ORIGINAL ARTICLE

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An Inverse Relationship between the Expression of the Gastric Tumor Suppressor RUNX3 and Infection with *Helicobacter pylori* in Gastric Epithelial Dysplasia

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Background/Aims: This study was performed to determine the association between RUNX3 expression and *Helicobacter pylori* infection in premalignant gastric lesions. **Methods:** We examined 107 patients with gastric epithelial dysplasia who had undergone endoscopic mucosal resection or submucosal dissection. All tissue samples were evaluated by RUNX3 staining and subclassified by immunophenotype. *H. pylori* infection in dysplastic lesions and the normal surrounding tissue was examined by silver staining, and cagA status was assessed by polymerase chain reaction. **Results:** The loss of RUNX3 expression was observed in 62 cases (57.9%), and an association with *H. pylori* infection was found in 54 cases (50.5%). The infection rate with the cagA-positive *H. pylori* strain was 63.0%. In RUNX3-negative lesions, the rate of *H. pylori* infection (p=0.03) and the frequency of category 4 lesions (according to the revised Vienna classification) were high (p=0.02). In addition, the gastric mucin phenotype was predominant. In RUNX3-negative category 4 lesions, the rate of cagA-positive *H. pylori* infection rate was high but not significantly increased (p=0.08). **Conclusions:** Infection with *H. pylori* is associated with inactivation of RUNX3 in early gastric carcinogenesis. This mechanism was prominent in gastric cancer with a gastric mucin phenotype. **(Gut Liver 2013;7:688-695)**

Key Words: *Helicobacter pylori*; CagA protein; Core binding factor alpha 3 subunit

INTRODUCTION

It has been accepted that the particular virulence strain of *Helicobacter pylori* is one of the factors that determine the clinical outcome of infection. Virulent *H. pylori* strains having cytotoxin-associated gene pathogenicity islands (cag PAI) have been associated with increased inflammatory responses of the gastric mucosa.[1-4] The *H. pylori* cag PAI encodes a type IV secretion system that injects the bacterial virulence factor cagA into host epithelial cells.[5] Although several studies have tried to establish an association between virulence markers and clinical outcomes, the results have been conflicting,[6-9] and the exact mechanism of carcinogenesis remains to be clarified.

Runt-related transcription factor 3 (RUNX3) is a member of the runt domain family of transcription factors. RUNX3 protein is expressed in the cytoplasm and nucleus of epithelial cells of the gastric mucosa, whereas there is a loss of RUNX3 expression in gastric cancer specimens. The loss of RUNX3 expression is inversely correlated with survival and is an independent predictor of poor prognosis.[10,11] It appears to occur both at an early stage as well as during progression of gastric cancer, and its expression correlates with the stage of the cancer; fewer late-stage tumors expressed RUNX3 than early-stage tumors.[12-14] There have been a few studies about the relationship between loss of RUNX3 expression and *H. pylori* infection in gastric cancer.[15,16] Now, in premalignant gastric lesions, whether or not *H. pylori* infection has an effect on the expression of RUNX3 should be elucidated.

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Gastric mucin as a major component of mucus is a large carbohydrate-rich glycoprotein that protects the surface of the stomach from the damage. The change of mucin expression by *H. pylori* infection could contribute to the development of atrophic gastritis or intestinal metaplasia and also to the dysplasia and cancer.[17,18] Several reports suggested that MUC5AC and MUC6 expressions were significantly down-regulated in *H. pylori*-positive precancerous mucosa.[19,20] To date, the clinical importance of mucin phenotype is still controversial.

In this study, we evaluated the association of RUNX3 expression and *H. pylori* infection state in premalignant gastric lesions. Also, we investigated the relationship between RUNX3 expression and mucin phenotypes of gastric epithelium in early gastric carcinogenesis.

MATERIALS AND METHODS

All tissues were excised by therapeutic endoscopic mucosal resection or submucosal dissection, and paired with adjacent normal tissue samples. They were examined and analyzed by two histopathologists. When they disagreed, the tissue sample was excluded from the study to clarify the diagnosis. The lesions were histopathologically assigned into two groups according to the revised Vienna classification system.[21,22]

All normal surrounding tissues were grossly intact mucosa at least 1 cm apart from the mucosal lesion and were taken by gastric biopsy specimen just after endoscopic mucosal resection or submucosal dissection. In the microscopic examination, there was no evidence of a malignant cell component. Each patient was diagnosed as *H. pylori* positive or negative by the histologic

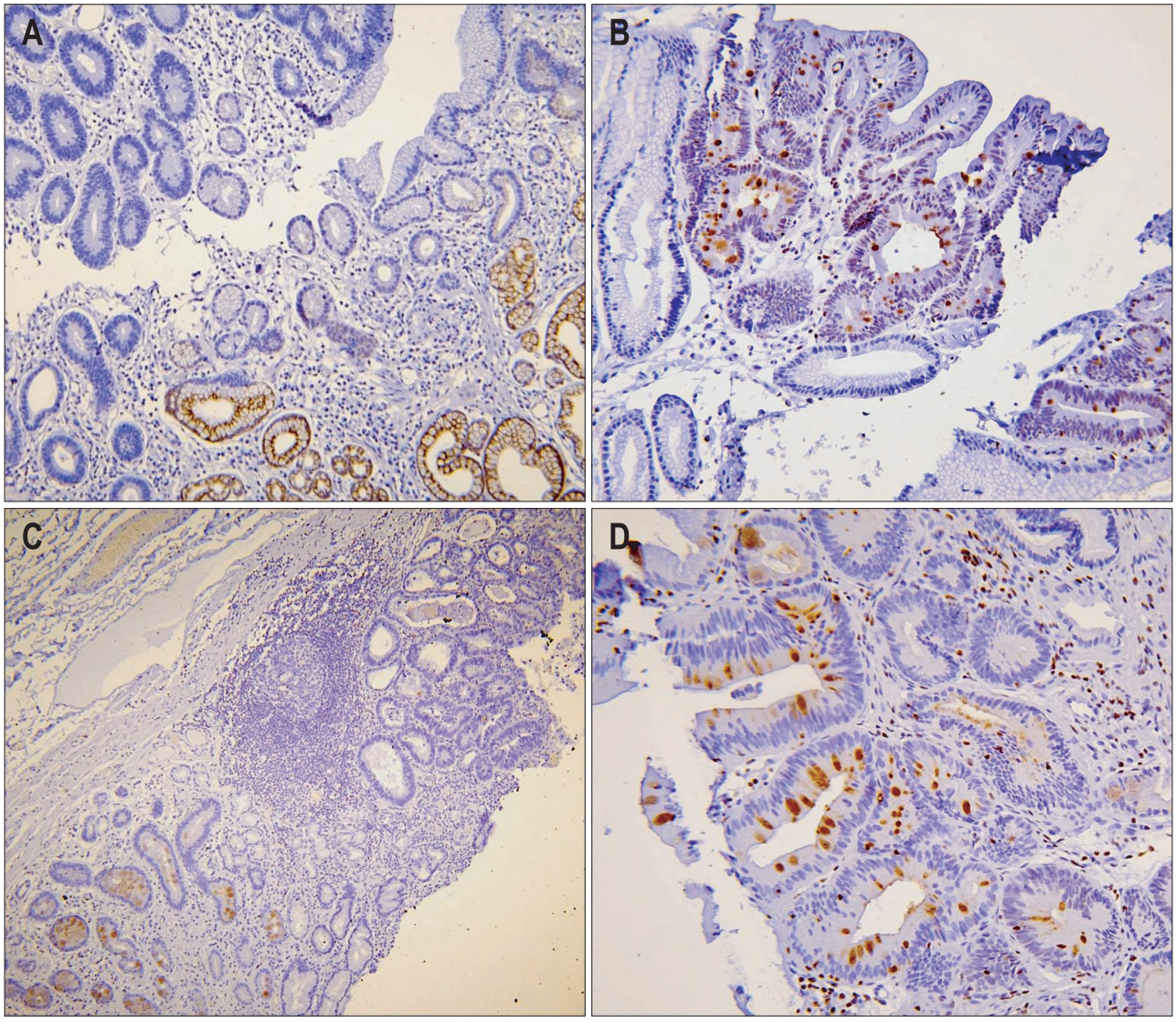

Fig. 1. Representative example of runt-related transcription factor 3 (RUNX3) expression in gastric epithelial neoplasia categorized by the revised Vienna classification (immunohistochemical staining findings). (A) Negative expression of RUNX3 in a category 3 lesion (×100). (B) Positive expression in category 3 lesion (×200). (C) Negative expression in a category 4 lesion (×100). (D) Positive expression in a category 4 lesion (×400).

results (silver stain).

1. Immunohistochemical stain of CD10, MUC2, MUC5AC, MUC6, and 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 in this study. 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 they were next incubated with the primary antibodies. The primary antibodies used were MUC2 (1:200, clone Ccp58, monoclonal; Novocastra Lab., Newcastle upon Tyne, UK) MUC5AC (1:100, clone CLH2, monoclonal; Novocastra Lab.), MUC6 (1:100, clone CLH5, monoclonal; Novocastra Lab.), CD10 (1:400, clone 56C6, monoclonal; Novocastra Lab.), RUNX3 (1:200, AMC-2/RUNX3 rabbit polyclonal antibody, Active Motif®; Active Motif, Carlsbad, CA, USA). Antibody detection was performed using the IMPRESS peroxidase reagent kit (VECTOR Laboratory, Burlingame, CA, USA) according to the manufacturer's protocol. Immunoreactive cells were identified by DAB peroxidase substrate kit (VECTOR Laboratory).

2. Assessment of immunohistochemical staining

The results of immunostaining for CA10, MUC2, MUC5AC, MUC6, and RUNX3 were considered to be positive if more than 10% of the tumor cells were stained. The microscopic features of RUNX3 are presented in Fig. 1. The MUC2 and CD10 expressions were examined as markers of the intestinal phenotypes and the MUC6 and MUC5AC expressions were examined as markers of the gastric phenotypes. The cellular mucin phenotypes were classified according to the combined expression patterns of the gastric markers and intestinal markers as four phenotypes: the gastric type (tumor cells were positive for either MUC6 or MUC5AC, and negative for both MUC2 and CD10, G type); the intestinal type (tumor cells were positive for either MUC2 or CD10, and negative for both MUC6 and MUC5AC, I type); the mixed type (tumor cells were positive for both gastric and intestinal markers, M type); and the unclassified phenotype (tumor cells were negative for both gastric and intestinal markers, N type).

3. Polymerase chain reaction for cagA associated with *H. pylori*

Polymerase chain reaction (PCR) analysis for cag PAI was performed to amplify the *cagA* gene. The primers used for detecting the *H. pylori*-specific cagA region were 5′-GATAAC AGG CAA GCT TTTGA-3′ (F)/ 5′-CCG AACGGA TCA AAA ATT CAT GG-3′ (R) (GenBank accession number, AF001357). The PCR reaction was setup using i-star Taq DNA polymerase (iNtRON, Seongnam, Korea). The PCR amplification protocol was as follows: 95°C for 5 minutes, then denaturing at 94°C for 30 seconds, annealing at 50°C for 30 seconds, extension at 72°C for 1 minute for 35 cycles, and then a final extension at 72°C for 10 minutes. The amplified products were electrophoresed on 2% agarose gels, and then visualized with ethidium bromide (Fig. 2).

4. Statistical analysis

In the quantitative variables, the mean and its standard deviation were calculated. In the qualitative variables, the percentage and its 95% confidence interval were calculated. For comparison of age, we used the unpaired t-test. The chi-square test or Fisher exact test was used to investigate the association with the other variables. SPSS statistical package version 12.0.1 (SPSS Inc., Chicago, IL, USA) was used for all analyses. Significance was defined as $p<0.05$.

5. Ethical statement

The informed consent was obtained from all patients and human samples were used according to the guidelines of the Ethical Committee of Catholic University of Korea. This study was approved by the Institutional Review Board of the Catholic University of Korea (VC09TISI0005).

RESULTS

A total of 107 patients with gastric epithelial dysplasia and intramucosal cancer were evaluated in this study. According to the revised Vienna classification, 51 category 3 (low grade dysplasia) and 56 category 4 (high grade dysplasia or intramucosal cancer) tissue samples were included. Category 3 tissue samples (low-grade adenoma; from 25 men and 26 women; mean age, 62.53±4.48 years) and category 4 tissue samples (from 40 men and 14 women; mean age, 63.66±4.09 years) were obtained. Of the category 4 gastric epithelial neoplasias, 35 tissues were high-grade adenomas (category 4.1) and 19 were intramucosal

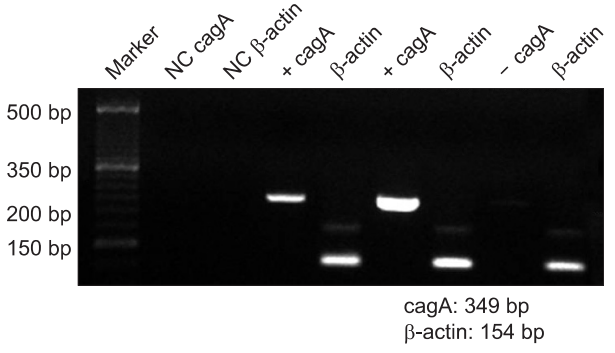

Fig. 2. Representative example of polymerase chain reaction for cagA, associated with *Helicobacter pylori*.
NC, negative control.

Table 1. Patient Characteristics, *Helicobacter pylori* Infection Status, cagA Positivity, and Mucin Positivity according to RUNX3 Expression

Characteristic	RUNX3 expression		p-value	OR	95% CI
	Positive	Negative			
No.	45	62			
Sex, male/female	30/15	35/27	0.28		
Age	64.11±6.11	61.93±6.94	0.09		
H. pylori			0.03*	2.438	1.109–5.359
Negative	28	25			
Positive	17	37			
cagA			0.15		
Negative	10	14			
Positive	7	23			
Vienna classification†			0.03*	2.375	1.083–5.209
Category 3	27	24			
Category 4	18	38			
Mucin phenotype			0.01*		
Gastric type	10	30			
Intestinal type	17	15			
Null type	12	16			
Mixed type	6	1			

Data are presented as mean±SD.
H. pylori, *Helicobacter pylori*; RUNX3, runt-related transcription factor 3; OR, odds ratio; CI, confidence interval.
*Statistically significant; †The revised Vienna classification of gastric epithelial dysplasia.[21,22]

carcinomas with adenomas (category 4.4).

Negative expression of RUNX3 was observed in 57.9% (62/107 cases) and association with *H. pylori* infection was seen in 50.5% (54/107 cases). In negative expression of RUNX3 lesions, *H. pylori*-positive rate was 59.7% (37/62), whereas in positive expression of RUNX3 lesions, *H. pylori*-positive rate was 37.8% (17/45). There was a significant difference (p=0.03). When the lesions were subgrouped by the revised Vienna classification, the frequency of category 4 of negative expression of RUNX3 lesion was 61.3% (38/62), whereas that of positive expression of RUNX3 lesion was 40.0% (18/45) (p=0.02) (Table 1). The infection rate with the cagA positive *H. pylori* strain was 28.0% (30/107). Among *H. pylori* infected cases, the frequency of RUNX3-negative, cagA-positive lesion was 42.6% (23/54), whereas that of RUNX3-positive, cagA-positive lesion was 13.0% (7/54). There was no statistically significant difference between RUNX3 expression and cagA state (p=0.15). In RUNX3-negative lesions, gastric mucin phenotype was significantly prominent (Table 1). In category 3, *H. pylori*-positive rate was 51.0% (26/51), and in category 4, 50.0% (28/56). The positive rate for cagA was 50.0% (13/26) in category 3, and 60.7% (17/28) in category 4 lesions (Table 2). In category 4 lesions, RUNX3-negative, cagA positive rate was 71.4% (15/21, p=0.008) (Table 3).

Taking into account the combinations of the expression of

Table 2. Patient Characteristics, *Helicobacter pylori* Infection Status, cagA Positivity, and Mucin Phenotype according to Gastric Epithelial Dysplasia as Categorized Based on the Revised Vienna Classification[21,22]

Characteristic	Category 3 lesion (n=51)	Category 4 lesion (n=56)	p-value
Sex, male/female	25/26	40/16	0.07
Age	62.53±6.87	63.66±6.54	0.49
H. pylori infection	26	28	0.27
cagA positive	13	17	0.43
RUNX3 expression in *H. pylori* infection			
Positive	10	7	0.28
Negative	16	21	
RUNX3 expression in *H. pylori* cagA(+) strain infection			
Positive	5	2	0.19
Negative	8	15	
Mucin phenotype			0.06
Gastric type	15	25	
Intestinal type	19	13	
Null type	16	12	
Mixed type	1	6	

H. pylori, *Helicobacter pylori*; RUNX3, runt-related transcription factor 3.

Table 3. RUNX3 Expression according to *Helicobacter pylori* cagA Status in Category 4 Lesions (Vienna Classification)

	RUNX3 expression		p-value	OR	95% CI
	Positive (n=7)	Negative (n=21)			
H. pylori cagA status			0.08	6.250	0.941–41.516
Negative	5	6			
Positive	2	15			

RUNX3, runt-related transcription factor 3; OR, odds ratio; CI, confidence interval; *H. pylori*, *Helicobacter pylori*.

Table 4. Patient Characteristics, *Helicobacter pylori* Infection Status, cagA Positivity, and RUNX3 Expression according to Mucin Phenotype

Characteristic	G type	I type	Null type	Mixed type	p-value
No.	40	32	28	7	
Sex, male/female	24/16	21/11	18/10	2/5	
Age	63.78±7.80	61.53±9.16	63.32±8.36	65.86±7.95	
H. pylori					
Positive	21	17	13	3	
Negative	19	15	15	4	
cagA positive	10	13	6	1	
RUNX3 expression in *H. pylori* cagA(+) strain infection					
Positive	0	5	5	1	<0.01*
Negative	10	8	1	0	
Vienna classification†					
Category 3	15	19	16	1	0.06
Category 4	25	13	12	6	

Data are presented as mean±SD.
H. pylori, *Helicobacter pylori*; RUNX3, runt-related transcription factor 3.
*Statistically significant; †The revised Vienna Classification of gastric epithelial dysplasia.[21,22]

the gastric (MUC6 and MUC5AC) and intestinal (MUC2 and CD10) markers, the gastric epithelial dysplasia was subclassified as four mucin phenotypes. *H. pylori*-positive rate was 52.5% (21/40), 53.1% (17/32), 46.4% (13/28), and 42.9% (3/7) in gastric, intestinal, null, and mixed mucin phenotypes, respectively. *H. pylori* cagA-positive rate was 47.6% (10/21), 76.5% (13/17), 46.2% (6/13), and 33.3% (1/3). Among them, the frequencies of RUNX3-negative expression were 100% (10/10), 61.5% (8/13), 16.7% (1/6), and 0% (0/1), respectively. Gastric mucin phenotype was predominant (p=0.01) (Table 4).

DISCUSSION

RUNX3 can regulate cell proliferation and apoptosis in gastric epithelial cells, and shows tumor suppressor activity by targeting transforming growth factor-β superfamily signaling.[12,23,24] Conflicting results have emerged on the role that RUNX3 plays in gastric carcinogenesis.[25] In the previous report, the expression of RUNX3 was absent in nonneoplastic epithelium, whereas the expression was occasionally found in tumor cells.[26] It might preclude a role for RUNX3 as a tumor suppressor gene. Recently, the meta-analysis of pooled data provides evidence to support a strong association between inactivation of the RUNX3 and gastric cancer.[27] Inactivation of the *RUNX3* gene can be caused by loss of heterozygosity, promoter hypermethylation, or protein mislocalization.[12] Although there were limited data on the association between RUNX3 expression and methylation in the precancerous gastric lesions, expression level of RUNX3 by immunohistochemical stain was well correlated with methylation status, as previously reported.[28,29] RUNX3 expression was negatively associated with increased frequency of RUNX3 promotor hypermethylation and it was associated with tumor progression and might provide useful clues for predicting the malignant behavior of gastric cancer. Our results could provide a strong evidence for RUNX3 as tumor suppressor gene. It showed that the loss of RUNX3 expression was distinct in category 4 lesions according to the revised Vienna classification (high grade dysplasia and intramucosal cancer).

Epidemiological studies have established a strong causal relationship between *H. pylori* infection and gastric cancer, and *H. pylori* has been classified as a group I carcinogen.[30,31] Several researchers hypothesized that *H. pylori*-induced chronic active

gastritis slowly progresses through the premalignant stages of atrophic gastritis, intestinal metaplasia and dysplasia to an intestinal type of gastric cancer.[32,33] To prove this concept, eradication of *H. pylori* will be an effective approach for prevention of gastric cancer, but it does not prevent the development of gastric cancer in all patients during long-term follow-up. Currently, it is thought that further progression of premalignant lesions to true cancer may be less dependent on *H. pylori* infection. Therefore, if *H. pylori* eradication therapy aimed at gastric cancer prevention is prescribed in patients with premalignant gastric lesions, proof of validity for eradication therapy should be obtained. In our results, the expression of RUNX3 had an inverse relationship with infection of the *H. pylori*. Unfortunately, we did not show strong association between the bacterial virulence factors of *H. pylori* and gastric carcinogenesis in our results. It was noteworthy that loss of expression of RUNX3 had a tendency with *H. pylori* cagA-positive state in gastric epithelial dysplasia. Broader studies will be required before any relatively concrete conclusions can be drawn.

Gastric cancers have been classified into two histological types, an intestinal type and a diffuse type, by Lauren.[34] It is well known that the intestinal type arises from gastric mucosa with intestinal metaplasia, and that the diffuse type arises from ordinary gastric mucosa.[34,35] This classification is based on the tendency of glandular formation and it is necessary to evaluate the neighboring mucosa for a correct diagnosis. In practice, it is very difficult for pathologists to discriminate between diffuse and intestinal type gastric cancer in the specimens of endoscopic resection. On the other hand, gastric cancers are classified according to the expression of mucin phenotypic markers. Several genes (MUC1 through to MUC17) encode mucin proteins. MUC1 and MUC5AC are expressed in the superficial foveolar epithelium, and MUC6 in the mucous neck cells of the fundus, and in antral-type glands.[36-41] *H. pylori* infection causes alterations in mucin expression, and it is suspected to be involved in the mechanisms of *H. pylori* induced gastric inflammation and carcinogenesis. Previously, several researchers have reported that patients with the gastric mucin phenotype had poorer prognosis.[42,43] On the contrary, a phenotype shift from gastric to intestinal mucin phenotype was associated with tumor progression.[44-46] Our results revealed that gastric mucin phenotype was predominant in RUNX3-negative gastric epithelial dysplasia. In high grade dysplasia and intramucosal cancer, there was an increasing frequency of RUNX3-negative lesions having gastric mucin phenotype. These results suggested that the mucin phenotype might influence the expression of RUNX3. Most importantly, the dysplastic lesions with the gastric mucin phenotype were closely associated with the *H. pylori* cagA state, although the sample size of this study was too small for this finding to be definitive.

Early *H. pylori* eradication could reduce the risk of gastric cancer development in an animal model, whereas the risk was reduced only in a minority of cases of human studies. Also, conflicting results have been reported on whether or not successful eradication of *H. pylori* infection prevents the progression of atrophic gastritis or intestinal metaplasia.[47-49] The reason is that the development of gastric cancer is a multi-factorial process related to interactions of *H. pylori* infection, host factors and environmental factors such as diet. We hypothesized that eradication of *H. pylori* would be an effective therapeutic strategy to prevent gastric cancer in selected cases of the patients with premalignant gastric lesions. On the basis of our results, it seemed to be beneficial especially in RUNX3-negative dysplastic lesions with gastric mucin phenotype.

In conclusion, the loss of RUNX3 expression and *H. pylori* state might play an important role in the progression of gastric carcinogenesis. Although *H. pylori* eradication did not completely prevent progression of gastric cancer, its eradication might be a promising approach of premalignant gastric lesions. Larger prospective and longer follow-up studies are needed to evaluate whether eradication of *H. pylori* infection will really diminish the risk of gastric type of gastric cancer.

CONFLICTS OF INTEREST

No potential conflict of interest relevant to this article was reported.

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