. (a) Irrespective of H. pylori infection, there were no differences of LINE-1 methylation level between LOH-L and LOH-H. (b) In dysplasia with H. pylori infection, LINE-1 methylation level of MSI is significantly reduced than that of MSS. Box plots illustrate median values, 25th and 75th percentiles, and outliers on a linear scale. The unpaired t test was applied for nonparametric statistical analysis, and * was considered statistically significant (P < 0.05).

Table 2: LINE-1 methylation level according to genetic instability (chromosomal instability and microsatellite instability) in gastric epithelial dysplasias categorized by the revised Vienna classification.

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<tr>
<th>Category 3 GED</th>
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<tr>
<td>n</td>
<td>LINE-1 methylation</td>
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<tr>
<td>Chromosomal instability</td>
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<td>LOH (-)</td>
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<tr>
<td>LOH-L</td>
<td>27</td>
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<td>LOH-H</td>
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<td>Microsatellite state</td>
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<td>MSS</td>
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LOH: loss of heterozygosity; LOH-L: LOH (+) < 3 loci; LOH-H: LOH (+) > 4 loci; MSS: microsatellite stable; MSI: microsatellite instable, * statistically significant.

MSI is a molecular phenotype for human cancers with defects in the postreplicative DNA mismatch repair system and its deficiency resulted in MSI phenotype [30, 31]. It is known that the frequency of MSI in gastric cancer is between 25% and 50% [25]. MSI-positive gastric cancers have been reported to be located in the distal stomach and associated with intestinal-type histology and favorable clinical features [32]. Despite of association with H. pylori and MSI, there was no difference of LINE-1 methylation level according to microsatellite state or H. pylori infection state in our results. However, in gastric epithelial dysplasia with H. pylori infection, MSI had a tendency of the reduced level of LINE-1 methylation. It suggested that H. pylori might appear as a cofactor for inducing gastric carcinogenesis.

To identify MSI, the five suitable markers including BAT25, BAT26, D2S123, D17S250, and D5S346 have been proposed [33]. Nevertheless, we use only two monomorphic mononucleotides (BAT25 and BAT26) in this study. Although the frequency for MSI-positive was considerably similar with the previous study [25], the potential pitfall to
define MSI existed because of a huge number and diversity of microsatellite regions in the human genome. However, previous studies show that BAT25 and BAT26 are more sensitive and better markers for microsatellite instability detection than their dinucleotide counterparts [34, 35]. These markers are considered to be sensitive in detecting MSI of tumors and can be testable even in the absence of normal tissue.

Gastric low-grade dysplasia can progress into an invasive form, but all cases of it do not transform to advanced carcinoma. It has been reported that approximately 15%–30% of low-grade dysplasia progress to high-grade dysplasia or adenocarcinoma [36–38]. To date, there is no doubt that H. pylori infection is a major risk factor in the pathogenesis of gastric cancer. The curious problem is which factor determines progression of gastric epithelial lesions. Whether H. pylori infection can contribute to the progression of low-grade dysplasia is debatable, and whether the eradication of H. pylori infection would reduce the risk of gastric cancer is also controversial. In this point of view, our results are very hopeful. It supports that progression of gastric epithelial dysplasia to true gastric cancer could be blocked after H. pylori eradication in the selected cases—MSI positive state.

In conclusion, MSI-positive gastric epithelial dysplasia with H. pylori infection is correlated with reduced LINE-1 methylation level. Coexistence of H. pylori infection and MSI might be a driving force of gastric carcinogenesis. To clarify these results, the investigators will conduct a prospective, randomized, and population-based study to determine whether H. pylori eradication can reduce the incidence of gastric cancer.

Figure 4: Level of LINE-1 hypomethylation in gastric epithelial neoplasias categorized by the revised Vienna classification. There were no significant differences of LINE-1 methylation level according to the degree of LOH and MSI state. Except in category 3, the lesions with MSI had the lower LINE-1 methylation level than that of MSS. Box plots illustrate median values, 25th and 75th percentiles, and outliers on a linear scale. The unpaired t test and one way ANOVA were applied for nonparametric statistical analysis, and * was considered statistically significant (P < 0.05).

References


