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헬리코박터 파일로리균이 상피-간엽전환을 통해 위암발생에 미치는 역할

The role of *Helicobacter pylori* on gastric carcinogenesis through epithelial-mesenchymal transition

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The role of *Helicobacter pylori* on Gastric Carcinogenesis through Epithelial-Mesenchymal Transition

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Abstract

The Role of *Helicobacter pylori* on Gastric Carcinogenesis through Epithelial-Mesenchymal Transition

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We know little concerning the expression of transforming growth factor-β1 (TGF-β1) and TGF-β1-induced epithelial-mesenchymal transition (EMT) markers in gastric mucosa and their changes after eradication of *Helicobacter pylori* infection have not yet been clarified. In the present study, we compared the time course of mRNA expression of TGF-β1 and 5 EMT markers (Twist, Snail, Slug, vimentin, and E-cadherin) in 111 controls, 55 patients with gastric dysplasia, and 71 patients with early gastric cancer, following eradication of *H. pylori*. mRNA levels in noncancerous gastric mucosa were measured using qRT-PCR and the histologic findings of gastric mucosa were compared before and after eradication. The average duration of follow-up was 46.7 months

(6.0–112.4). The levels of TGF-β1, Twist, Snail, Slug, and vimentin mRNA, in addition to levels of CD44 and Leucine-rich repeat-containing G-protein coupled receptor 5 (Lgr5) detected by immunohistochemistry, showed all up-regulation in patients with dysplasia or early gastric cancer compared with controls ($P < 0.05$); moreover, the mRNA levels of E-cadherin, an epithelial marker, were decreased in these patients compared with the control group ($P < 0.001$). Eradication of *H. pylori* reduced the expression of TGF-β1, Twist, Snail, Slug and vimentin mRNA (*P*-value for slope < 0.001), as well as the immunohistochemical expression of CD44 ($P = 0.014$), whereas it enhanced the expression of E-cadherin (*P*-value for slope < 0.05). Thus, *H. pylori* infection may trigger the TGF-β1-induced EMT pathway and the emergence of gastric cancer stem cells. Its eradication may prevent the carcinogenesis of gastric cancer by inhibiting these 2 pathways.

Keywords: *Helicobacter pylori*, epithelial-mesenchymal transition, cancer stem cells, CD44, Leucine-rich repeat-containing G-protein coupled receptor 5

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1. Introduction

Gastric cancer (GC) is still a leading cause of cancer-related mortality worldwide. GC is the third leading cause for men and the fifth leading cause for women of global cancer mortality (1). Within the last decade, both incidence and mortality have been declining substantially, particularly in developed countries. For example, GC is responsible for 3.7% (male) and 3.0% (female) of cancer-related deaths in Germany in 2008 (www.krebsdaten.de), respectively.

Due to the development of diagnostic and therapeutic strategies there is excellent survival for patients with early GC while the prognosis for the advanced cancers is not favorable. The five-year survival rate is less than 30% in most countries because of a rather late diagnosis for the majority of patients (2). Among GC patients, 90% develop adenocarcinomas; whereas 10% develop lymphomas and stromal tumors. Adenocarcinomas were classified histologically into the “intestinal” (50%, differentiated), the “diffuse” (33%, poorly differentiated), and the mixed or unclassified types (17%) (3-5).

GC is promoted by environmental factors including high-salt diet, smoked food or meat/nitrates/nitrites, chili peppers, alcohol, smoking, as well as a diet low in fruits and vegetables, and low fiber intake (6). However, the most striking risk factor for GC is infection with

Helicobacter pylori, which was discovered in 1984 by Marshall and Warren and classified since 1994 by the WHO as a class I carcinogen (7, 8). For the intestinal type of gastric adenocarcinomas, a well-defined multi-step process was described in 1988 on the base of chronic inflammation, known as the "Correa cascade", starting from chronic gastritis and proceeding to gastric atrophy, intestinal metaplasia (IM), and dysplasia. Only in 1992 was this model extended by introducing *H. pylori* as a major causative agent of chronic inflammation and GC. In contrast, the diffuse type of gastric adenocarcinomas, which can also result from *H. pylori* infection, arises in the absence of metaplasia (9).

Although *H. pylori* is assumed to trigger the early stages of this process, the exact initiation mechanism or the origin of GC has not been identified.

The cancer stem cell (CSCs) hypothesis has emerged as a likely theory for the initiation of cancer. The available data suggest that CSCs are present in many solid tumors and cancer cells are derived from a stem cell compartment which undergoes an abnormal replicative process to form a non-stem cell component of the tumor (10). Although CSCs with different specific cell surface markers have been characterized, CD44 is usually present and has also been identified as a marker of gastric CSCs (11).

Epithelial-mesenchymal transition (EMT) is considered to be a key process of cancer spreading (12). During EMT, the epithelial cell detaches from adjacent cells, and infiltrates surrounding tissue, resulting in the invasion or metastasis of cancer. Consequently, loss of E-cadherin, the

prototypical epithelial cell marker, and increase in intermediate filament, vimentin and several EMT-inducing transcription factors, such as Snail, Slug, Twist are observed (13). Interestingly, recent studies have suggested that cells undergoing the EMT acquire stem cell-like properties (14, 15). Mani et al. (15) showed that the induction of EMT in immortalized mammary epithelial cells generated a subpopulation of CD44+/CD24- cells with both the phenotype and properties of breast CSCs. Considering the evidence indicating EMT and a stem cell as an origin of breast cancer (14, 16), it is of interest to investigate whether *H. pylori* is a common triggering factor for both EMT and CSCs in gastric cancer. Transforming growth factor-β (TGF-β) is known to be potent inducer of EMT (17), and *H. pylori* infection promotes the expression of TGF-β1 in both gastric epithelial cell lines and human gastric tissue (18-20).

Recently, cancer has been thought to derive from different "cells of origin" and tumors consist of a heterogeneous population of cancer cells which originate from a small subset of CSCs (21-23). According to this concept, only CSCs can initiate tumor formation being the basis of the CSC theory (21). Although many proteins have been proposed as markers for gastric cancer stem cells, the identification and confirmation of them in normal human tissues remains tricky. CD44 and leucine-rich repeat-containing G-protein coupled receptor 5 (LGR5) are the most promising putative cancer

stem cell markers related with gastric cancer (10, 24).

Although EMT could represent a convergence point of *H. pylori* infection and gastric cancer initiation, there is only limited experimental data supporting the association, particularly in breast cancer. Moreover, the influence of *H. pylori* infection and its eradication on EMT or the emergence of CSCs has not been investigated to date.

Here, we hypothesized that *H. pylori* infection triggers EMT and emergence of gastric cancer stem cells contributing to gastric carcinogenesis. Non-cancerous tissue, not tumor tissue was evaluated in order to elucidate whether this marker may reflex early event of carcinogenesis. Specifically, we evaluated the possible carcinogenic effects of TGF-β1-induced EMT and CSCs by comparing the levels of mRNAs encoding TGF-β1 and 5 EMT markers (Twist, Snail, Slug, vimentin, and E-cadherin) in non-cancerous mucosa from control subjects to those in patients with dysplasia or gastric cancer. For comparison at the protein level, Snail, E-cadherin, CD44, LGR5 were analyzed by immunohistochemistry (IHC). We also investigated long-term changes in expression before and after *H. pylori* eradication in order to evaluate whether anti-*H. pylori* therapy may suppress EMT. Additionally, since *H. pylori* infection has been implicated as a major cause of IM (25, 26), the possible relationship between expression of EMT markers and IM was assessed.

2. Materials and Methods

2.1 Patient Selection

Study subjects were consecutively enrolled at Seoul National University Bundang Hospital from February 2006 to December 2013. We invited subjects with current *H. pylori* infection were to be participating. After exclusion of patients who refused to receive anti-*H. pylori* therapy for various reasons, potential adverse effects, cost, or infirmity, all participants underwent anti-*H. pylori* treatment. We divided patients into 3 groups according to the pathologic results, namely control, dysplasia, and early gastric cancer. Controls were selected from those who when upper GI endoscopy showed no evidence of malignant lesions or peptic ulcer. Patients with dysplasia or early gastric cancer underwent endoscopic submucosal dissection or mucosal resection. Patients with advanced gastric cancer were excluded since the aim was to assess EMT in the early stages of carcinogenesis.

The first-line therapy for *H. pylori* infection included 7-day triple therapy (esomeprazole 40 mg b.i.d., clarithromycin 500 mg b.i.d., and amoxicillin 1000 mg b.i.d.). To evaluate whether *H. pylori* was eradicated, a 13C-urea breath test was performed at least 4 weeks after the completion of therapy. If *H. pylori* were not eradicated, 2 weeks of second-line treatment (tripotassium dicitrate bismuthate 300 mg q.i.d., metronidazole 500 mg t.i.d., tetracycline 500 mg q.i.d., and esomeprazole 20 mg b.i.d.)

was administered. A total of 237 patients in whom *H. pylori* had been eradicated and who had been followed for more than 6 months were enrolled. Among these, 111 controls, 55 subjects with gastric dysplasia, and 71 patients with early gastric cancer were included. In order to compare the mRNA or IHC expression of EMT markers between *H. pylori*-positive and-negative subjects, non-cancerous gastric tissue from 14 *H. pylori*-negative, stage I gastric cancer patients who were enrolled in the same period and from 20 *H. pylori*-naive control subjects was analyzed. The mean duration of follow-up was 46.7 months (range: 6.0–112.4), with mean number of 4.10 follow-up visits (range: 1−9). The study protocol was approved by the Ethics Committee at Seoul National University Bundang Hospital (IRB number B1301/186-111). All subjects provided written informed consent and were asked to complete a questionnaire that included questions regarding demographic information and socioeconomic habits.

2.2 *H. pylori* testing and histology

During endoscopy, biopsies were obtained from both the gastric antrum and body for determining *H. pylori* infection status and histology. Baseline *H. pylori* infection status was determined based on modified Giemsa staining, culture, rapid urease test (CLO test, Delta West, Bentley, Australia) (25). If one of these invasive tests was positive, the patient was judged to be currently *H. pylori* infected. As for the histologic feature of

the gastric mucosa, the degree of inflammatory cell infiltration, atrophy, and IM was recorded using the updated Sydney scoring system (0 = none, 1 = slight, 2 = moderate, and 3 = marked) (27). If the histologic grade of IM or glandular atrophy continuously decreased, participants were considered to have improved. If the IM increased or did not change, the case was considered as non-improved. If IM grade changed inconsistently, the patient was excluded.

Fasting serum was collected from study subjects and serum concentrations of pepsinogen (PG) I and II were measured using a latex-enhanced turbidimetric immunoassay (Shima Laboratories, Tokyo, Japan). Based on the results of serum PG tests, patients were categorized as having no, mild to moderate, or severe gastric atrophy, according to the definition of Kato et al (28).

2.3 Real-time quantitative polymerase chain reaction

Total RNA was extracted directly from noncancerous gastric body specimens with TRIzol® reagent (Invitrogen, Carlsbad, CA, USA), as recommended by the manufacturer. Next, 1500 ng of RNA was reverse transcribed to cDNA with oligo (dT) and M-MLV reverse transcriptase (Invitrogen, CA), according to the manufacturer's instructions. Quantitative PCR was performed in 96-well reaction plates using 2 μl of cDNA in a 20 μl reaction mix containing 2× SYBR® Premix Ex TaqTM (Takara Bio Inc., Otsu, Japan). Samples were run on an Applied

Biosystems 7500/7500 Fast Real-Time PCR instrument (Applied Biosystems, Foster City, California, USA), The PCR cycling conditions were as follows: an initial denaturation step for 30 s at 95℃ and then 40 cycles of denaturation at 95℃ (5 s) and annealing at 60℃ (34 s) with the final dissociation stage of 15 s at 95℃, 1 min at 60℃ and 15 s at 95℃. The primer sequences for PCR are shown in Table 1. Expression levels of mRNA of the target gene were compared with the endogenous control β-actin using the 2-ΔΔCt method (29).

2.4 Immunohistochemistry

Immunostaining of CD44, Snail, and E-cadherin was also performed. Paraffin-embedded non-cancerous mucosal tissue distant from tumor sites was analyzed from 50, 29, and 38 subjects in the control, dysplasia, and cancer groups, respectively. For the dysplasia and cancer groups, the area of the stomach where the lesion was located was chosen. For the control group, 24 specimens were obtained from the antrum and 26 specimens were obtained from the body.

Core tissue biopsies (2 mm in diameter) were obtained from paraffin-embedded gastric mucosa. Cores were arranged in recipient paraffin blocks (tissue array blocks) using a trephine. The test procedure used a human control slide for IHC analysis (Superbiochips Laboratories, Seoul, Korea). Antibodies against CD44 (H-CAM) (1:200 dilution; Leica

Biosystems, Breckland, UK), Snail (NBP1-19529) (1:100 dilution; Novus Biologicals, Littleton, CO, USA), and E-cadherin (610181) (1:400 dilution; BD Biosciences, San Jose, CA, USA) were used. Staining of sections (4 μm thick) from tissue array blocks was performed using the BenchMark XT staining system and the ultraVIEW Universal DAB Detection Kit (Ventana Medical Systems Inc., Tucson, AZ, USA). Snail is aberrantly expressed in the nuclei of gastric cancer cells, whereas it is rarely expressed in non-neoplastic gastric epithelial cells. Moreover, aberrant expression of CD44 is observed in the membrane and cytoplasm of cancer cells. E-cadherin was strongly expressed in the membrane of non-neoplastic glands (30). Scoring for the expression of CD44, LGR5 Snail, and E-cadherin in gastric epithelium was determined using light microscopy with a magnification of 200, by multiplying the intensity times the area (%) where staining was observed in epithelial glands; the possible scores ranged from 0 to 300. Area was defined as a 'stained cells in a gland/total cells in a gland'. More than 5 glands were evaluated. The intensity of staining was scored as 0, no staining; 1+ faint/barely perceptible partial staining; 2+, weak to moderate staining; 3+, strong staining (31). Tests were repeated three times and mean values were described. Each sample was scored by a blind reviewer.

2.5 Statistical analysis

The $\chi 2$ test and Fisher's exact test were used for the analysis of categorical

variables. To find the best model for the time course of mRNA levels of TGF-β1 and EMT markers after *H. pylori* eradication, a linear mixed model (LMM) was applied. There was no significant difference in the results between the LMM and generalized additive mixed model (32). Moreover, LMM is appropriate for the data since it incorporates a generic correlation structure of longitudinal data by considering both within-subject and between-subject variations (33). After the model selection process was completed by comparing Akaike Information Criteria, a random intercept model was employed. The mRNA levels encoding TGF-β1 and 5 EMT markers were compared between groups using one-way analysis of variance (ANOVA) followed by Scheffe's or Tamhane's tests. Analysis of covariance was used for the adjustment of *H. pylori* infection status and development of cancer. For the analysis of changes in histologic grades after the eradication of *H. pylori*, a paired t-test or Wilcoxon rank sum test was used. All analyses were performed using either R (version 2.13.0, The R Foundation for Statistical Computing) or SPSS (version 21.0, IBM, NY, USA).

3. Results

3.1 Demographic characteristics

Table 2 shows the demographic and histologic characteristics of *H. pylori*-positive subjects (n = 237). Patients with dysplasia or gastric cancer

were significantly older than controls. In terms of histology, neutrophil infiltration of the antrum was more prominent in the control group than in the dysplasia or cancer groups (Table 2). Atrophic gastritis (determined by histology and serum PG I/II ratio) and IM were more prevalent in patients with dysplasia and gastric cancer than controls (Table 2).

3.2 Expression of mRNAs encoding EMT-markers and CD44, and IHC analysis in *H. pylori*-positive subjects

The expression of mRNAs encoding TGF-β1 and 5 EMT markers were measured before *H. pylori* eradication, to determine whether EMT is involved in the dysplasia–cancer sequence. The levels of TGFB1 mRNA in the control group were significantly lower than those in the cancer group (1.66 ± 0.18 vs. 3.07 ± 0.46, P = 0.005) (Figure 1A). Similarly, mRNAs encoding Twist, Snail, Slug, and vimentin were more up-regulated in patients with early gastric cancer than control subjects (Fig. 1B-E). In contrast, the expression of E-cadherin mRNA was significantly lower in patients with dysplasia and early gastric cancer than in controls (3.75 ± 0.71 vs. 0.17 ± 0.07 and 0.28 ± 0.09, all P < 0.001) (Figure 1F).

In addition, there was a trend toward increased expression of mRNAs encoding TGF-β1, Snail, slug and vimentin along the normal–dysplasia–cancer sequence (TGF-β1, P trend = 0.003; Twist, P trend = 0.004; Snail, P trend = 0.002; Slug, P trend < 0.001 and vimentin, P trend = 0.008,

respectively). In contrast, a sequential decreasing trend in the mRNA levels of E-cadherin was observed as the disease progressed toward cancer (P trend < 0.001). When CD44 mRNA was investigated as a possible gastric CSC marker, patients with cancer or dysplasia showed lower expression of CD44 mRNA compared with the control subjects (0.72 ± 0.10 and 0.48 ± 0.04 vs. 1.62 ± 0.16, all P < 0.001) (Figure 1G).

IHC staining of Snail, CD44, and E-cadherin was analyzed. Tissues in the cancer group showed strong staining of Snail and CD44 compared with the control group (Snail: 167.88 ± 14.70 vs. 110.05 ± 12.39, P = 0.002) (Figure 1H and 2A, upper row) (CD44: 67.50 ± 12.39 vs. 27.40 ± 6.52, P = 0.007) (Figure 1I and 2C, upper row).

Since most epithelia stained markedly with a membranous pattern, relative comparison of E-cadherin by IHC was not possible.

3.3 Association between genes encoding EMT markers and CD44 expression

To investigate the relationship between EMT-related genes and CD44 mRNA, the expression levels were compared using Spearman's rho test (Table 3). The expression of TGF-β1 mRNA significantly correlated with the expression of the Twist, Snail, Slug, and vimentin genes (all *P* < 0.001). Messenger RNAs encoding EMT-acquired markers (Twist, Snail, Slug and vimentin) showed significant positive correlations with each

other. Slug mRNA expression had an inverse correlation with the expression of E-cadherin mRNA (Spearman's rho = -0.180, $P = 0.015$).

Unexpectedly, the expression of CD44 mRNA did not correlate with the expression of the 4 EMT-inducing genes or with the CD44 staining (Table 3) (CD44 IHC: Spearman's rho = -0.032, $P = 0.734$). Instead, CD44 mRNA expression positively correlated with that of E-cadherin (Spearman's rho = 0.184, $P = 0.005$).

The intensity of CD44 IHC had significant positive correlations with the expression of Snail and Slug mRNAs (Spearman's rho = 0.396, $P < 0.001$ and Spearman's rho = 0.372, $P < 0.001$, respectively).

3.4 Expression of TGF-β1 and EMT markers between the *H. pylori*-positive and *H. pylori*-negative groups

The expression of mRNAs encoding TGF-β1 and EMT markers was compared between the *H. pylori*-positive and *H. pylori*-negative groups (Table 4). *H. pylori*-infected control subjects showed significantly elevated expression levels of TGF-β1, Twist, and vimentin mRNAs compared to *H. pylori*-negative controls (Figure 2, A, B and E) (TGF-β1: 1.30 ± 0.42 vs. 2.87 ± 0.57; Twist: 1.02 ± 0.08 vs. 2.77 ± 0.45; vimentin: 0.98 ± 0.09 vs. 1.99 ± 0.30). Snail (Figure 2C) and Slug (Figure 2D) also presented similar tendencies, whereas the expression of the E-cadherin gene was suppressed to a greater extent in *H. pylori*-positive controls than in *H. pylori*-negative subjects (1.54 ± 0.47 vs. 0.39 ± 0.11, $P = 0.001$)

(Figure 2F).

In the cancer group, markedly more enhanced expression of mRNAs encoding TGF-β1 and EMT markers was seen in the *H. pylori*-positive group than the *H. pylori*-negative group, although the differences in Snail mRNA expression did not reach statistical significance (TGF-β1: 1.42 ± 0.64 vs. 5.94 ± 1.09; Twist: 0.58 ± 0.09 vs. 5.32 ± 0.96; Snail: 5.97 ± 2.94 vs. 14.50 ± 3.38; Slug: 0.53 ± 0.10 vs. 2.34 ± 0.30; vimentin: 0.53 ± 0.79 vs. 3.89 ± 0.67) (Figure 2, A-E). E-cadherin mRNA expression in *H. pylori*-infected cancer tissues was lower than that in *H. pylori*-negative cancers (0.67 ± 0.17 vs. 0.14 ± 0.07, $P = 0.068$) (Figure 2F).

In multiple regression analyses, both *H. pylori* infection and cancer were independent factors for the induction of TGF-β1, Twist, Slug, and vimentin mRNA expression (Table 5). In contrast, both *H. pylori* infection and the development of cancer independently reduced the expression of E-cadherin mRNA. The expression of CD44 mRNA was suppressed to a greater extent than in control subjects (Table 5).

The IHC expression of Snail and CD44 in *H. pylori*-negative tissues showed weaker staining than in in *H. pylori*-positive tissues ($P < 0.001$ and $P = 0.077$, respectively) (Figure 3A vs. Figure 3B, upper row and Figure 3C vs. Figure 3D, upper row). Multiple regression analyses of the immunoreactivity of Snail and CD44 were also performed. Both *H. pylori* infection and cancer were independently associated with enhanced immunoreactivity of Snail and CD44 in comparison with control and *H.*

pylori-negative tissues (Snail IHC: cancer, β = 0.334 and $P < 0.001$, *H. pylori*-positive: β = 0.402 and $P < 0.001$; CD44 IHC: cancer, β = 0.310 and $P = 0.001$, *H. pylori*-positive: β = 0.210 and $P = 0.018$) (Table 6).

3.5 Effects of *H. pylori* eradication on histologic grade in gastric mucosa

At the time of the first follow-up (median time: 13.3 months after *H. pylori* eradication), infiltration of both neutrophils and mononuclear cells in the antrum and body was markedly reduced compared with the baseline values (Table 7). The grade of atrophic gastritis decreased only in the body ($P = 0.008$), whereas the PGI/PGII ratio was significantly increased ($P < 0.001$). The degree of IM was marginally improved in the antrum ($P = 0.069$), but did not show any significant changes in the body (Table 7). However, at the last follow-up (mean duration of follow-up: 44.47 months after *H. pylori* eradication), a significant reduction in IM was seen in both the antrum and body (reaching 0.94 ± 0.06 in the antrum and 0.52 ± 0.06 in the body, $P = 0.013$ and $P = 0.028$).

3.6 Effects of *H. pylori* eradication on the expression of TGF-β1, EMT markers, and CD44

We then evaluated the effects of *H. pylori* eradication on the mRNA expression of TGF-β1 and 5 EMT markers. The expression of TGF-β1,

Twist, vimentin, Snail, and Slug mRNA was markedly reduced at approximately 1 year after *H. pylori* eradication (Table 7). Moreover, the expression of these genes decreased continuously after *H. pylori* eradication for several years (*P*-value for slope < 0.001, Figure 4). In contrast, the temporal profiles of E-cadherin mRNA showed a steady increase following eradication of *H. pylori* (*P*-value for slope < 0.05) (Figure 4).

To determine whether the reversibility of EMT occurs regardless of the development of cancer or dysplasia, we examined the changes in genes encoding EMT markers after *H. pylori* eradication in the 3 groups. In this subgroup analysis, the mRNA levels of TGF-β1, Twist, Snail, Slug, and vimentin were found to be reduced after *H. pylori* eradication in all groups (*P*-value for slope < 0.05) (Figure 5A-5O). The increased expression of E-cadherin mRNA was statistically significant in the dysplasia and cancer groups (Figure 5, Q and R), but not in the control group (Figure 5P).

The immunohistochemical results for CD44, Snail, and E-cadherin were compared before and after *H. pylori* eradication (Figure 3A and 3F). After eradication, all 3 groups showed a significant reduction in the expression of Snail (Figure 3A; upper to bottom row). For CD44, the cancer group showed a marked reduction in expression after eradication and resection, while the differences in the dysplasia and control groups did not reach statistical significance (Figure 3C; upper to bottom row). In contrast, *H. pylori*-negative tissues did not show any difference in staining

of either Snail or CD44 at the 1-year follow-up (Figure 3B and 3D; upper to bottom row).

3.7 Relationship between TGF-β1/EMT markers and intestinal metaplasia

To further investigate whether IM might correlate with EMT, the initial mRNA levels of *TGFB1* and 5 EMT markers were compared according to the severity of IM. Overall, quantitative RT-PCR analyses revealed that increased expression of the TGF-β1, Twist, Snail, Slug, and vimentin genes tended to correlate with increased severity of IM (Figure 6, A-E) (*P* trends for TGF-β1, Twist, Snail, Slug, and vimentin mRNA: 0.005, 0.020, 0.002, 0.051, and 0.021, respectively). However, no such trend was seen for E-cadherin mRNA.

The expression of the genes was compared according to the severity of IM in the 3 groups. The control group showed a trend towards increased expression of Twist, vimentin, and Slug mRNA (*P* trends for TGF-β1, Twist, Snail, Slug, vimentin, and E-cadherin mRNAs: 0.178, 0.020, 0.285, < 0.001, 0.030, and 0.920, respectively). In contrast, no such trend was seen in the dysplasia and cancer groups (*P* trends for TGF-β1, Twist, Snail, Slug, vimentin, and E-cadherin mRNAs in the dysplasia group: 0.866, 0.857, 0.831, 0.545, 0.174, and 0.639 respectively; in the cancer group: 0.311, 0.857, 0.676, 0.898, 0.127, and 0.735, respectively). With regard to

the possible association between gastric CSCs and IM, IHC staining of CD44 showed a trend toward increased expression in more severe IM grades (Figure 6G).

Additionally, we assessed whether improvement of IM in the body was associated with a reduction in mRNA expression of *TGFB1* or EMT markers. Among the 237 subjects, 55 showed improvement of IM, 58 had an increase or no change in the Sydney score, and the remaining 7 patients showed a fluctuation. As a result, regardless of improvement of IM, the levels of mRNAs encoding EMT-acquired markers and TGF-β1 were significantly reduced, whereas those of E-cadherin were increased (all $P < 0.05$) (Figure 7) describes Snail (A-C) and E-cadherin mRNA (D-F) expression).

3.8 Association between LGR5 immunoreactivity and gastric carcinogenesis

The IHC expressions of LGR5 were compared between *H. pylori*-negative and *H. pylori*-positive tissue according to the normal-dysplasia and adenocarcinoma sequence.

Noncancerous tissue of cancer patients showed markedly stronger staining than that of control subjects regardless of *H. pylori* status (*H. pylori*-positive: 29.0 ± 4.36 vs. 72.70 ± 7.20, $P < 0.001$; *H. pylori*-negative: 17.60 ± 5.56 vs. 51.60 ± 6.40, $P = 0.002$) (Figure 8).

Increasing intensity of LGR5 staining was observed from normal, dysplasia to gastric cancer (29.00 ± 4.36, 43.20 ± 8.51 and 72.70 ± 7.20, *P* for trends < 0.001).However, there were any significant differences in LGR5 IHC between *H. pylori*-negative and positive subjects neither in control nor in cancer group.

3.9 Effects of *H. pylori* eradication on the expression of LGR5

We then evaluated the effects of *H. pylori* eradication on the LGR5 IHC. The immunointensity of LGR5 was markedly reduced at approximately 1 year after *H. pylori* eradication (45.36 ± 3.54 to 32.45 ± 2.82, $P < 0.001$).

To determine whether the change in LGR5 expression occurs regardless of the development of cancer or dysplasia, we examined the changes in immunohistochemical results for LGR5 IHC after *H. pylori* eradication in the 3 groups. After eradication, control and cancer groups showed a significant reduction in the expression of LGR5 IHC (control: 29.59 ± 4.41 to 15.25 ± 2.43, $P = 0.004$; cancer: 72.73 ± 7.01 to 49.9 ± 5.64, $P = 0.004$) (Figure 9A and 9C). Contrary to this, non-cancerous mucosa of *H. pylori*-positive subjects with dysplasia and *H. pylori*-negative subjects showed no significant

changes of LGR5 IHC (Figure 9B, 9 D-E).

3.10 Association between expression of LGR5 and CD44

To investigate the relationship between LGR5 IHC and CD44 IHC, the expression levels were compared using Spearman's rho test. The score of LGR5 IHC significantly correlated with the expression of CD44 immunoreactivity (surface epithelium: Spearman's rho = 0.354, $P < 0.001$).

3.11 Expression of LGR5 IHC according to the location of gastric mucosa and intestinal metaplasia

Immunoreactivity of LGR5 in the body was significantly reduced than in the antrum (29.45 ± 4.40 vs. 56.21± 4.21, $P < 0.001$) (Figure 10A). While antral mucosa did not show any significant difference in LGR5 IHC according to presence or absence of IM (54.58 ± 8.93 vs. 61.03 ± 6.51, $P = 0.637$) (Figure 10B), gastric body mucosa with IM showed an elevated immunointensity than in that without IM (53.98 ± 7.29 vs. 7.11 ± 2.17, $P < 0.001$). Increasing intensity of LGR5 staining in gastric body was observed when the severity of IM was increased (mild IM: 43.13 ± 8.33; moderate/severe IM: 57.60 ± 9.29, P for trends < 0.001) (Figure 10C).

4. DISCUSSION

We set out to determine the possibility of evaluating the relationship between EMT markers and initiation of gastric cancer and stem cell and investigated changes in long-term expression of EMT markers during long-term post-eradication of *H. pylori*. In this study, up-regulation of mRNAs encoding EMT-acquired markers (vimentin, Twist, Snail and Slug) and loss of E-cadherin mRNA correlated with the dysplasia–cancer sequence. Similarly, immunoreactivity of CD44 and LGR5 progressively increased in sequence, from normal to dysplasia to cancer, although CD44 mRNA did not. Following *H. pylori* eradication, there was a marked reduction in mRNA of EMT-acquired markers and recovery of E-cadherin mRNA was seen. In the case of the cancer group with *H. pylori*-eradication after endoscopic resection of cancer, a significant reduction in both CD44 and LGR5 IHC was detected, while *H. pylori*-eradicated control subjects showed a reduction only in LGR5 immunostaining. However, IHC staining of CD44 correlated well with the expression of both Snail and Slug mRNAs, whereas that of CD44 mRNA did not.

Other studies have shown that *H. pylori* promotes EMT in gastric

cancer cells. For example, CagA-positive *H. pylori* stabilizes Snail (34), and Yu et al. reported that CagA induces both TWIST1 and vimentin and inhibits E-cadherin by down-regulation of programmed cell death protein 4 (35). In agreement with these studies, we observed enhanced mRNA expression of 4 EMT-mesenchymal markers in *H. pylori*–positive subjects compared with those who were negative for *H. pylori*. Since this result was observed even in control subjects, we gave more attention to the possible contribution of EMT to early carcinogenesis of gastric cancer related with CSCs or IM. Since CagA positivity was high (89.5%) in our sample (36), further analysis according to the presence or absence of the toxin was not performed.

Besse`de et al. (37) recently demonstrated that *H. pylori*, via CagA, is responsible for an EMT phenotype associated with an increase in EMT markers and CD44 expression in gastric epithelial cell lines. In particular, cells expressing high levels of CD44 were highly tumorigenic in a xenograft mouse model, and higher expression of CD44 and EMT markers was seen in human and mouse gastric mucosa in *H. pylori*-positive gastric dysplasia and carcinoma (37).

Our study showed a step-wise increase in CD44 immunostaining

in the normal, dysplasia, and cancer sequence (*P* trend < 0.001). In the multiple regression model, both *H. pylori* infection and cancer development independently predicted increased expression of CD44 by IHC. Taken together, these observations indicate that *H. pylori* infection promotes EMT process, which may be associated with the initiation of tumorigenesis via gastric CSCs.

CD44 has been identified as a well-known marker for CSCs in gastric cancer, even if it is expressed ubiquitously in various cell types, including hematopoietic cells (38). Unexpectedly, the levels of CD44 mRNA did not show any correlation with other EMT-inducing genes, but IHC analysis of CD44 did correlate with the levels of Snail and Slug mRNA in the present study. A possible reason for this discrepancy is that immunostaining can detect cell morphology and discriminate inflammatory cells from possible CSCs unlike qRT-PCR. The application of CD44 mRNA as the CSCs marker may work well for in vitro studies, but is of limited value in application to clinical samples, although further study is needed to clarify this issue. Previous studies have suggested that CD44v8-10 was a specific maker for gastric CSCs, but not CD44s (29, 39). Other study reported that different CSC markers such as ALDH1, LGR5, and CD166 are increased in *H. pylori*–associated

gastritis and gastric adenocarcinomas (40). Furthermore, expression of CD44 is known to be closely linked with the Wnt/β-catenin pathway (41). True markers for gastric CSCs still need to be identified.

For the question concerning the origin of cancer or cancer stem cells, bone marrow-derived cells (BMDCs) have been thought to be recruited to the stomach and repopulate the gastric mucosa after chronic *H. pylori* infection (42, 43). About 25% of the dysplastic lesions included cells that originated from the bone marrow (43). This implies that majority of dysplastic lesions do not originate from the bone marrow and adult stem cells are still attractive candidate for source of gastric CSCs (21). Lgr5 is an adult stem cell marker expressed in the small intestine, colon, stomach, and hair follicles (44). Currently, *in vivo* lineage tracing revealed that a group of Lgr5-postive cells at the base of the pyloric glands were multipotent stem cells that contributed to daily epithelial renewal (45). However, investigations ofLGR5 expression in human tissues, have been limited, and an appropriate histological method to identify Lgr5- cells in human organs has not been established (46).

In the present study, non-cancerous tissue of *H. pylori*–positve control, patients with dysplasia and cancer showed an increasing

tendency of LGR5 immunoreativity in accordance with CD44 IHC. This suggests that CD44 and LGR5-posive cells may be involved in early gastric carcinogenesis related with *H. pylori* infection.

Even though accumulative evidence supports the existence of CSCs that have the capacity to generate tumor, the stem cell hypothesis in carcinogenesis is under debate. Nonetheless, EMT process has been regarded to be associated with both inflammation and initiation of tumor. It has been reported that the protein of *H. pylori*, HP0157, induced gastric Th17 responses with up-regulation of matrix metalloproteinase (MMP)-2 and MMP-9, which are related to malignancy and invasion, in patients with distal gastric adenocarcinoma (47). While TGF-β1 induced expression of Twist and Smad interacting protein 1 (SIP1), Snail enhanced the secretion of interleukin (IL)-1, IL-6 and IL-8 (48). Up-regulation of EMT-acquired markers with gaining of migratory properties by *H. pylori* infection was recently demonstrated (49). Consequently, EMT can be a link between *H. pylori* infection and gastric carcinogenesis, facilitating de-differentiation or re-differentiation of tissue.

Of note in our study is that mRNA expression of TGF-β1, EMT-inducing transcription factors and mesenchymal markers, as well as IHC staining of CD44, continuously decreased following

eradication of *H. pylori*. Although there was no significant difference in the expression of CD44 by IHC before and after *H. pylori* eradication in control subjects, this may be due to the small number of control subjects who were positive for *H. pylori* at the start of the study. Although it is still debatable whether *H. pylori* eradication can reduce the risk of gastric cancer (50-53), several studies have also reported that gastric cancers with high expressions of EMT-inducing transcription factor or mesenchymal marker is associated with poor survival along with advanced stage, undifferentiated histologic type, and vascular or neural invasion (30). Although it is unclear whether these results derive from the invasive nature of EMT itself or accompanying gastric CSCs, considering the decrease in EMT markers after *H. pylori* eradication in all groups, anti-*H. pylori* therapy is beneficial for most subjects infected with *H. pylori*. In Japan, its eradication has been recommended for all individuals with *H. pylori*-positive gastritis (http://www.mhlw.go.jp/seisakunitsuite/bunya/kenkou_iryou/iryouhouken).

In addition, the reduction in Sydney grade of corporal glandular atrophy and antral IM after the *H. pylori* eradication was shown in the present study, similar to a previous report (54). Moreover,

improvement of IM in the gastric body was observed when follow-up was extended.

However, since the degree of improvement was modest (mean change in Sydney score of -1.41 ± 0.08) and most of improved cases initially had mild degree of IM, it appears that radical improvement of IM rarely occurs. Heterogeneous histological distribution of IM lesions also demands precautious interpretation of this result.

As to a potential association between IM and EMT, or CSCs, information is limited. Expression of the Cdx gene is closely related with IM (55). Given the abundant expression of Cdx1 and Cdx2 in IM lesions in the stomach and the multipotentiality of stem cells, it can be hypothesized that the intestinalization of gastric stem cells is the initiating event in IM (24). On the other hand, an indirect association between EMT and IM through bone morphogenetic protein (BMP) has been reported in previous studies. BMP pathway induces the expression of Cdx2 (44), and EMT is also activated by the BMP family (56, 57).

In the present study, the expression of mRNAs encoding EMT-inducing transcription factors or mesenchymal markers and IHC staining of CD44 and LGR5 were both increased in proportion to

the grade of IM. Interestingly, immunoreactivity of LGR5, known for the stem cell marker of antrum, was detected in gastric body with IM.

Although we did not demonstrate that IM arises from CSCs, our data can be considered as preliminary evidence that provides further insights into the possibility that the pathogenesis of IM is associated with EMT or CSCs.

The present work has several limitations. Since we did not confirm stem cell properties by cell culture or animal models, it is not clear whether the elevated number of CD44 or LGR5-positive epithelial cells reflects true gastric CSCs. Since mucosal resection was performed in the dysplasia and cancer groups, marked reduction of these EMT markers at follow-up cannot be solely attributable to *H. pylori* eradication.

Nonetheless, this is one of the few studies that imply that the EMT may favor development of gastric cancer and that this event is likely associated with gastric CSCs and *H. pylori* at an early stage of tumorigenesis. Moreover, the observed reduction of EMT-inducing transcription factors, mesenchymal markers and IHC expression of CD44 and LGR5 after *H. pylori* eradication suggests that eradicating *H. pylori* will benefit most individuals with *H.*

pylori infection and aid in the prevention of gastric cancer.

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초 록

서론: Transforming growth factor-β1 (TGF-β1)과 TGF-β1-유래 상피-간엽전환을 위암의 발생의 측면에서, 헬리코박터균의 감염과 관련하여 인체조직에서 연구한 것은 매우 드물다. 또한 최근 상피-간엽전환이 암줄기세포의 출현과 관련있다는 보고들이 있어 이번 연구에서는 정상인과 이형성증, 조기위암을 가진 환자의 정상 위점막조직에서 대조군에서 TGF-β1및 상피-간엽전환의 표지자 발현양을 비교해 보고 제균 전후를 비교함으로써 위암발생과 상피-간엽전환, 위암줄기세포와의 연관성을 헬리코박터 파일로리 균의 감염과 관련지어 이해해 보고자 한다.

방법: 2006년 2월부터 2013년 12월까지 분당 서울대학교 병원을 방문하여 내시경을 시행한 환자 중 6개월 이상 추적 관찰된 111명의 대조군과, 55명의 이형성증, 71명의 조기위암환자를 분석하였다. 또한 같은 기간에 등록된 연구대상자중 14명의 헬리코박터 파일로리 균 음성, 제1병기 위암환자와 20명의 대조군이 따로 분석되었다. TGF-β1및 상피-간엽전환의 표지자 mRNA는 내시경 중에 얻은 중부 체부의 대만곡의 점막에서 Real-time quantitative PCR을 이용하여 측정하였고, CD44 및 LGR5의 면역염색은 병변이 있는 위치의 정상조직을 얻어 시행되었다.

결과: TGF-β1및 상피-간엽전환의 표지자의 mRNA발현 및 면역염색으로 평가한 CD44와 LGR5의 발현은 이형성증이나 위암을 가진 환자의 정상 위점막에서 대조군의 위 점막에서보다 유의하게 높았다($P < 0.05$). 반대로 E-cadherin mRNA의 발현은 이들 환자에서 대조군에 비해 낮게 나타났다($P < 0.001$).

헬리코박터 파일로리균의 제균은 상피-간엽전환의 표지자의 mRNA발현을 억제하였고 (P-value for slope < 0.001), CD44 및 LGR55 IHC를 감소시킨 반면(all P < 0.05), E-cadherin mRNA의 발현을 증가시켰다(P-value for slope < 0.05).

결론: 헬리코박터 파일로리균의 감염은 TGF-β1-유래 상피-간엽전환 및 위암줄기세포의 발현을 촉발한다. 헬리코박터 파일로리균을 제균하는 것은 상기 두 경로를 억제하여 위암의 발생을 예방하는데 기여할 수 있다.

주요어: 상피-간엽전환, 위암줄기세포, 헬리코박터 파일로리, CD44, Leucine-rich repeat-containing G-protein coupled receptor 5

학 번: 2014-30682

Table 1. Primer sequences for qRT- PCR

Gene name	Forward primer	Reverse primer	Size (bp)
TGFB1 (Tumor growth factor-ß1)	5-AGG GCT ACC ATG CCA ACT TCT-3	5-CCG GGT TAT GCT GGT TGT ACA-3	101
TWIST1 (Twist homolog 1)	5-ACA AGC TGA GCA AGA TTCA GAC C-3	5-TCC AGA CCG AGA AGG CGT AG-3	133
VIM (vimentin)	5-AAA ACA CCC TGC AAT CTT TCA GA-3	5-CAC TTT GCG TTC AAG GTC AAG AC-3	170
SNAI1 (Snail)	5-CCC CAA TCG GAA GCC TAA CT-3	5-GGT CGT AGG GCT GCT GGA A-3	73
SNAI2 (Slug)	5-AAA AGC CAA ACT ACA GCG AAC TG-3	5-AGG ATC TCT GGT TGT GGT ATG ACA-3	99
CDH1(E-cadherin)	5-ACA CCA TCC TCA GCC AAG A-3	5-CGT AGG GAA ACT CTC TCG GT-3	115
CD44s	5-TGC CGC TTT GCA GGT GTA T-3	5- GGC CTC CGT CCG AGA GA-3	66
ACTB (β-actin)	5-TTC GAG CAA GAG ATG GCC AC-3	5-CGG ATG TCC ACG TCA CAC TT-3	202

Table 2. Baseline characteristics of 237 *H. pylori*-infected subjects

	No. of Subjects (%)			P^1
	Controls (n = 111)	Dysplasia (n = 55)	Early gastric cancer (n = 71)	
Women	51 (45.9)	17 (30.9)	24 (33.8)	0.357
Age (year, mean ± SD)	53.9 ± 10.9	61.6 ± 7.5	59.6 ± 10.5	< **0.001**
Smoking	(n = 106)	(n = 52)	(n = 62)	0.104
Nonsmoker	57 (53.8)	21 (40.4)	24 (38.7)	
Current/ex-smoker	49 (46.2)	31 (59.6)	38 (61.3)	
Drinking	(n = 105)	(n = 52)	(n = 61)	0.418
Never/rare drinker	66 (62.9)	27 (51.9)	37 (60.7)	
Current/ex-drinker	39 (37.1)	25 (48.1)	24 (39.3)	
Pepsinogen I/II ratio (mean ± SD)	3.65 ± 1.66	2.96 ± 1.23	2.77 ± 1.64	**0.001**
Histology [2]				
Neutrophil infiltration				
Antrum	1.73 ± 0.08	1.13 ± 0.12	1.45 ± 0.11	< **0.001**
None	10 (9.5)	16 (29.6)	12 (18.7)	**0.001**
Mild	19 (18.1)	17 (31.5)	14 (21.9)	
Moderate/severe	76 (72.4)	21 (38.9)	38 (59.4)	
Body	1.63 ± 0.08	1.74 ± 0.10	1.88 ± 0.10	0.136
None	13 (12.3)	5 (9.3)	6 (9.4)	0.411

Mild	20 (18.9)	7 (13.0)	6 (9.4)	
Moderate/severe	73 (68.9)	42 (77.8)	52 (81.3)	
Monocyte infiltration				
Antrum				
None	1 (0.9)	0	1 (1.6)	0.106
Mild	7 (6.6)	9 (16.7)	12 (18.8)	
Moderate/severe	98 (92.5)	45 (83.3)	51 (79.7)	
Body				
None	1 (0.9)	0	0	0.870
Mild	18 (17.0)	9 (16.7)	8 (12.5)	
Moderate/severe	87 (82.1)	45 (83.3)	56 (87.5)	
Atrophic gastritis[3]				
Antrum	(n = 83)	(n = 40)	(n = 52)	
None	31 (37.3)	9 (22.5)	22 (42.2)	**0.019**
Mild	37 (44.6)	13 (32.5)	15 (28.9)	
Moderate/severe	15 (18.1)	18 (45.0)	15 (28.9)	
Body	(n = 83)	(n = 42)	(n = 51)	
None	56 (67.5)	24 (57.1)	26 (51.0)	**0.045**
Mild	15 (18.0)	4 (9.5)	9 (17.6)	
Moderate/severe	12 (14.4)	14 (33.3)	16 (31.4)	
Intestinal metaplasia				

Antrum	(n = 111)	(n = 55)	(n = 71)	< **0.001**
None	54 (47.7)	10 (18.2)	15 (21.1)	
Mild	31 (27.9)	22 (40.0)	18 (25.4)	
Moderate/severe	27 (24.3)	23 (41.8)	38 (53.5)	
Body	(n = 111)	(n = 55)	(n = 71)	< **0.001**
None	83 (74.8)	25 (45.5)	34 (47.9)	
Mild	14 (12.6)	12 (21.8)	20 (28.2)	
Moderate/severe	14 (12.6)	18 (32.7)	17 (23.9)	

[1]*P*-values for Chi-square test or one-way ANOVA analysis for comparison of variables across groups. [2]Some of the specimens were inadequate for histologic evaluation for glandular atrophy because their paraffin sections were not parallel to the vertical axis of the stomach. [3]No atrophy was defined as PG I > 70 and PG I/II ratio > 3.0; severe atrophy was defined as PG I ≤ 30 and PG I/II ≤ 2.0; all other subjects were found to have mild to moderate atrophy. Non-atrophic gastritis indicates that there is no evidence of atrophy in histology as well as in serum pepsinogen tests. Bold style indicates clinical significance.

Table 3. Correlation coefficients of expression status between EMT-related genes

	Correlation coefficient (*rho*)					
	TGFB1	Twist	vimentin	Snail	Slug	E-cadherin	CD44s
TGFB1	1	0.343*	0.420*	0.288*	0.283*	0.045	-0.057
Twist		1	0.525*	0.316*	0.421*	0.040	-0.025
vimentin			1	0.311*	0.458*	0.049	0.038
Snail				1	0.280*	-0.089	-0.093
Slug					1	-0.180**	0.117
E-cadherin						1	0.184**

Spearman's *rho* test was performed, * $P < 0.001$, ** $P < 0.05$

EMT, epithelial-mesenchymal transition.

Table 4. Baseline characteristics between *H. pylori*-positive and *H. pylori*-negative groups

	H. pylori-negative		*H. pylori*-positive		
	Control (n = 20)	Cancer (n =14)	Control (n = 111)	Cancer (n = 71)	*P*
Women	8 (40.0)	4 (28.6)	51 (45.9)	24 (33.8)	0.241
Age[1] (year, mean ± SD)	56.80 ± 10.64	58.71 ± 3.09	53.9 ± 10.9	59.6 ± 10.5	0.003
Current/ex-smoker	33 (42.3)	6 (31.6)	49 (46.2)	38 (61.3)	0.081
Current/ex-drinker	45 (57.7)	6 (31.6)	39 (37.1)	24 (39.3)	0.326

[1] post-*hoc* analysis showed the difference was between *H. pylori*-positive control and cancer groups

Table 5. Relative expression of *TGFB1* and EMT-related mRNAs in multiple regression analyses

mRNA expression	Cancer (vs. control)		*H. pylori* (vs. negative)	
	β	*P*	β	*P*
TGF-β1	0.011	**0.011**	0.137	**0.050**
Twist	0.162	**0.013**	0.163	**0.013**
vimentin	0.171	**0.009**	0.154	**0.018**
Snail	0.130	0.152	0.096	0.288
Slug	0.143	**0.034**	0.168	**0.013**
E-cadherin	- 0.150	**0.018**	- 0.263	< **0.001**
CD44	- 0.335	< **0.001**	- 0.086	0.165

EMT, epithelial-mesenchymal transition.

Both cancer and *H. pylori* infectious status were taken into a linear regression analyses model. Bold style indicates clinical significance.

Table 6. Immunohistochemistrial reactivity of Snail and CD44 in multiple regression analysis

	Mean ± SE[1]	β	*P*
Snail IHC			
Cancer			
Control (reference)	91.03 ± 10.27		**< 0.001**
Cancer	133.24 ± 10.72	0.334	
H. pylori infection			
Negative (reference)	77.59 ± 10. 81		**< 0.001**
Positive	139.69 ± 8.15	0.402	
CD44 IHC			
Cancer			
Control (reference)	24.03 ± 5.23		**0.001**
Cancer	52.39 ± 7.46	0.310	
H. pylori infection			
Negative (reference)	29.69 ± 5.78		**0.018**
Positive	43.97 ± 5.55	0.210	

IHC, immunohistochemistry

[1]Multiplying the intensity and the area (%), where stained among all of the epithelial glands. The intensity of staining was classified as 0, no staining; 1+ faint/barely perceptible partial staining; 2+, weak to moderate staining; 3+, strong staining.

Table 7. Histology and expression of EMT-related genes between base-line and after *H. pylori* eradication

	Before	After[1]	*P*[2]
Histology (N = 237)			
Neutrophil infiltration			
Antrum	1.52 ± 0.06	0.33 ± 0.04	< **0.001**
Body	1.73 ± 0.05	0.33 ± 0.04	< **0.001**
Mononuclear cell infiltration			
Antrum	1.98 ± 0.04	1.51 ± 0.04	< **0.001**
Body	1.99 ± 0.04	1.49 ± 0.04	< **0.001**
Glandular Atrophy[3]			
Antrum	1.02 ± 0.08	1.10 ± 0.09	0.472
Body	0.71 ± 0.09	0.46 ± 0.07	**0.008**
Intestinal metaplasia			
Antrum	1.11 ± 0.06	1.00 ± 0.06	0.069
Body	0.66 ± 0.06	0.64 ± 0.06	0.721
PGI/PGII ratio	3.35 ± 0.15	4.73 ± 0.20	< **0.001**
mRNA expression [3] (N = 237)			
TGF-ß1 mRNA (mean ± SE)	17.64 ± 2.74	10.14 ± 4.30	< **0.001**
Twist mRNA	10.96 ± 1.63	2.06 ± 0.19	< **0.001**
Vimentin mRNA	6.69 ± 0.73	2.30 ± 0.29	< **0.001**
Snail mRNA	54.37 ± 29.81	39.22 ± 18.03	< **0.001**
Slug mRNA	8.87 ± 2.47	7.58 ± 2.46	< **0.001**
E-cadherin mRNA	15.30 ± 4.00	166.99 ± 54.96	< **0.001**

EMT, epithelial-mesenchymal transition.

[1]First follow-up after *H. pylori* was eradicated. Mean follow-up duration was 13.3 months after completion of *H. pylori* eradication. [2]Paired t test was done. [3] Some of the specimens were inadequate for histologic evaluation for glandular atrophy because their paraffin sections were not parallel to the vertical axis of the stomach. Bold style indicates clinical significance.

Figure 1. Expression of TGF-β1, EMT markers and CD44 in controls, dysplasia, and early gastric cancer by qRT-PCR and immunohistochemistry. mRNA expression of TGF-β1 (A), Twist (B), Snail (C), Slug (D), and vimentin (E) in patients with dysplasia and cancer was markedly enhanced, while that of E-cadherin in patients with dysplasia was significantly reduced compared with controls (F). The expression of CD44 mRNA was lower in cancer and dysplasia than in control subjects (G). Immunoreactivity of Snail (H) and CD44 (I) was higher in cancer than in control samples. Data shown are means ± standard errors (SE). $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ vs. control. Results are representative of 2−3 experiments.

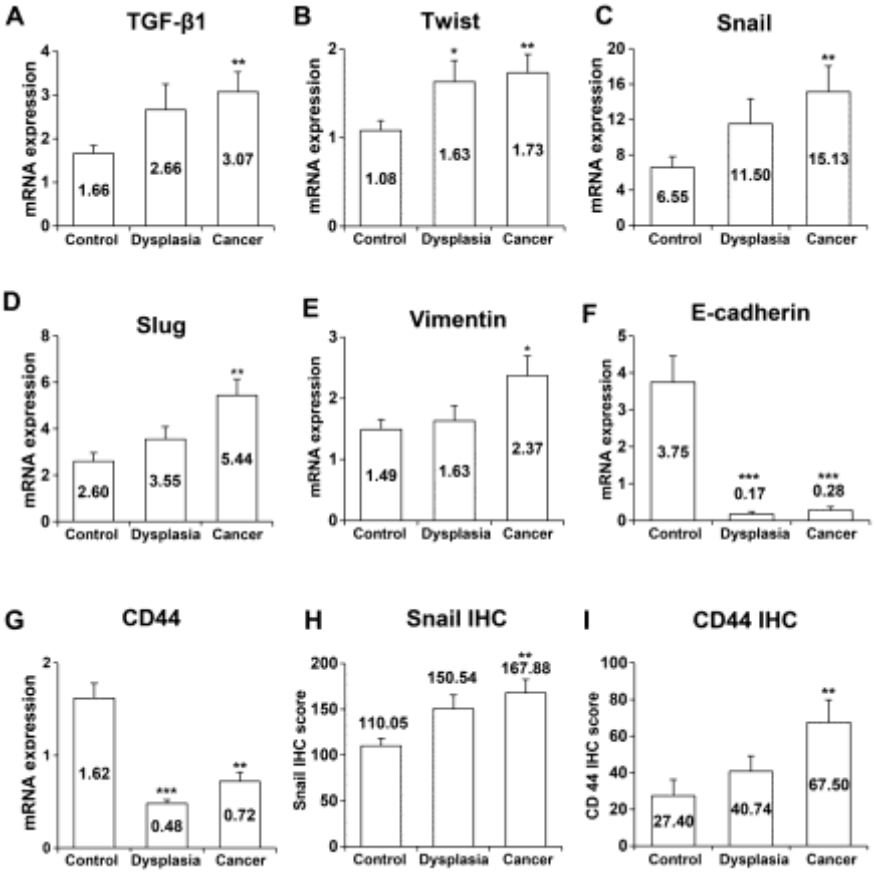

A
TGF-β1
mRNA expression
1.66
2.66
3.07
**
Control
Dysplasia
Cancer
B
Twist
mRNA expression
1.08
1.63
1.73
*
**
C
Snail
mRNA expression
6.55
11.50
15.13
**
D
Slug
mRNA expression
2.60
3.55
5.44
**
E
Vimentin
mRNA expression
1.49
1.63
2.37
*
F
E-cadherin
mRNA expression
3.75
0.17
0.28
***
G
CD44
mRNA expression
1.62
0.48
0.72
***
**
H
Snail IHC
Snail IHC score
110.05
150.54
167.88
**
I
CD44 IHC
CD 44 IHC score
27.40
40.74
67.50
**

Figure 2. Relative mRNA expression of TGF-β1 and 5 EMT markers in controls and patients with cancer according to *H. pylori* infection status. *H. pylori*-negative: 14 patients with early gastric cancer and 20 control subjects were included. In each control and cancer group, the *Hp*-infected group showed elevated expression of TGF-β1 (A), Twist (B), Snail (C), Slug (D) and vimentin (E) mRNAs compared with the *Hp*-negative group, whereas the expression of the E-cadherin (F) gene was more suppressed in the *Hp*-positive group than in the *Hp*-negative group. Each point represents the average of duplicates ± SE. $^{**}P < 0.01$ by ANOVA and post-hoc analysis with Tamhane's test was used. HP, *H. pylori.*

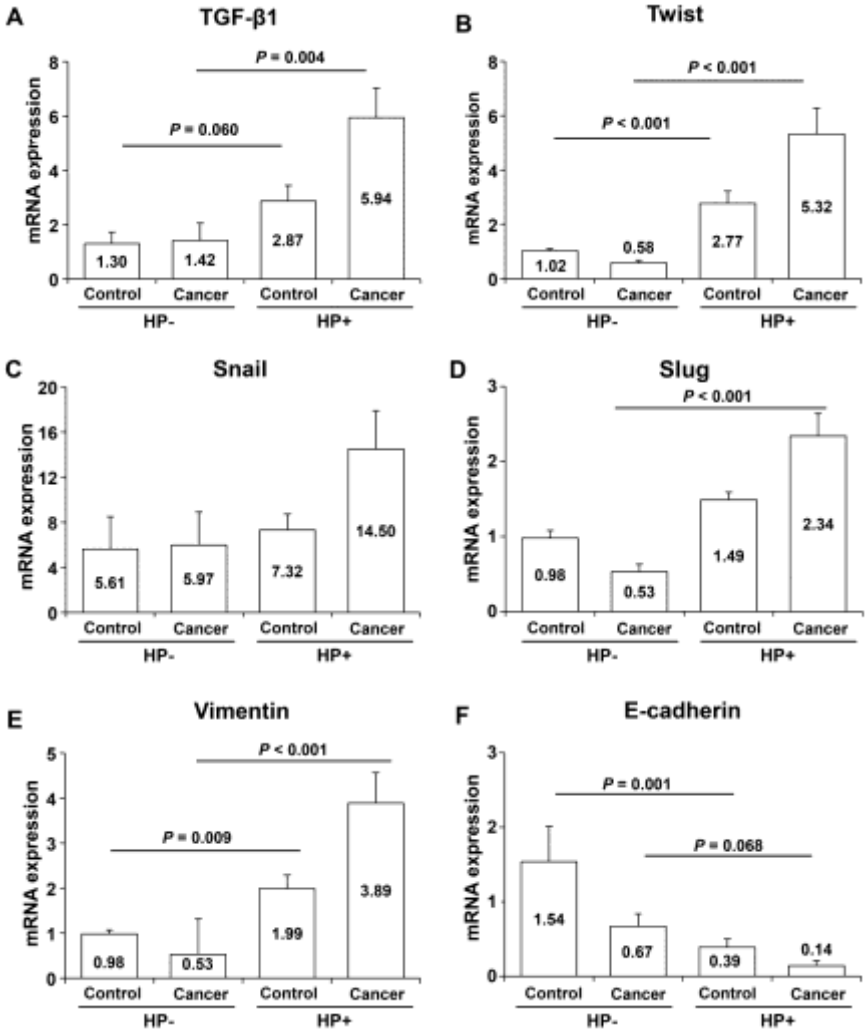

A
TGF-β1
P = 0.004
P = 0.060
mRNA expression
1.30
1.42
2.87
5.94
Control
Cancer
Control
Cancer
HP-
HP+
B
Twist
P < 0.001
P < 0.001
1.02
0.58
2.77
5.32
C
Snail
5.61
5.97
7.32
14.50
D
Slug
P < 0.001
0.98
0.53
1.49
2.34
E
Vimentin
P < 0.001
P = 0.009
0.98
0.53
1.99
3.89
F
E-cadherin
P = 0.001
P = 0.068
1.54
0.67
0.39
0.14

Figure 3. Representative photomicrographs of immunohistochemical staining for Snail and CD44 in non-cancerous gastric mucosal tissue (original magnification A, B, D ×200; C ×400). Staining of Snail (A) and CD44 (C) in representative control, dysplasia, and cancer samples at baseline and at 1 year after eradication, as indicated in *H. pylori*-positive patients. The immunoreactivity of both Snail and CD44 decreased after *H. pylori* eradication. Staining of Snail (B) and CD44 (D) in representative control, dysplasia, and cancer samples at baseline and after 1 year, as indicated in patients who were negative for *H. pylori*. There was no significant difference between those at baseline and after 1 year. The arrows indicate intense nuclear staining with Snail and staining of cell membranes with CD44. Immunoreactivity scores of Snail (E) and CD44 (F) were analyzed with a paired t-test. In the case of *H. pylori*-negative cancer, 5 patients were lost during follow-up. The white bars indicate the initial score of immunostaining, while the black bars indicate staining at approximately 1 year after successful *H. pylori* eradication in *H. pylori* -positive subjects. The gray bars indicate staining intensity at approximately 1 year after enrollment of *H. pylori*-negative subjects. ** $P < 0.01$ by paired t-test. Con, control; Dys, dysplasia; Can, cancer; HP, *H. pylori.*

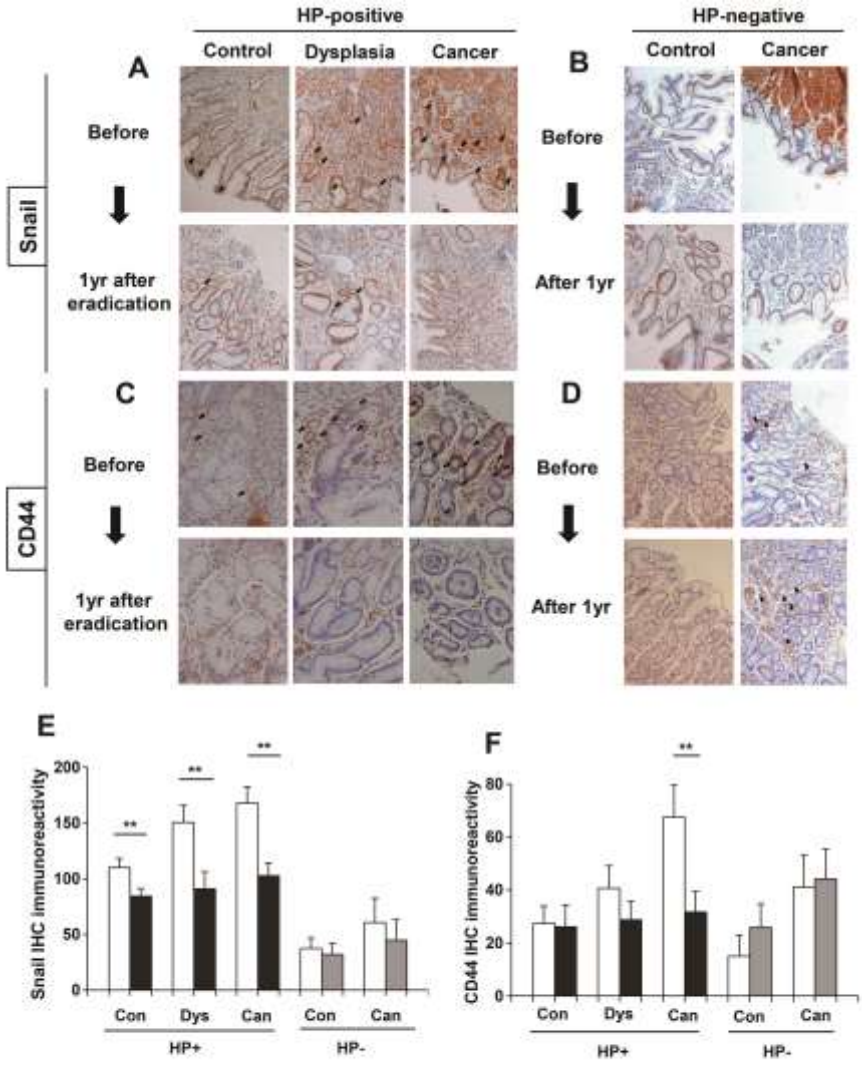

HP-positive
HP-negative
Control
Dysplasia
Cancer
Control
Cancer
A
B
Before
Before
1yr after eradication
After 1yr
Snail
C
D
Before
Before
1yr after eradication
After 1yr
CD44
E
Snail IHC immunoreactivity
200
150
100
50
0
**
**
**
Con
Dys
Can
Con
Can
HP+
HP-
F
CD44 IHC immunoreactivity
80
60
40
20
0
**
Con
Dys
Can
Con
Can
HP+
HP-

Figure 4. Effects of *H. pylori* eradication on mRNA expression of TGF-β1 and 5 EMT markers during long-term follow-up. *H. pylori* eradication decreased the mRNA levels of TGF-β1, Twist, Snail, Slug, and vimentin. Expression of E-cadherin mRNA increased following *H. pylori* eradication.

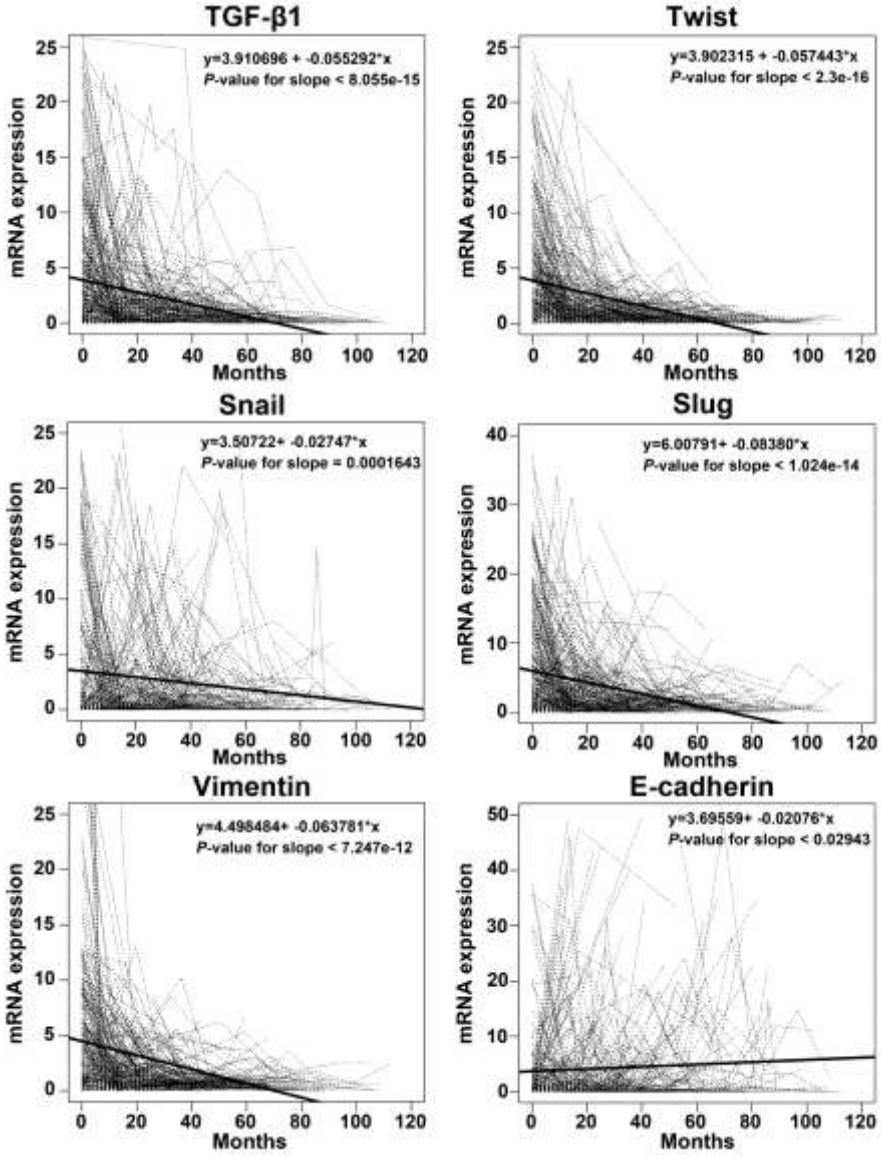

TGF-β1
y=3.910696 + -0.055292*x
P-value for slope < 8.055e-15
Twist
y=3.902315 + -0.057443*x
P-value for slope < 2.3e-16
Snail
y=3.50722+ -0.02747*x
P-value for slope = 0.0001643
Slug
y=6.00791+ -0.08380*x
P-value for slope < 1.024e-14
Vimentin
y=4.498484+ -0.063781*x
P-value for slope < 7.247e-12
E-cadherin
y=3.69559+ -0.02076*x
P-value for slope < 0.02943
mRNA expression
Months

Figure 5. Time course of gene expression encoding TGF-β1 and 5 EMT markers following *H. pylori* eradication in controls, dysplasia, and early gastric cancer. Regardless of groups, relative mRNA expression of TGF-β1, Twist, Snail, Slug, and vimentin were significantly reduced after *H. pylori* eradication (A-O). In contrast, expression of E-cadherin mRNA was up-regulated following *H. pylori* eradication (P-R), which was statistically significant in dysplasia (Q) and early gastric cancer (R).

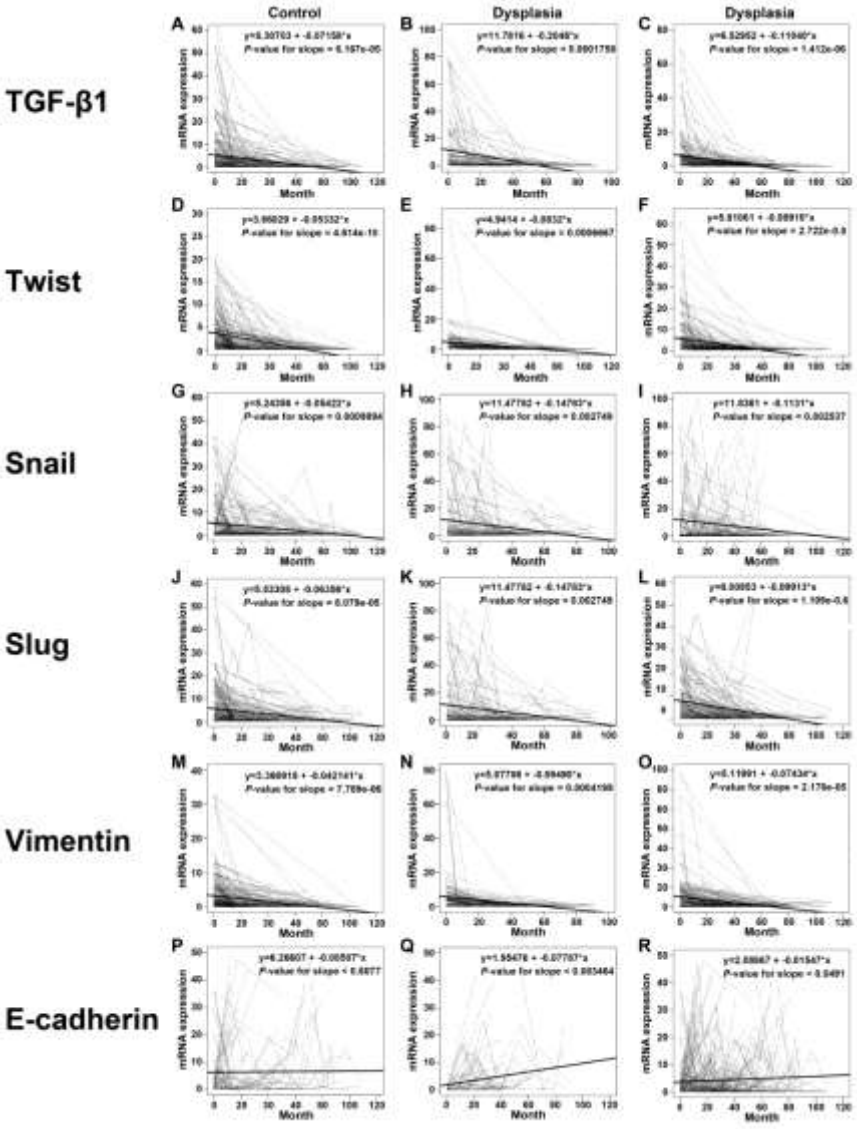
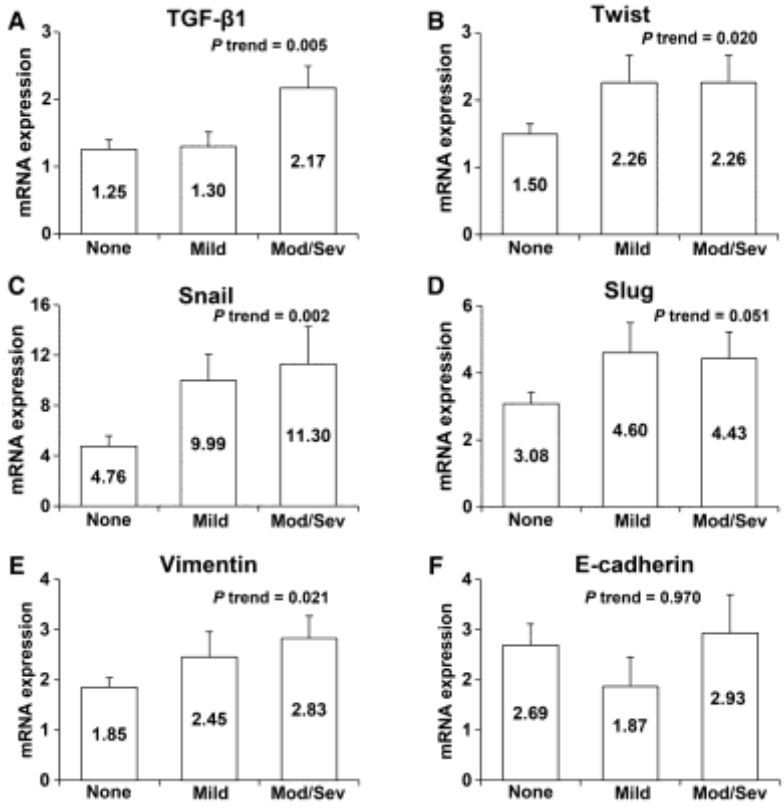

Control
Dysplasia
Dysplasia
TGF-β1
Twist
Snail
Slug
Vimentin
E-cadherin
mRNA expression
Month

Figure 6. Expression of mRNAs encoding TGF-β1 and 5 EMT markers and CD44 immunoreactivity according to the intensity of intestinal metaplasia (IM). mRNA expression levels of TGF-β1 (A), Twist (B), Snail (C), Slug (D), and vimentin (E), as well as the immunohistochemical staining of CD44 (G), tended to increase with increasing severity of IM. There was no significant trend in E-cadherin mRNA (F). $^{*}P < 0.05$ by ANOVA followed Scheffe's test. Mod, moderate; Sev, severe.



G

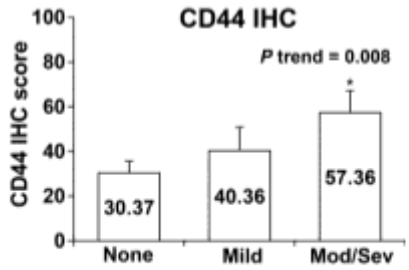

CD44 IHC
P trend = 0.008
CD44 IHC score
100
80
60
40
20
0
30.37
40.36
57.36
*
None
Mild
Mod/Sev

Figure 7. Relative changes in mRNA levels of Snail after *H. pylori* eradication according to improvement in intestinal metaplasia (IM).

There was a marked decrease in the expression of Snail mRNA and an increase in E-cadherin mRNA after *H. pylori* eradication in subjects without IM (A, D), improved IM (B, E) and non-improved IM (C, F).

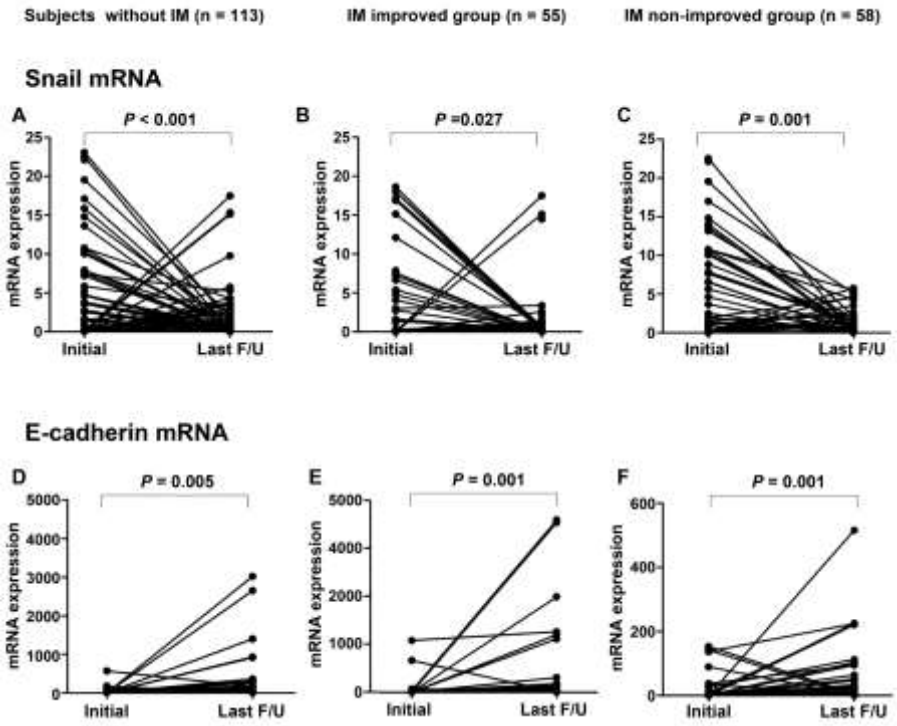


Figure 8. Immunoreactivity of LGR5 according to *H. pylori* infection and cancer. Immunoreactivity of LGR5 was higher in cancer than in control samples. Data shown are means ± standard errors (SE). $^{*}P <$ 0.05 HP, *H. pylori*

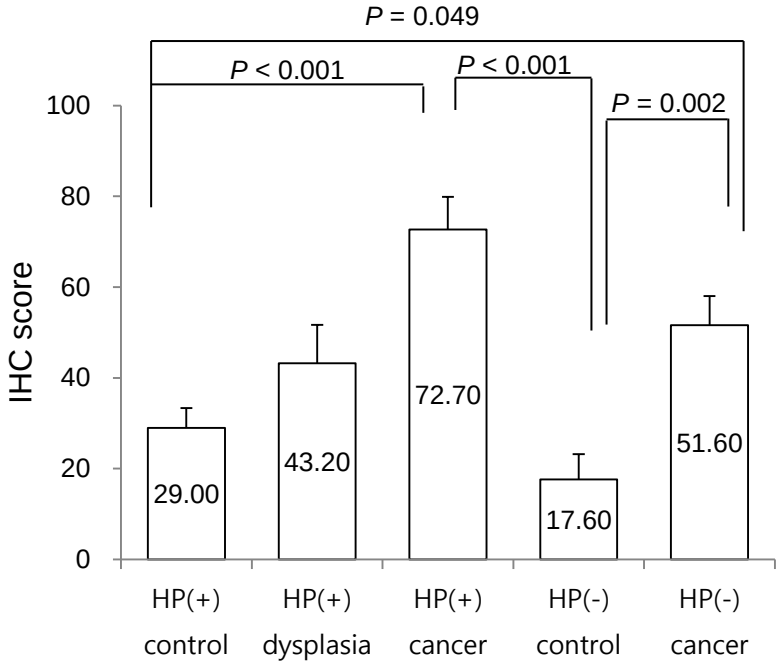


Figure 9. Effects of *H. pylori* eradication on the LGR5 immunohistochemistry. After eradication, control (A) and cancer (C) groups showed a significant reduction in the expression of LGR5 IHC. Non-cancerous mucosa of *H. pylori*-positive subjects with dysplasia (B) and *H. pylori*-negative subjects (D and E) showed no significant changes of LGR5 IHC. Data shown are means ± standard errors (SE). HP, *H. pylori.*

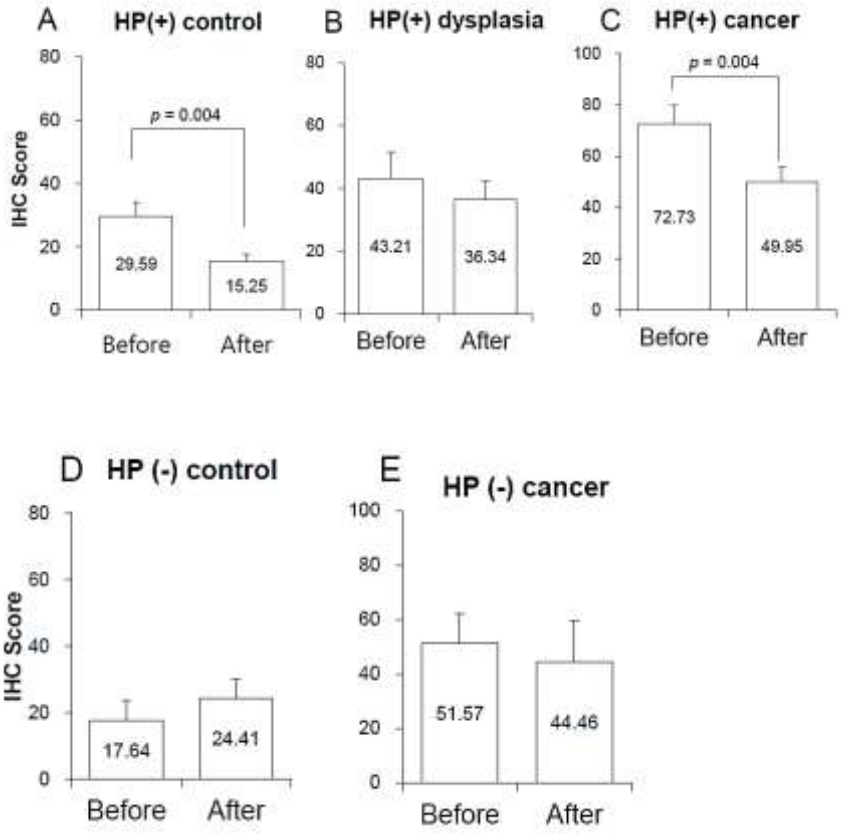


Figure 10. Immunoreactivity of LGR5 according to the location of gastric mucosa and intestinal metaplasia. (A) LGR5 IHC in the body was significantly reduced than in the antrum. Antral mucosa (B) did not show any significant difference in LGR5 IHC according to presence or absence of IM. Gastric body mucosa with IM (C). showed an elevated immunointensity than in that without IM.

Data shown are means ± standard errors (SE).