Differential LINE-1 Hypomethylation of Gastric Low-Grade Dysplasia from High Grade Dysplasia and Intramucosal Cancer

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Background/Aims: Gastric epithelial dysplasia is considered a precancerous lesion with a variable clinical course. There is disagreement, however, regarding histology-based diagnoses, which has led to confusion in choosing a therapeutic plan. New objective markers are needed to determine which lesions progress to true malignancy. We measured LINE-1 methylation levels, which have been reported to strongly correlate with the global methylation level in gastric epithelial dysplasia and intramucosal cancer.

Methods: A total of 145 tissue samples were analyzed by two histopathologists. All tissues were excised by therapeutic endoscopic mucosal resection and paired with adjacent normal tissue samples. A modified long interspersed nucleotide elements–combined bisulfite restriction analysis (COBRA-LINE-1) method was used.

Results: Gastric epithelial dysplasia and intramucosal cancer tissues had significantly lower levels of LINE-1 methylation than adjacent normal gastric tissues. High-grade dysplasia and intramucosal cancer were distinguishable from low-grade dysplasia based on LINE-1 methylation levels. Furthermore, the distinction could be determined with high sensitivity and specificity, as shown by the receiver operating characteristic (ROC) curve (AUC, 0.82; 95% confidence interval, 0.74 to 0.88).

Conclusions: LINE-1 methylation levels may provide a diagnostic tool for identifying high-grade dysplasia and intramucosal cancer.

Key Words: LINE-1 methylation; Gastric epithelial dysplasia; Intramucosal cancer

INTRODUCTION

Gastric epithelial dysplasia is considered to be a precursor lesion of gastric cancer, and has various clinical courses. Some progress to adenocarcinoma, whereas others persist unchanged for many years.¹,² Moreover, without uniform and definite criteria for the diagnosis of gastric epithelial dysplasia, treatment is inconsistent and often results in over-treatment of gastric epithelial dysplasia by surgical resection.³,⁴ In an attempt to resolve this problem, the revised Vienna classification has been proposed,⁵,⁶ providing a consensus on guidelines for clinical management. The removal of category 3 (low grade dysplasia) lesions may not be necessary, whereas the removal of category 4 lesions (high grade dysplasia and intramucosal cancer) must be obligatory. But this classification is not perfect and there are frequently cases that do not easily fit into the diagnostic categories of the revised Vienna classification. Approximately 15% to 30% of low grade dysplasia progress to high grade lesions and/or adenocarcinomas,⁵,⁸ so this classification system needs a complementary marker. Moreover, the inter-observer and intra-observer variation of histopathologists should be never neglected in the diagnosis of gastric epithelial dysplasia and intramucosal cancer.

The goal of this study was to investigate the level of LINE-1 methylation in gastric epithelial dysplasia and intramucosal cancer samples as well as the level of LINE-1 methylation in the adjacent mucosa. The results could potentially be of clinical interest and we hoped to provide the pathologist with an objective marker to distinguish between low grade dysplasia and high grade dysplasia or between high grade dysplasia and intramucosal cancer.
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. The lesions were histopathologically assigned to divide into 3 groups according to the revised Vienna classification system: low grade dysplasia (category 3), high grade dysplasia (category 4.1) and intramucosal cancer (categories 4.2, 4.3, and 4.4).

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 microscopic examination showed no evidence of malignant cells. Each patient was classified as Helicobacter pylori (H. pylori) positive or negative according to the histological results. In the present study, the resection specimen and gastric biopsy of surrounding mucosa were stained with hematoxylin and eosin and silver stains. To assess H. pylori state accurately, two biopsies were taken both from antrum and corpus after 4 weeks of the endoscopic resection. We evaluated with CLO test or histological examination.

1. DNA extraction

Four-micrometer-thick tissue sections from the dysplasia/cancer and normal tissues were placed on a glass slide and stained with hematoxylin and eosin. The diagnosis of the tissue samples was confirmed by two different histopathologists. Prior to DNA extraction, all the microdissected tumor sites were checked for the tumor cell contents ≥70% using a stereomicroscope under a ×40 magnification.

Two ten-micrometer-thick tissue sections from cancer samples and normal tissues were placed on glass slides. The tissue sections were added by xylene (1 mL), incubated for 10 minutes and repeated 3 times. These were then dehydrated in graded ethanol solutions (100% ethanol, 1 mL), dried without a cover glass for 10 minutes and repeated three times. The DNA was extracted from the tissues with 20 uL of extraction buffer (100 mmol/L Tris-HCl; 2 mmol/L ethylene diamine tetraacetic acid [EDTA], pH 8.0; 400 ug/mL of proteinase K) at 55°C overnight. The tubes were boiled for 7 minutes to inactivate the proteinase K, and cooling on ice. The solution was added by 20 uL of 100% ethanol (1 mL, 100%) and the tube was gently inverted. It was incubated at -20°C for 10 minutes and centrifuged at 12,000 rpm, 4°C for 5 minutes. DNA pellet was made by washing with ethanol (1 mL, 75%) and centrifuged at 14,000 rpm for 5 minutes. The pellet was dissolved by adding 20 uL of dextrose water, and then 1 uL of this extract was used for each polymerase chain reaction (PCR) amplification.

2. 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 and normal mucosa.9–11 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, LINE3 (5V-GYGTAAGGGTGAGGAGTTTTT) and LINE4 (5V-AARCTAAACCTCCCCRAAACCAATATAAAA), 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, Hercules, CA, USA) and scoring with Quantity One Software (Bio-Rad). 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.

3. Statistical analysis

For the quantitative variables, the mean and its standard error were calculated. For the qualitative variables, the percent and its 95% confidence interval (CI) were calculated. We used the χ² 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 and one way ANOVA. The diagnostic yield of the level of LINE–1 methylation, for the prediction of premalignant or early cancer lesions, was calculated using the area under the receiver operating characteristic (ROC) curve. The area under the ROC curve (AUC) is the measure of separation of two probability distributions: excellent for AUC values greater than 0.9, good for 0.8 to 0.9, fair for 0.7 to 0.8, poor for 0.6 to 0.7, and failure for values less than 0.6. We used the SPSS statistical package (version 12.0.1; SPSS Inc., Chicago, IL, USA) for all analyses.

RESULTS

Total 145 tissue samples were examined and thirteen tissue samples were excluded because of disagreement on diagnosis and inappropriate preparation of tissue. The concordance rate between the two histopathologists was 91.0% and 132 patients...