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H. pylori-Eradication Therapy Increases RUNX3 Expression in the Glandular Epithelial Cells in Enlarged-Fold Gastritis

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Summary ***Helicobacter pylori*** **(HP)-eradication therapy increases Runt domain transcription factor 3 (RUNX3) expression in the glandular epithelial cells in enlarged-fold gastritis. The aim of this study is to evaluate expression of the RUNX3 protein, the product of a gastric tumor suppression gene, and mutagenic oxidative stress in human gastric mucosal specimens obtained from patients with HP-induced enlarged-fold gastritis. Methods. RUNX3 expression was immunohistochemically scored and the degree of the mucosal oxidative stress was directly measured by the chemiluminescense (ChL) assay in the biopsy specimens. Results. RUNX3 expression was detected in the gastric epithelial cells. HP-eradication significantly increased RUNX3 expression in the glandular epithelium of the corpus, however, no change was observed in those of the antrum. A fourfold higher mucosal ChL value was observed in the corpus as compared with that in the antrum. HP-eradication significantly decreased the mucosal ChL values in both portions of the stomach to nearly undetectable levels. Conclusion. The glandular epithelium is exposed to a high level of carcinogenic oxidative stress and shows low levels of expression of the tumor suppressive molecule, RUNX3; however, this expression was restored after HP-eradication, suggesting the high risk of carcinogenesis associated with HP-induced enlarged-fold gastritis of the corpus.**

Key Words**: *H. pylori*, RUNX3, gastritis, oxidative stress**

Introduction

Gastric cancer is the most prevalent gastrointestinal (GI) malignancy in Japan, while in other developed countries, the prevalence of colon cancer is higher than that of gastric cancer. Recent clinical studies have clearly demonstrated that patients with chronic gastritis associated with *Helicobacter pylori* (HP) infection have an approximately three-fold higher risk of developing gastric cancer than those without HP infection [*1*]. Furthermore, eradication of HP infection by antibiotics has been shown to be associated with a definite decrease in the risk of gastric cancer [*2*, *3*]. Thus, while the carcinogenic role of HP is now widely accepted, the underlying pathogenetic mechanism remains to be clarified. Recently, enlarged-fold gastritis has attracted much attention, because the risk of carcinogenesis associated with this type of gastritis has been suggested to be much higher than that associated with atrophic gastritis, the commonly observed type of chronic gastritis in the Japanese population [*4*]. This type of gastritis is characterized by enhanced inflammation of the corpus mucosa, however the

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mechanism involved in the carcinogenic potential of this type of gastritis has not been fully elucidated.

Oxidative stress, now acknowledged as one of the major carcinogenic process, directly modifies genetic nucleotides, followed by activation of many tumor suppressor genes [*5*]. On the other hand, the Runt domain transcription factors (RUNX) have also been linked to gastric cancer development [*6*]. Among the members of the RUNX family, RUNX3 has been proposed as a gastric tumor suppressor gene and its activity has been shown to be influenced by DNA methylation [*7*].

This study was aimed at clarifying the carcinogenic mechanism of enlarged-fold gastritis by measuring the levels of oxidative stress and RUNX3 expression in the gastric mucosa of patients with HP-induced enlarged-fold gastritis.

Methods

Patients and sampling

Thirteen patients with HP-induced enlarged-fold gastritis (fold width ≥5 mm) who visited the Gastroenterology Division of the National Hospital Organization Tokyo Medical Center with upper GI symptoms were enrolled in this study. All of the subjects provided written informed consent for this study, and the study protocol was approved by the Ethics Committee of Tokyo Medical Center. Upper GI endoscopy was performed in the patients. Two biopsy specimens were obtained from the antrum and two from greater curvature of the upper corpus. One of these was processed for histological assessment (Hematoxylin-Eosin stain (HE) and immunohistochemistry for RUNX3) and the other was used for assay of oxygen-free radical production. Eradication for HP was performed using routine one week course of triple therapy with proton pump inhibitor, clarithromycin, and amoxicillin. Success of eradication therapy was confirmed by ^{13}C-urea breath test.

Histological assessment

Immunohistochemistry for RUNX3 was performed according to a previously described method. [*8*] The histological assessment was performed in a blinded manner, and the degree of staining in the surface epithelial cells and glandular epithelial cells was scored as follows: intensity of gastric RUNX3 expression was graded in the epithelial cells of the mucosa as follows: 0 (negative), 1 (<33% cells showing positive staining), 2 (33–66% cells showing positive staining), or 3 (>66% cells showing positive staining). The degree of inflammatory cell infiltration was scored on the HE-stained sections according to the Updated Sydney System [*9*].

Oxygen-derived free radical measurement

Luminol-dependent chemiluminescence (ChL) assay was used for direct assessment of oxygen-derived free radical production from the gastric mucosa, according to a previously reported method [*10*, *11*]. Briefly, biopsy specimens (approximately 10 mg in wet weight) were placed, immediately after sampling, in a scintillation vial containing 0.5 ml of Eagle's minimum essential medium (MEM; pH 7.4) and 20 μg of luminol. The average ChL value (count/10 s/mg wet weight) for the first 5 min was used for the analysis.

Statistical analysis

All the data were expressed as mean ± SD and analyzed by Wilcoxon's rank sum test. Significance was set at $p<0.05$.

Results

Endoscopic appearance

The characteristic endoscopic features of enlarged-fold gastritis are shown in Fig. 1. Before the HP eradication, enlarged folds with thick mucus were observed in the greater curvature at the corpus, which clearly improved after HP eradication therapy.

RUNX3 expression in the gastric mucosa

The results of immunohistochemistry for RUNX3 protein in the gastric mucosa are illustrated in Fig. 2 (antrum) and Fig. 3 (corpus). In the antrum, RUNX3 protein expression was observed in the epithelial cells as well as the infiltrating leukocytes. Although the nuclei of epithelial cells were counterstained with hematoxylin, strong RUNX3 expression was still observed in some nucleus. While high immunoreactivity was observed in the infiltrating leukocytes, dramatic weakening of the staining intensity was observed after HP eradication. High level of the RUNX3 expression was also observed in the glandular epithelium in the corpus, which further increased following HP eradication.

Figs. 4 and 5 show the immunohistochemical scores for RUNX3 expression in the epithelial cells. Semi-quantitative analysis demonstrated that the RUNX3 expression in the surface epithelium was unchanged following HP eradication (antrum: 1.15 ± 0.55 to 1.00 ± 0.41, n.s., corpus: 1.31 ± 0.63 to 1.08 ± 0.28, n.s.). While no increase of the protein expression in the glandular epithelium was also observed in the antrum (1.38 ± 0.65 to 1.54 ± 0.52, n.s.), that in the corpus significantly increased following HP eradication (1.85 ± 0.55 to 2.54 ± 0.52, $p<0.01$).

Table 1 shows the leukocyte infiltration score as assessed according to the Updated Sydney System. Significant decrease in the number of infiltrating leukocytes (polymorphonuclear and mononuclear cells) was observed following HP eradication.

Fig. 6 illustrates the ChL activity of the gastric mucosa,

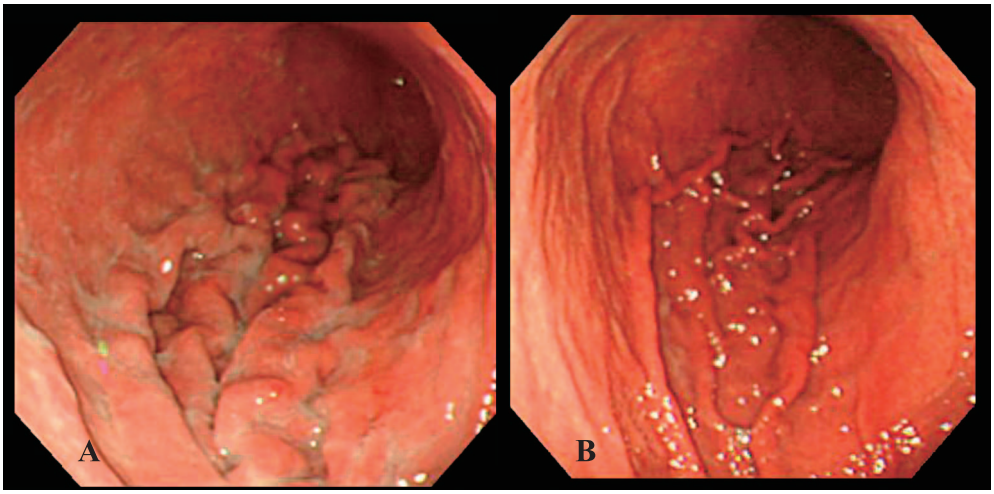

Fig. 1. Endoscopic images before (A) and after (B) HP eradication in patients with enlarged-fold gastritis. HP eradication was followed by a reduction of the enlarged folds with thick mucus.

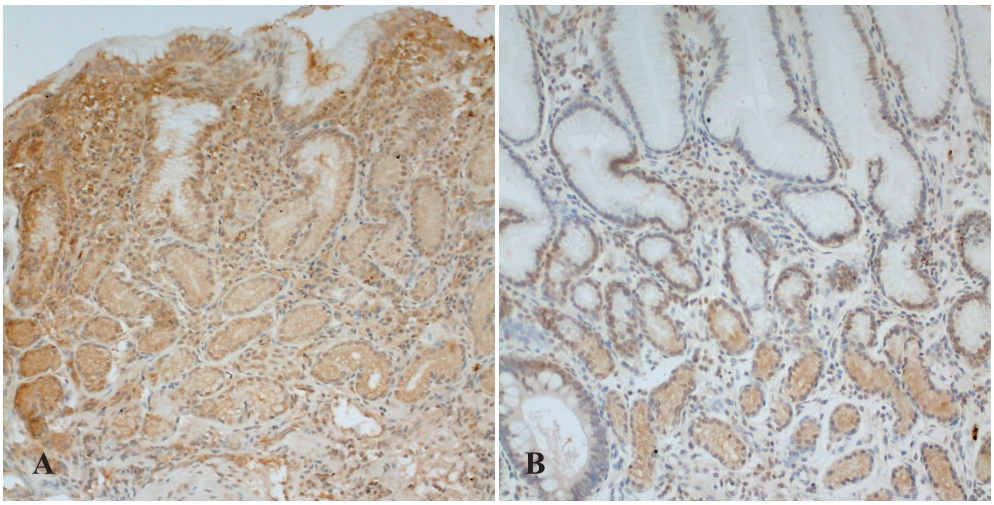

Fig. 2. Immunohistochemistry for RUNX3 protein expression in the antral mucosa pre-(A) and post-(B) HP eradication (counterstaining was also done with hematoxylin). Pre-eradication, RUNX3 expression was observed in epithelial cells as well as infiltrating leukocytes, and the strong expression was observed in the nuclei of the epithelial cells. Post-eradication, no significant change was observed in the surface and glandular epithelium.

which is a direct measure of oxidative stress. The ChL value was fourfold higher in the corpus mucosa as compared with that in the antral mucosa. Significant decrease of the ChL values to undetectable levels was observed in both portions of the gastric mucosa after HP eradication (antrum: 72.7 ± 110 to 2.89 ± 0.94, $p<0.01$; corpus: 309 ± 251 to 3.90 ± 1.92, $p<0.01$).

Discussion

Gastric carcinoma is reported as the first or second leading cause of cancer-related death in Japan. HP infection now generally accepted as one of the major factors involved in the development of gastric carcinoma. This bacterium reportedly modifies the cellular functions related to cell growth, which is mainly regulated by the cagA-protein [*12*].

Nishibayashi *et al.* reported detecting enlarged-fold gastritis (fold width ≥5 mm) in 81% of patients with gastric carcinoma, while it was detected in only 46% of HP-positive controls. Furthermore, mucosal levels of 8-OHdG, another measure of oxidative stress, were higher in specimens from enlarged-fold gastritis than those in the controls [*13*]. The finding in the present study of enhanced levels of oxidative stress as measured by tissue-associated ChL activity lend

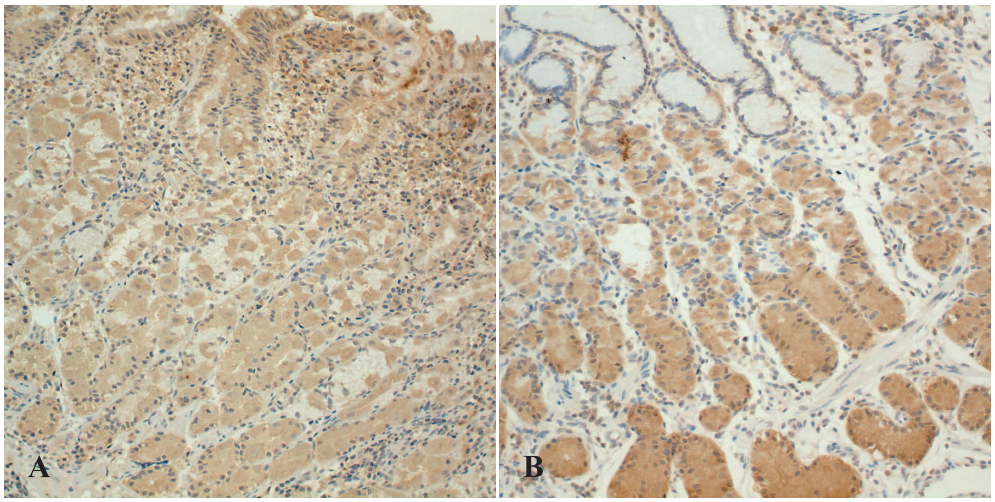

Fig. 3. Immunohistochemistry for RUNX3 protein expression in the corpus mucosa pre-(A) and post-(B) HP eradication (counterstaining was also done with hematoxylin). Pre-eradication, strong RUNX3 expression was observed in the epithelial cell as well as infiltrating leukocytes. Post-eradication, RUNX3 expression was not changed in the surface epithelial cells, while the increase was observed in the glandular epithelium.

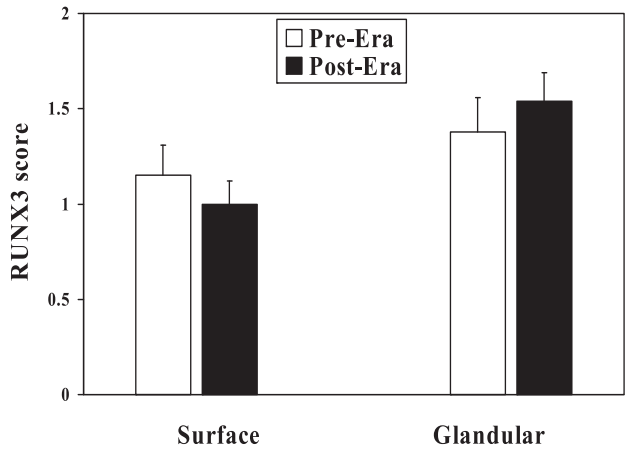

Fig. 4. Histological score for RUNX3 expression in the antral epithelial cells pre- and post-HP eradication. The protein expression in the surface and glandular epithelial cells was unchanged after HP eradication.

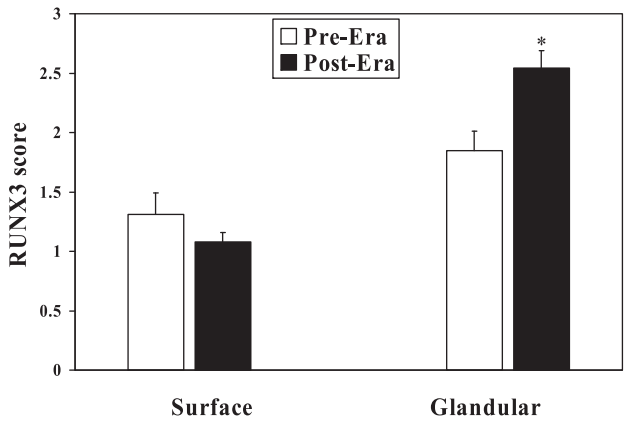

Fig. 5. Histological score for RUNX3 expression in the corpus epithelial cells pre- and post-HP eradication. RUNX3 expression in the glandular epithelial cells increased significantly after HP eradication (*p<0.01), while that in the surface epithelium was unchanged.

Table 1. Alterations of the histological score pre- and post-HP eradication in patients with enlarged-fold corpus gastritis.

	Antrum		Corpus	
	PMN	Mo	PMN	Mo
Pre-eradication	1.23 ± 0.73	1.92 ± 0.76	2.00 ± 0.82	2.23 ± 0.60
Post-eradication	0.46 ± 0.66	1.31 ± 0.95	0.46 ± 0.88	0.62 ± 0.87
p value	n.s.	n.s.	p<0.01	p<0.01

(n = 13) PMN: polymorphonuclear cell, Mo: mononuclear cell.

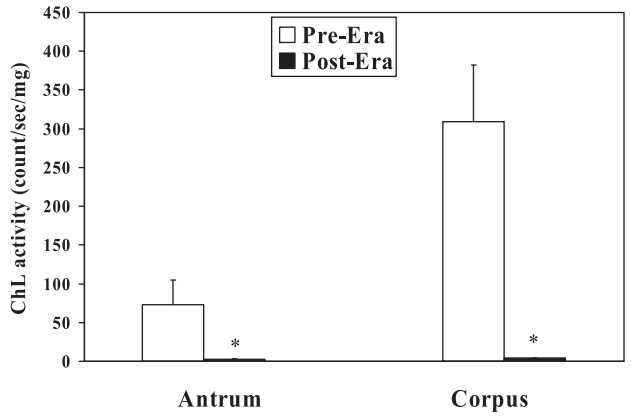

Fig. 6. Chemiluminescence (ChL) activity of the gastric mucosa (antrum and corpus) pre- and post-HP eradication. Marked ChL activity was observed in the corpus mucosa, which decreased dramatically after HP eradication. (*p<0.01 compared to each pre-eradication value)

support to these previous reports. Our previous data demonstrated that the corpus ChL activity in HP-positive non-enlarged-fold gastritis (DU and GU) was approximately 70% lower than that in enlarged-fold gastritis [*10*]. Since this enhanced ChL activity was dramatically reduced by eradication of HP, it was considered that elevated levels of oxidative free radical production may be one of the possible carcinogenic mechanisms resulting in gastric cancer. Significant reduction in the degree of inflammatory cell infiltration (polymorphonuclear and mononuclear cells) was also observed (antrum: 30–60%, corpus: 70–75% reduction) following HP eradication, although the magnitude of this decrease was much lower than that of ChL, suggesting that enhanced oxidative stress caused by HP infection may be regulated by not only the number of inflammatory cells, but also by other factors, including the activity of each leukocyte and tissue anti-oxidant molecules.

The Runt domain transcription factors (RUNX1, 2 and 3) have been reported to play key roles in developmental pathways in mammalian cells. Among this gene family, RUNX3 has been proposed as a gastric tumor suppressor gene. Enhanced expression of RUNX3 has been demonstrated to inhibit gastric carcinoma cell growth *in vitro*, as well as tumorigenicity and metastasis [*14*, *15*]. Friedrich *et al.* [*16*] examined RUNX3 protein expression in HP-infected non-cancerous gastric mucosa and found that the protein expression was prominently detected in the infiltrating leukocytes in the lamina propria, but not in the human gastric epithelium, as confirmed by a low mRNA expression in quantitative reverse transcriptase-polymerase chain reaction (RT-PCR). However, Nakase *et al.* [*17*] demonstrated RUNX3 expression in the normal epithelial cells of the remnant stomach by in situ hybridization as well as RT-PCR. Ito *et al.* [*8*] performed immunohistochemical staining of RUNX3 in the normal human stomach epithelial cells with R3-6E9, and found that while the chief cells and surface epithelial cells were strongly stained, the parietal cells showed only weak staining. In the present study, RUNX3 expression was clearly detected in the gastric epithelium as well as infiltrating leukocytes. Furthermore, semi-quantitative analysis revealed no significant change of RUNX3 expression in the surface epithelial cells, whereas a significant increase of the protein expression was observed in the glandular epithelium of the corpus, which is composed of chief cells and parietal cells, after HP eradication.

RUNX3 expression has often been reported to be downregulated in human gastric carcinoma owing to epigenetic silencing by promoter hypemethylation [*7*]. Kim *et al.* [*18*] demonstrated RUNX3 methylation in 8.1% of chronic gastritis specimens, 28.1% of intestinal metaplasia specimens, 27.3% of gastric adenoma specimens and 64% of gastric carcinoma specimens, suggesting increase of RUNX3 methylation with progression of the lesion along the path of multistep gastric carcinogenesis. Kitajima *et al.* [*19*] reported that RUNX3 methylation is induced by HP infection, and that subsequent loss of RUNX3 expression may affect gastric carcinogenesis. Miyazaki *et al.* [*20*] found a decrease of the immunoreactivity for E-cadherin in HP-positive patients with enlarged-fold gastritis as compared with that in HP-negative patients, and suggested that this reduction might be due to the high methylation percentage of E-cadherin.

DNA methylation is closely associated with free radical injury [*21*], and methylation of E-cadhelin has been shown to be enhanced by reactive oxygen species (ROS) [*19*]. In the present study, extensive increase of the oxidative stress in the corpus mucosa was demonstrated, thus RUNX3 expression may be partially downregulated by oxidant-induced DNA methylation. Further study including direct assessment of methylation process is necessary to clarify this issue.

In the present study, RUNX3 immunoreactivity was restored in the corpus glandular epithelium (probably chief cells) after HP eradication, while those in the other epithelium were unchanged. It is postulated that greater RUNX3 gene methylation may be induced in the glandular epithelium than in the surface epithelium by HP infection, resulting in a relatively lower expression level of RUNX3 in the glandular epithelium than in the surface epithelium, lending support to the hypothesis that carcinogenic transformation may occur more frequently in the glandular epithelium than in the surface epithelium in HP-induced enlarged-fold gastritis.

In conclusion, the low level of RUNX3 expression in the glandular epithelium and high level of oxidative stress in the corpus mucosa may contribute to the high risk of carcinogenesis associated with enlarged-fold gastritis.

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