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Helicobacter pylori infection is an independent risk factor for Runx3 methylation in gastric cancer

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Abstract. Runx3, a member of the human runt-related transcription factor family, is known as a possible tumor suppressor gene for gastric cancer. Runx 3 expression is frequently suppressed by the promoter hypermethylation in gastric cancer cell lines and tissues. However, the precise mechanism of the induction of Runx3 methylation, which is considered to be a critical step in gastric carcinogenesis, remains to be elucidated. In the present study, we evaluated *runx3* gene methylation in 57 resected early gastric cancer specimens. Then, we correlated Runx3 methylation in the cancer tissue specimens with clinicopathological factors as well as the mucosal backgrounds, such as intestinal metaplasia surrounding the cancer cells and *Helicobacter pylori* (*H. pylori*) infection. Runx3 methylation was observed in 30 of the 57 (52.6%) cancer specimens, whereas methylation was detected in 10 of the 57 (17.5%) corresponding non-cancerous mucosae. In comparison to the clinicopathological factors, Runx3 methylation was significantly correlated with both age and tumor location. A multivariate analysis demonstrated that age and tumor location as well as *H. pylori* infection were independent risk factors for Runx3 methylation. We demonstrated for the first time that *H. pylori* infection contributes to Runx3 methylation in gastric cancer tissues. When a persistent infection by *H. pylori* continues in the middle/lower stomach for a long period, Runx3 methylation may be induced and the subsequent loss of Runx3 expression may therefore affect gastric carcinogenesis.

Introduction

The human runt-related transcription factors (Runx) are important targets of transforming growth factor (TGF)-ß superfamily signaling (1). The Runx genes *runx1*, *runx2* and *runx3* have been identified as human homologs of the *Drosophila* genes runt (1). In a recent study, the gastric mucosa of a Runx3 knockout mouse exhibited hyperplasia due to stimulated proliferation and suppressed apoptosis, which was accompanied by reduced sensitivity to TGF-ß (2). This finding identified Runx3 as a tumor suppressor gene for genesis of gastric cancer. In addition, intestinal metaplasia (IM) is generally regarded as a precancerous gastric lesion for intestinal type gastric cancer (3). In the Runx3 knockout mouse gastric epithelial cells, some cells differentiated into intestinal type cells suggesting that the loss of Runx3 expression triggered precancerous IM, which possibly leads to cancer development in the stomach (4).

In gastric cancer cell lines and tissues, the promoter region of the Runx3 gene is frequently methylated and such methylation is correlated with the loss of Runx3 expression (5). Runx3 methylation has also been found in non-cancerous diseases such as chronic gastritis, IM and gastric adenoma with less frequency than cancer (6). These studies demonstrate one possible carcinogenic pathway, namely that the loss of Runx3 expression by promoter methylation causes chronic gastritis or IM in gastric mucosa, which leads to the development of gastric cancer.

Helicobacter pylori (*H. pylori*) infection is a major causative factor for gastric cancer, chronic gastritis, peptic ulcers, atrophic gastritis and IM (7-9). The risk of patients with *H. pylori* infection developing gastric cancer is in the order of two- to six-fold according to most retrospective case-control and prospective epidemiologic studies (7). Several factors have been reported which affect gastric carcinogenesis by *H. pylori* infection (8,9). For instance, the cytotoxin-associated gene (cag) of *H. pylori* is one of the most studied virulences and has been reported to have oncogenic effects (8,10). On the other hand, several current reports have demonstrated that high levels of aberrant methylation of several CpG islands were isolated in *H. pylori* infected gastric mucosae and its possible association with gastric cancer risk (11). However, whether or not *H. pylori* infection directly induces Runx3 methylation in the gastric mucosa remains to be elucidated.

In the present study, we investigated Runx3 methylation in 57 early gastric cancer tissues and corresponding non-cancerous tissues. The existence of IM was histologically determined on adjacent mucosa surrounding the cancer cells. H. pylori infection in the samples was assessed by PCR of the 16S rRNA and cagA region in *H. pylori* DNA according to a

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previous study (12). The methylation status of Runx3 was compared with clinicopathological factors and the mucosal background such as IM or *H. pylori* infection. The aim of this study was to clarify whether or not *H. pylori* infection directly affects IM or Runx3 methylation in gastric cancer tissues.

Materials and methods

Patients and tissue samples. Fifty-seven patients with early gastric cancer, who underwent gastrectomy from March 2001 to July 2006 at Saga University Hospital, were eligible for the present study. The patients gave informed consent for specimen collection, which is required by the Ethics Committee. All of the specimens were examined macroscopically and histologically based on criteria proposed by the Japanease Research Society for Gastric Cancer (13). Intestinal metaplasia (IM) on the 57 cancer specimens was histologically assessed by two pathologists (D.M. and K.K.). Samples from cancer tissues and the corresponding non-cancerous mucosa were freshly obtained from the resected stomach and stored at -80˚C until use. DNA was purified using the EZ1 DNA tissue kit in BioRobot®EZ1 workstation (Qiagen, GmbH Hilden, Germany).

Bisulfite modification and methylation-specific PCR (MSP). Treatment of DNA samples with bisulfite converts all unmethylated cytosines to uracils, while leaving the methylcytosines unaltered. This allows for the subsequent differentiation of methylated and unmethylated sequences by methylation-specific PCR (MSP). Bisulfite modification of DNA (1 μg) was carried out according to the method described previously (14). Then, 30 μl of the modified DNA solution was obtained and 1 μl of 30 μl was subjected to MSP. The methylated (M) and unmethylated (U)-specific primers for Runx3 were synthesized according to a previous report (15). M-specific primers: 5'-TTCGTTTATTTTGTCGTCG TCGT-3' (forward), 5'-CGCTATTATACGTATTCCCG-3' (reverse). U-specific primers: 5'-TTTGGGTTTATGGGAAT ATG-3' (forward), 5'-TTCTACAACAACAACAACC-3' (reverse). PCR was performed using the exTaq polymerase system (Takara, Tokyo, Japan) under the following conditions. One cycle at 96˚C for 3 min, 35 cycles at 96˚C for 30 sec, 55˚C for 30 sec, 72˚C for 30 sec and 1 cycle at 72˚C for 5 min. An artificial methylated DNA (universal methylated DNA, Invitrogen, Purchase, NY, USA) and an unmethylated DNA isolated from normal lymphocyte were used for the methylation- and unmethylation-specific reactions, respectively. Distilled water without DNA was used as a negative control. PCR products were analyzed in 1.5% agarose gel and stained with ethidium bromide. The proposed size of the PCR product from the methylation-specific primer is 100 bp, whereas that from the unmethylation primer is 120 bp.

PCR detection of H. pylori infection. DNA of *H. pylori* on both of the cancer and the corresponding non-cancerous tissues was amplified using PCR. The primers were designed for the 16rDNA and cagA region in the *H. pylori* genome as previously reported (12). 16r DNA-specific primers: 5'-TGC GAAGTGGAGCCAATCTT-3' (forward), 5'-GGAACGTA TTCACCGCAACA-3' (reverse). CagA-East Asian-specific primers: 5'-AAAGGAGTGGGCGGTTTCA-3' (forward), 5'-CCTGCTTGATTTGCCTCATCA-3' (reverse). The experimental conditions for PCR were the same as for the MSP analysis. The proposed sizes were 118 bp for 16S rRNA and 91 bp for CagA-East Asian, respectively.

Table I. Relationship between Runx3 methylation and clinicopathological factors.

	Runx3		
	U	M	P-value
Age	62.5±2.32	68.6±1.82	0.04
Gender			
M	14	20	
F	13	10	0.26
Location			
U	8	1	
M/L	19	29	0.01
Histology			
Diff	16	16	
Undiff	11	14	0.65
T			
m	14	11	
sm	13	19	0.25
N			
-	24	25	
+	3	5	0.71
ly			
-	24	22	
+	3	8	0.19
v			
-	25	30	
+	2	0	0.22

U, upper stomach; M/L, middle/lower stomach; Diff, differenetiated carcinoma; Undiff, undifferentiated carcinoma; T, depth of cancer invasion; N, lymph node metastasis; ly, invasion to lymphatic vessel; v, invasion to venus vessel.

Statistical analysis. The statistical analysis was performed using the χ^2 test and the Mann-Whitney U test. The student's t-test was used to determine group differences. For all statistical analyses, the StatView® 5.0 software program (SAS Institute, Cary, USA) was used. Multivariate logistic regression analysis was used to analyze the risk factor for Runx3 methylation. Statistical significance was established at a value of $P<0.05$.

Results

MSP analysis of the runx3 gene in early gastric cancer tissues. Fifty-seven paired DNA from early gastric cancer and the

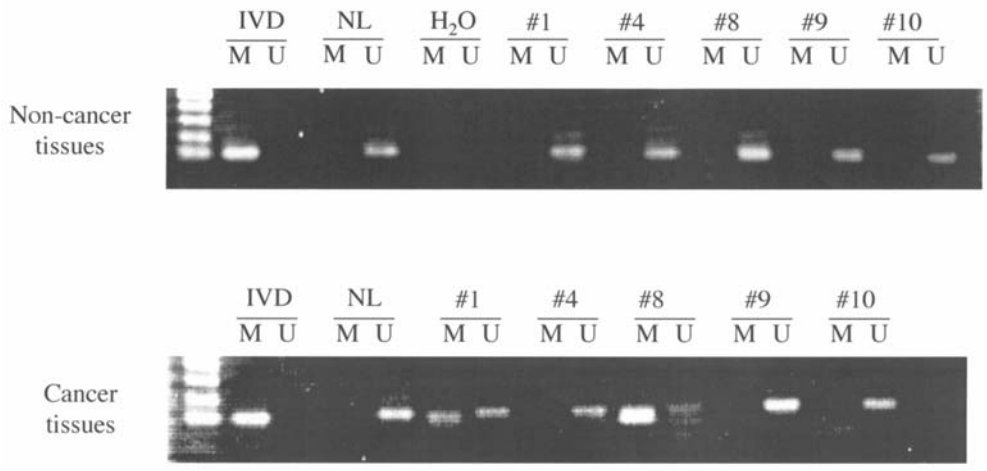

Figure 1. An MSP analysis of the *runx3* gene using 5 paired cancer and corresponding non-cancerous tissues (#1, #4, #8, #9 and #10). IVD: *in vitro* methylated DNA NL: DNA from normal lymphocyte. M, methylated; U, unmethylated.

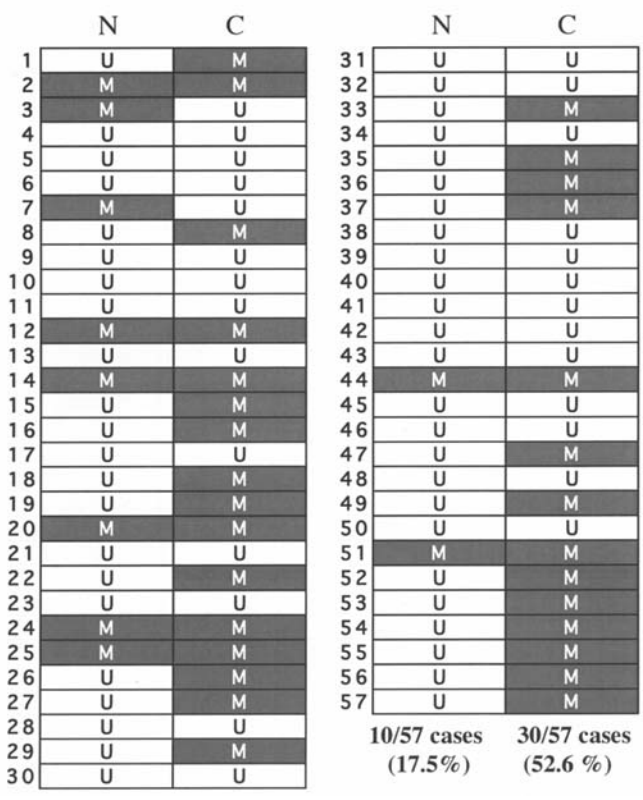

Figure 2. Summarized results of an MSP analysis using the 57 gastric cancer tissues and the corresponding non-cancerous mucosa. N, non-cancerous tissues; C, cancer tissues; M, methylated; U, unmethylated.

corresponding non-cancerous mucosa were treated with bisulfite and subjected to methylation-specific PCR (MSP) for detecting *runx3* gene methylation. As shown in Fig. 1, the PCR product at 100 bp from the methylation-specific primer was observed in a positive control IVD, whereas the DNA fragment at 120 bp length from unmethylation-specific primers was found in the normal lymphocyte (NL). No signal from the water sample was detected. In all 5 samples from the non-cancerous mucosa, there were unmethylation- and non-methylation-specific signals. In contrast, methylation-specific signals were found in cases 1 and 8 from the cancer specimens. A summary of the result of MSP is shown in Fig. 2. Thirty of the 57 cancer tissues (52.6%) were assessed as methylation positive, while 10 of the 57 (17.5%) normal mucosae were also assessed as methylated. Of the 10 non-cancerous mucosa with Runx3 methylation, cases 3 and 7 did not show the methylation in the corresponding cancer specimens.

Table II. PCR detection of 16S rRNA and cagA from *H. pylori* DNA in gastric mucosae.

	Non-cancerous tissues			Cancer tissues		
	16S rRNA			16S rRNA		
	-	+	P	-	+	P
cagA						
-	15	14		17	22	
+	3	25	0.001	0	18	<0.001

Relationship between Runx3 methylation and clinicopathological factors. Table I shows the relationship between Runx3 methylation in the 57 cancer tissues and the clinicopathological factors. Runx3 methylation was significantly correlated with age and tumor location, whereas the methylation did not show correlation with gender, depth of cancer invasion (T), histological type, lymph node metastases (N) and vessel invasion (ly and v).

Relationship between H. pylori infection and Runx3 methylation. We assessed *H. pylori* infection on the 57 paired samples by PCR of 16S rRNA and the cagA region in *H. pylori* DNA. Fig. 3 shows the PCR results of 16S rRNA and East-Asian cagA in 5 gastric cancer tissues. 16S rRNA at 118 bp length were observed in 4 of the 5 DNA samples, whereas the products at 91 bp from East-Asian cagA were 2 of the 5 samples. As summarized in Table II, DNA from 16S rRNA was amplified in 39 of the 57 (68.4%) non-cancerous

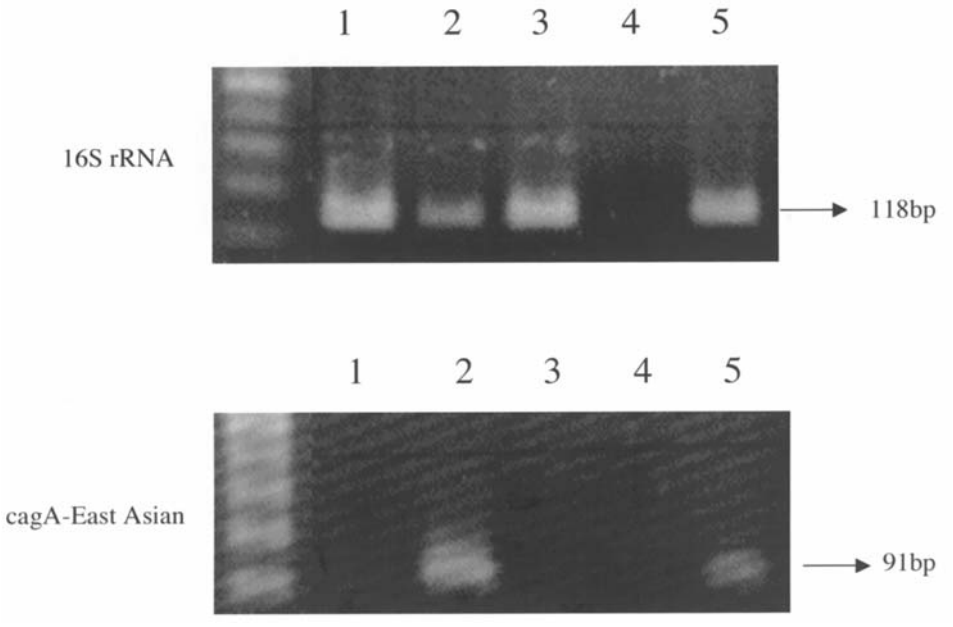

Figure 3. PCR amplification of 16S rRNA and East-Asian cagA in *H. pylori* DNA. Asterisk: positive signal. Arrows: PCR products from 16S rRNA at 118 bp and cagA at 91 bp length.

Table III. Relationship between Runx3 methylation and mucosal background.

	Runx-3		
	U	M	P
IM			
-	10	4	
+	17	26	0.06
H. pylori infection			
16S rRNA			
-	11	5	
+	16	24	0.09
CagA			
-	19	20	
+	8	10	0.76

IM, intestinal metaplasia.

Table IV. Multivariate logistic regression analysis for the risk factor affecting Runx-3 methylation in cancer tissues.

	Risk ratio	95% CI	P-value
Age	6.04	0.84-0.98	0.014
Location	8.26	3.21-477	0.004
IM	0.11	0.13-4.26	0.74
16S rRNA	8.37	2.27-70.7	0.004

CI, confidence interval.

mucosae while that from East-Asian cagA was 28 of the 57 (49.1%). These two factors showed significant correlation (p=0.001). Similarly, 16S rRNA East-Asian cagA were detected in 40 of the 57 (70.2%) and 18 of the 57 (31.6%) cancer tissues, respectively. There was a strong correlation between 16rDNA and cagA in the 57 cancer tissues (p<0.001).

Analysis of the risk factor for Runx3 methylation. In order to clarify whether or not mucosal background affects Runx3 methylation, the relationship between the methylation status in the cancer tissues and IM, *H. pylori* infection was analyzed (Table III). Although no statistical significance was found, Runx3 methylation showed a trend of positive correlation with the existence of IM (p=0.06) and 16S rRNA (p=0.09). We then performed a multivariate analysis using logistic regression analysis to isolate the risk factors for Runx3 methylation (Table III). As a result, age, tumor location and 16S rRNA were independent risk factors for Runx3 methylation in the cancer tissues.

Discussion

Gastric cancer is one of the most common malignancies in the world. Various studies on oncogenes, tumor suppressor genes, cell adhesion molecules and cell cycle regulators have been performed in order to clarify the carcinogenesis of gastric cancer (3). Among these studies, one notable experiment using knockout mice indicated the *runx3* gene as a possible tumor suppressor gene of gastric cancer (2). The aberrant methylation of the *runx3* gene, which is the main mechanism of deficient Runx3 expression in gastric cancer tissues, has been studied in various literature (5,6). Moreover, the aberrant methylation of Runx3 in non-cancerous gastric

as been reported (6). Kim *et al* reported that Runx3 ion was found in 64% of gastric cancer, 8% of chronic gastritis, 28% of IM and 27% of gastric adenoma (6). These results suggest that Runx3 is a target for epigenetic gene silencing in gastric carcinogenesis and is proposed to play an important role in the carcinogenic sequence from the precancerous state to carcinoma (6). However, the precise mechanism by which Runx3 methylation occurs in carcinoma cells or non-cancerous gastric mucosa remains to be determined.

In the present study, we analyzed the aberrant methylation of Runx3 in 57 early gastric cancers and the corresponding non-cancerous mucosa. Furthermore, the mucosal background such as IM and *H. pylori* infection in the 57 gastric cancer tissues was investigated and correlated with the methylation status. In particular, we focused on the critical issues that H. pylori infection, diagnosed by the PCR method, contributes to Runx3 methylation in gastric cancer cells. As a result, Runx3 methylation was frequently observed in 37 of the 57 (52.6%) cancer tissues in comparison to 10 of the 57 (17.5%) non-cancerous tissues (Fig. 2). In a comparative analysis of the clinicopathological factors, Runx3 methylation in the cancer tissues did not correlate with the depth of cancer invasion, lymph node metastasis or vessel invasion, indicating the involvement of Runx3 in gastric carcinogenesis. On the contrary, age and cancer location were both significantly correlated with methylation. Previous studies have demonstrated a significant correlation between aberrant gene methylation in non-cancerous gastric mucosa and aging (16,17). Kang *et al* demonstrated using gastric epithelium with chronic gastritis that 5 of the 11 genes showed a general increase in the methylation frequency as a function of aging (17). Our study might support this link of aging to Runx3 methylation, although we did not find any significant correlation in the non-cancerous tissues. Runx3 methylation in the 57 cancer tissues was more frequently found in the middle/lower region of the stomach than the upper region. We speculated that the difference in the methylation frequency might depend on the mucosal background surrounding the cancer cells. To study this, we analyzed the association between IM and Runx3 methylation in the 57 cancer tissues. As shown in Table III, IM exhibited the trend of a positive correlation with Runx3 methylation in the cancer tissues, although there was no statistical significance (p=0.06). The relationship between IM and cancer location was also assessed and revealed a statistically significant correlation (p=0.03) (data not shown). These results indicated that IM occurs more frequently in the M/L region of the stomach and also that Runx3 methylation may affect IM formation surrounding cancer cells.

H. pylori infection is known as an important aetiological risk factor in gastric cancer and has been classified as a group I or defined carcinogen by the World Health Organization's International Agency for Research on Cancer (18). *H. pylori* infection causes chronic active inflammation in the gastric mucosa and may cause mucosal atrophy and intestinal metaplasia (IM), which are known as risk factors for gastric cancer (9). Uemura *et al* followed up 1,526 Japanese patients with upper gastrointestinal diseases for 7.8 years and observed that gastric cancer developed only in *H. pylori* infected patients, especially those with severe gastric atrophy and intestinal metaplasia (IM) (9). These studies prompted a possible scenario that *H. pylori* infection in gastric mucosa initiates Runx3 methylation, followed by IM formation and leads to cancer development. No study has yet revealed the critical attribution of *H. pylori* infection to Runx3 methylation in gastric cancer. *H. pylori* infection is usually diagnosed by a histological examination, a serum anti-*H. pylori* IgG antibody test, rapid urease test or culture test (8). Recently, it has been reported that the PCR method developed a new diagnostic tool to detect the *H. pylori* gene with both high sensitivity and specificity (12,19). In the present study, we thus assessed *H. pylori* infection in the cancer as well as the non-cancerous mucosa by PCR amplification of 16S rRNA and the East-Asian cagA region as described (12). As shown in Table II, 16S rRNA was highly detected in the normal (68.4%) and cancer (70.2%) tissues. East-Asian cagA DNA was also amplified in both the normal (49.1%) and cancer tissues (31.6%). A statistical analysis showed a strong correlation between 16S rRNA and East-Asian cagA in the normal and cancer tissues. These results indicate that *H. pylori* are frequently infected in gastric cancer tissues and the strains were considerably integrated by East-Asian cagA. Using this highly sensitive method, we revealed that *H. pylori* infection, which was estimated by 16S rRNA, showed a higher tendency of Runx3 methylation (p=0.09). In the multivariate analysis, we demonstrated that *H. pylori* infection was one of the independent risk factors for runx-3 methylation. Several studies have reported similar aspects that aberrant DNA methylation in gastric mucosa is induced by *H. pylori* infection (11,20). Maekita *et al* quantitatively estimated the methylation levels of 8 CpG islands (CGIs) in gastric mucosa, which were collected from healthy volunteers with or without *H. pylori* infection (11). The results demonstrated that the methylation levels of all of the 8 CGI regions were significantly higher in the *H. pylori* positive than in the *H. pylori* negative mucosa. Chan *et al* showed a significant relationship between the methylation of E-cadherin and *H. pylori* infection in gastric mucosa from dyspeptic patients, indicating that the promoter methylation of E-cadherin associated with *H. pylori* infection occurs as an early event in gastric carcinogenesis (20). These findings might support our notion that runx-3 methylation is induced by *H. pylori* infection and the subsequent silencing of runx-3 expression may correlate with gastric carcinogenesis. The cagA protein, which is encoded by the *cagA* gene is a highly immunogenic protein and is one of the most studied virulence factors of *H. pylori* (8,21,22). In the present analysis, cagA appeared not to affect Runx3 methylation (Table III). An alternative mechanism other than cagA may induce Runx3 methylation in *H. pylori*-infected gastric mucosa.

In conclusion, we demonstrated for the first time that *H. pylori* infection is an independent risk factor for aberrant methylation of the *runx3* gene in gastric cancer. A multivariate analysis also revealed that age and cancer location were independent factors for Runx3 methylation. *H. pylori* infection persisting for many years may induce Runx3 methylation in the epithelial cells at the M/L region of the stomach. However, we did not observe any correlation beween Runx3 methylation and *H. pylori* infection in the

non-cancerous mucosae. It is therefore plausible that some molecular alteration may be additionally necessary for developing gastric cancer in non-cancerous mucosa with Runx3 methylation. Conversely, the non-cancerous mucosa with Runx3 methylation may be reversibly brought back to a normal state with the eradication therapy of *H. pylori*.

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