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Long interspersed nuclear element (LINE)-1 methylation level as a molecular marker of early gastric cancer

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ABSTRACT

Background/aims: This study was performed to examine the state of long interspersed nuclear element (LINE)-1 methylation level in gastric epithelial dysplasias (GEDs) and evaluate as a molecular marker for gastric carcinogenesis when it was compared with RUNX3 expression.
Methods: We examined 89 patients with GEDs subcategorized by the Vienna classification – 41 category 3 (low grade) and 48 category 4 (high grade/intramucosal carcinoma) lesion. All tissue samples were evaluated for RUNX3 immunohistochemical staining and the level of LINE-1 methylation.
Results: The rate of negative expression of RUNX3 in category 4 lesion was significant higher than category 3 ($P < 0.01$). LINE-1 methylation level was statistically different between category 3 and category 4 lesion ($P < 0.01$). Between positive and negative expression of RUNX3 in GEDs, there was a significant difference of LINE-1 methylation level ($P < 0.01$). The area under the ROC curve (AUC) of LINE-1 methylation level for diagnosis of category 4 lesion was 0.88 (95% CI, 0.76–1.00).
Conclusions: LINE-1 methylation level was well correlated with the Vienna classification of GED and it had a close relationship with the negative expression of RUNX3 in category 4 lesion. LINE-1 methylation level could be a good candidate for a molecular marker of early gastric cancer.

1. Introduction

In general, differential DNA methylation levels are observed during cancer initiation and development. The 2 types of methylation change in cancer include global DNA hypomethylation events and hypermethylation at specific promoter CpG islands of tumor suppressor genes. Hypermethylation and hypomethylation contribute separately to the process of carcinogenesis, but these two types of methylation alterations are not completely exclusive [1–4]. Global DNA hypomethylation is associated with genomic instability and a common finding in early carcinogenesis and progression [5,6]. Long interspersed nuclear element (LINE)-1, a highly repeated interspersed human retrotransposon, is ubiquitous and constitutes approximately 17% of the human genome. Its methylation status reflects the genome wide methylation level [7–9]. Therefore, LINE-1 has been shown to be representative for the overall losses of DNA methylation. Recent studies reported that decreased level of LINE-1 methylation was associated with a poor prognosis in advanced gastric cancer [10,11]. We hypothesized that LINE-1 would be a potential biomarker for early change of gastric cancer.

Runt-related transcription factor 3 (RUNX3) has been recognized as a tumor suppressor gene for gastric cancer. Excellent correlation between Runx3 mRNA and protein expression was observed [12]. Cytoplasmic staining of RUNX3 indicates that there is no functional RUNX3 in normal gastric epithelium [13]. Recently, the meta-analysis of pooled data supported a strong association between inactivation of the RUNX3 and gastric cancer [14]. Inactivation of the RUNX3 gene can be detected by promoter hypermethylation, or cytoplasmic mislocalization of RUNX3 which are the mechanisms for its inactivation [15,16]. Negative expression of RUNX3 by immunohistochemical (IHC) stain is well correlated with methylation status [17,18]. In this study, we choose the more easy method for diagnosis – IHC stain of RUNX3. Moreover, it is associated with tumor progression and provides useful clues for predicting the malignant behavior of gastric cancer [19].

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We aimed to examine the state of LINE-1 methylation level in gastric epithelial dysplasias (GEDs) subcategorized according to the Vienna classification as category 3 (low grade) and category 4 (high grade/intramucosal carcinoma) lesion [20]. Also, we would evaluate as molecular marker for gastric carcinogenesis, when it was compared with RUNX3 expression.

2. Materials and methods

All tissues were excised by therapeutic endoscopic mucosal resection, and paired with adjacent normal tissue samples. Tissues were examined by 2 histopathologists and further analyzed. In cases with lack of consensus, the tissue sample was excluded from the study to clarify the diagnosis. The diagnosis of *Helicobacter pylori* infection was based on histological results of Warthin-Starry silver stain in 2 random specimens from the antrum and body. The lesions were histopathologically assigned to 2 groups – category 3 (low grade) and category 4 (high grade/intramucosal carcinoma) according to the revised Vienna classification of GED.

2.1. The Vienna classification of gastrointestinal epithelial neoplasia

The Vienna classification was proposed in 2002 [20]. It distinguished five categories and correlated with therapeutic option: (1) negative for neoplasia/dysplasia, (2) indefinite for neoplasia/dysplasia, (3) mucosal low-grade neoplasia (low-grade adenoma/dysplasia), (4) mucosal high-grade neoplasia (high-grade adenoma/dysplasia, non-invasive carcinoma, suspicion of invasive carcinoma, and intramucosal carcinoma), and (5) submucosal invasion by carcinoma.

2.2. Immunohistochemical stain of RUNX3

All mucosal biopsies were fixed immediately in 10% buffered formalin and processed routinely. For each biopsy, serial 4-μm-thick, paraffin-embedded tissue sections were cut and stained with hematoxylin and eosin. Well oriented sections that contained glandular epithelium were selected. They were deparaffinized in xylene and rehydrated in graded ethanol. Endogenous peroxidase activity was blocked with 3% H_2O_2 in phosphate-buffered saline. For antigen retrieval, the sections were incubated in 10 mM citrate buffer (pH 6.0) using a microwave and then incubated with the primary antibodies. RUNX3 (1:200, AMC-2/RUNX3 Rabbit Polyclonal antibody, Active Motif®) was used as the primary antibody. Antibody detection was performed using the IMPRESS peroxidase reagent kit (VECTOR laboratory, Burlingame, CA) according to the manufacturer's protocol. Immunoreactive cells were identified by DAB peroxidase substrate kit (VECTOR laboratory, Burlingame, CA). The results of immunostaining for RUNX3 were considered positive if >10% of the tumor cells were stained. The microscopic features of RUNX3 were presented in Fig. 1.

2.3. 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 2 histopathologists. 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 resection and the microscopic examination showed no evidence of malignant cells. Prior to DNA extraction, all the microdissected tumor sites were checked for ≥70% tumor cell content using a stereomicroscope under a 40× magnification. Two 10-micrometer-thick tissue sections from cancer samples and normal tissues were placed on glass slides. The tissue sections were incubated with xylene (1 mL) for 10 min and the process was repeated thrice. The sections were then dehydrated in graded ethanol solutions (100% ethanol, 1 mL), dried without a cover glass for 10 min, repeated thrice. The DNA was extracted from the tissues with 20 μL of extraction buffer (100 mmol/L Tris–HCl; 2 mmol/L ethylene diamine tetraacetic acid, pH 8.0; 400 μg/mL of proteinase K) at 55 °C overnight. The tubes were boiled for 7 min to inactivate the proteinase K, and cooled on ice. A 20 μL (phenol:chloroform:isoamyl alcohol, 25:24:1) solution was added and centrifuged at 12,000 rpm, 4 °C for 5 min. This process was a de-proteinization to extract tissue DNA. The supernatant was added to ethanol (1 mL, 100%) and the tube was gently inverted. It was incubated at −20 °C for 10 min and centrifuged at 12,000 rpm, 4 °C for 5 min. DNA pellet was washed with ethanol (1 mL, 75%) and centrifuged at 14,000 rpm for 5 min. The pellet was dissolved by adding 20 μL of dextrose water; and 1 μL of this extract was subsequently used for amplification in each polymerase chain reaction (PCR).

2.4. 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 [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

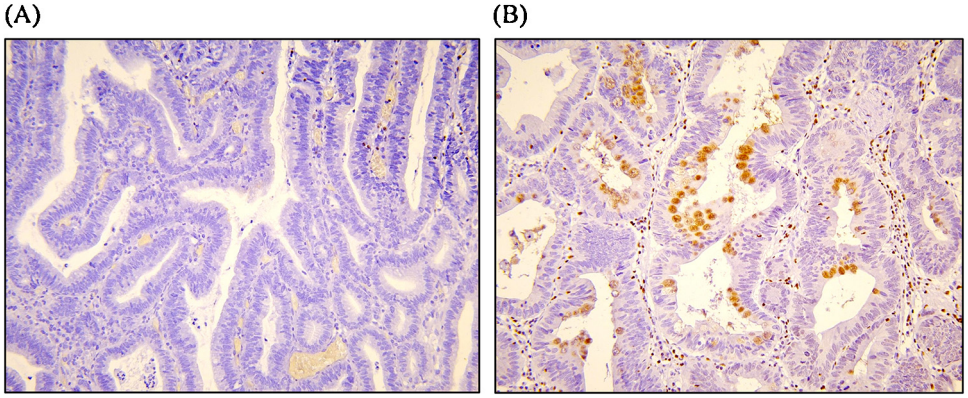

Fig. 1. Representative example of RUNX3 expression in gastric epithelial neoplasia categorized by the Vienna classification (Immunohistochemical staining findings). A: negative expression of RUNX3 in category 4 lesion (200×), B: positive expression in category 4 lesion (200×).