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Helicobacter pylori-induced epithelial-mesenchymal transition, a potential role of gastric cancer initiation and an emergence of stem cells

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Abstract

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 messenger RNA (mRNA) expression of TGF-β1 and five 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 non-cancerous gastric mucosa were measured using quantitative real time-polymerase chain reaction 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 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 (CSCs). Its eradication may prevent the carcinogenesis of gastric cancer by inhibiting these two pathways.

Introduction

Gastric cancer is a major cause of cancer-related death worldwide, and its incidence is particularly high in Asia. In South Korea, in 2010, the age-standardized incidence rate was 62.3 in males and 24.9 in females per 100 000 individuals (1). *Helicobacter pylori* is the primary etiological factor for gastric cancer (2,3). Gastric cancer is classified into two histologic types based on the Lauren system—namely, the intestinal and diffuse types (4). Intestinal type gastric cancer is thought to undergo multistep carcinogenesis, known as Correa's pathway, in which chronic inflammation eventually develops into gastric cancer via

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Abbreviations:

CagA	cytotoxin-associated gene A
CSCs	cancer stem cell
EMT	Epithelial-mesenchymal transition
IHC	immunohistochemistry
IM	intestinal metaplasia
mRNA	messenger RNA
TGF-β1	transforming growth factor-β1

atrophic gastritis, intestinal metaplasia (IM) and dysplasia (5). Although *H.pylori* is assumed to trigger the early stages of this process, the exact initiation mechanism 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 (6). 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 (7).

Epithelial-mesenchymal transition (EMT) is considered to be a key process of cancer spreading (8). 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 (9). Interestingly, recent studies have suggested that cells undergoing the EMT acquire stem cell-like properties (10,11). Mani *et al.* (11) 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 (10,12), 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 (13), and *H.pylori* infection promotes the expression of TGF-β1 in both gastric epithelial cell lines and human gastric tissue (14–16). 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.

We evaluated the possible carcinogenic effects of TGF-β1-induced EMT and CSCs by comparing the levels of messenger RNAs (mRNAs) encoding TGF-β1 and five 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 and CD44 were analyzed by immunohistochemistry (IHC). We also investigated long-term changes in expression before and after *H.pylori* eradication. Additionally, since *H.pylori* infection has been implicated as a major cause of IM (17,18), the possible relationship between expression of EMT markers and IM was assessed.

Materials and methods

Study subjects

Study subjects with current *H. pylori* infection were consecutively enrolled at Seoul National University Bundang Hospital from February 2006 to December 2013. 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 three groups according to the pathologic results, namely control, dysplasia and early gastric cancer. Controls were selected from those who when upper gastrointestinal 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 ^{13}C-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.

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) (17). 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) (19). 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 I and II were measured using a latex-enhanced turbidimetric immunoassay (Shima Laboratories, Tokyo, Japan). Based on the results of serum pepsinogen tests, patients were categorized as having no, mild to moderate or severe gastric atrophy, according to the definition of Kato *et al.* (20).

Quantitative real-time polymerase chain reaction

Total RNA was extracted directly from non-cancerous corporal biopsy specimens with TRIzol® reagent (Invitrogen, Carlsbad, CA), as recommended by the manufacturer. Next, 1500 ng of RNA was reverse transcribed to complementary DNA with oligo (dT) and M-MLV reverse transcriptase (Invitrogen), according to the manufacturer's instructions. Quantitative PCR was performed in 96-well reaction plates using 2 µl of complementary DNA in a 20 µl reaction mix containing 2× SYBR® Premix Ex Taq™ (Takara Bio, Otsu, Japan). Samples were run on an Applied Biosystems 7500/7500 Fast Real-Time PCR instrument (Applied Biosystems, Foster City, CA), The PCR cycling conditions were as follows: an initial denaturation step for 30 s at 95°C and then 40 cycles of denaturation at 95°C (5 s) and annealing at 60°C (34 s) with the final dissociation stage of 15 s at 95°C, 1 min at 60°C and 15 s at 95°C. The primer sequences for PCR are shown in Supplementary Table 1, available at *Carcinogenesis* Online. Expression levels of mRNA of the target gene were compared with the endogenous control β-actin using the $2^{-\Delta\Delta Ct}$ method (21).

IHC

Immunostaining of CD44, Snail and E-cadherin was also performed. Paraffin-embedded 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) and E-cadherin (610181) (1:400 dilution; BD Biosciences, San Jose, CA) 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, Tucson, AZ). 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 (22). Scoring for the expression of CD44, Snail and E-cadherin in gastric epithelium was determined using light microscopy, by multiplying the intensity times the area (%) where staining was observed in epithelial glands; the possible scores ranged from 0 to 300. The intensity of staining was scored as 0, no staining; 1+ faint/barely perceptible partial staining; 2+, weak to moderate staining; 3+, strong staining (23). Each sample was scored by a blind reviewer.

Statistical analysis

The χ^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 was applied. There was no significant difference in the results between the linear mixed model and generalized additive mixed model (24). Moreover, linear mixed model is appropriate for the data since it incorporates a generic correlation structure of longitudinal data by considering both within-subject and between-subject variations (25). 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 five EMT markers were compared between groups using one-way analysis of variance 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).

Results

Demographic characteristics

Table 1 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 1). Atrophic gastritis (determined by histology and serum pepsinogen I/II ratio) and IM were more prevalent in patients with dysplasia and gastric cancer than controls (Table 1).

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

The expression of mRNAs encoding TGF-β1 and five EMT markers were measured before *H.pylori* eradication, to determine whether EMT is involved in the dysplasia–cancer sequence. The levels of TGF-β1 mRNA in the control group were significantly lower than those in the cancer group (1.66 ± 0.18 versus 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 (Figure 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 versus 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 versus 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 versus 110.05 ± 12.39, P = 0.002) (Figures 1H and 2A, upper row) (CD44: 67.50 ± 12.39 versus 27.40 ± 6.52, P = 0.007) (Figures 1I and 2C, upper row).

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

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 (Supplementary Table 2, available at *Carcinogenesis* Online). 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 four EMT-inducing genes or with the CD44 staining (Supplementary Table 2, available at *Carcinogenesis* Online) (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 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).

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 (Supplementary Table 3, available at *Carcinogenesis* Online). *H.pylori*-infected control subjects showed significantly elevated expression levels of TGF-β1, Twist and vimentin mRNAs compared with *H.pylori*-negative controls (Figures 3A, B and E) (TGF-β1: 1.30 ± 0.42 versus 2.87 ± 0.57; Twist: 1.02 ± 0.08 versus 2.77 ± 0.45; vimentin: 0.98 ± 0.09 versus 1.99 ± 0.30). Snail (Figure 3C) and Slug (Figure 3D) also presented

Table 1. Baseline characteristics of 237 *Helicobacter pylori*-infected subjects

	No. of subjects (%)			
	Controls (*n* = 111)	Dysplasia (*n* = 55)	Early gastric cancer (*n* = 71)	*P*[a]
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
Non-smoker	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)	
PG I/II ratio (mean ± SD)	3.65 ± 1.66	2.96 ± 1.23	2.77 ± 1.64	**0.001**
Histology[b]				
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[c]				
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)	

PG, Pepsinogen; SD = standard deviation.

[a]P-values for Chi-square test or one-way analysis of variance for comparison of variables across groups.

[b]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.

[c]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 PG tests. Bold style indicates clinical significance.

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 versus 0.39 ± 0.11, *P* = 0.001; Figure 3F).

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 versus 5.94 ± 1.09; Twist: 0.58 ± 0.09 versus 5.32 ± 0.96; Snail: 5.97 ± 2.94 versus 14.50 ± 3.38; Slug: 0.53 ± 0.10 versus 2.34 ± 0.30; vimentin: 0.53 ± 0.79 versus 3.89 ± 0.67; Figure 3A–E). E-cadherin mRNA

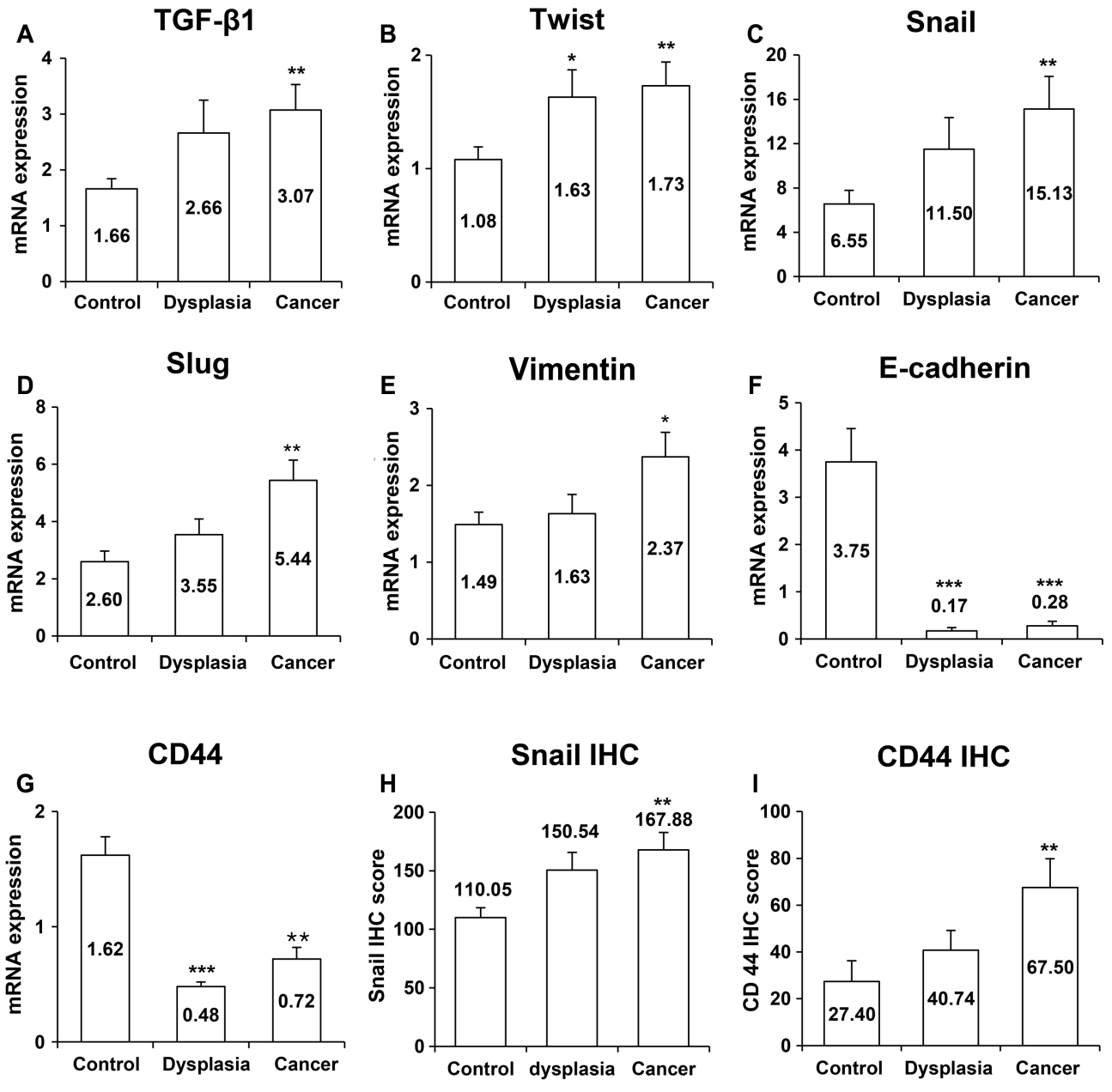

Figure 1. Expression of TGF-β1, EMT markers and CD44 in controls, dysplasia and early gastric cancer by quantitative real-time polymerase chain reaction and IHC. mRNA expression of TGF-β1 (A), Twist (B), Snail (C), Slug (D) and vimentin (E) in patients with dysplasia and cancer was markedly enhanced, 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 of the means (SEM). $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ versus control. Results are representative of 2–3 experiments.

expression in *H.pylori*-infected cancer tissues was lower than that in *H.pylori*-negative cancers (0.67 ± 0.17 versus 0.14 ± 0.07, $P = 0.068$) (Figure 3F).

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 (Supplementary Table 4, available at *Carcinogenesis* Online). 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 (Supplementary Table 4, available at *Carcinogenesis* Online).

The IHC expression of Snail and CD44 in *H.pylori*-negative tissues showed weaker staining than in *H.pylori*-positive tissues ($P < 0.001$ and $P = 0.077$, respectively) (Figure 2A versus Figure 2B upper row and Figure 2C versus Figure 2D, 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, $\beta = 0.334$ and $P < 0.001$, *H.pylori*-positive: $\beta = 0.402$ and $P < 0.001$; CD44 IHC: cancer, $\beta = 0.310$ and $P = 0.001$, *H.pylori*-positive: $\beta = 0.210$ and $P = 0.018$) (Supplementary Table 5, available at *Carcinogenesis* Online).

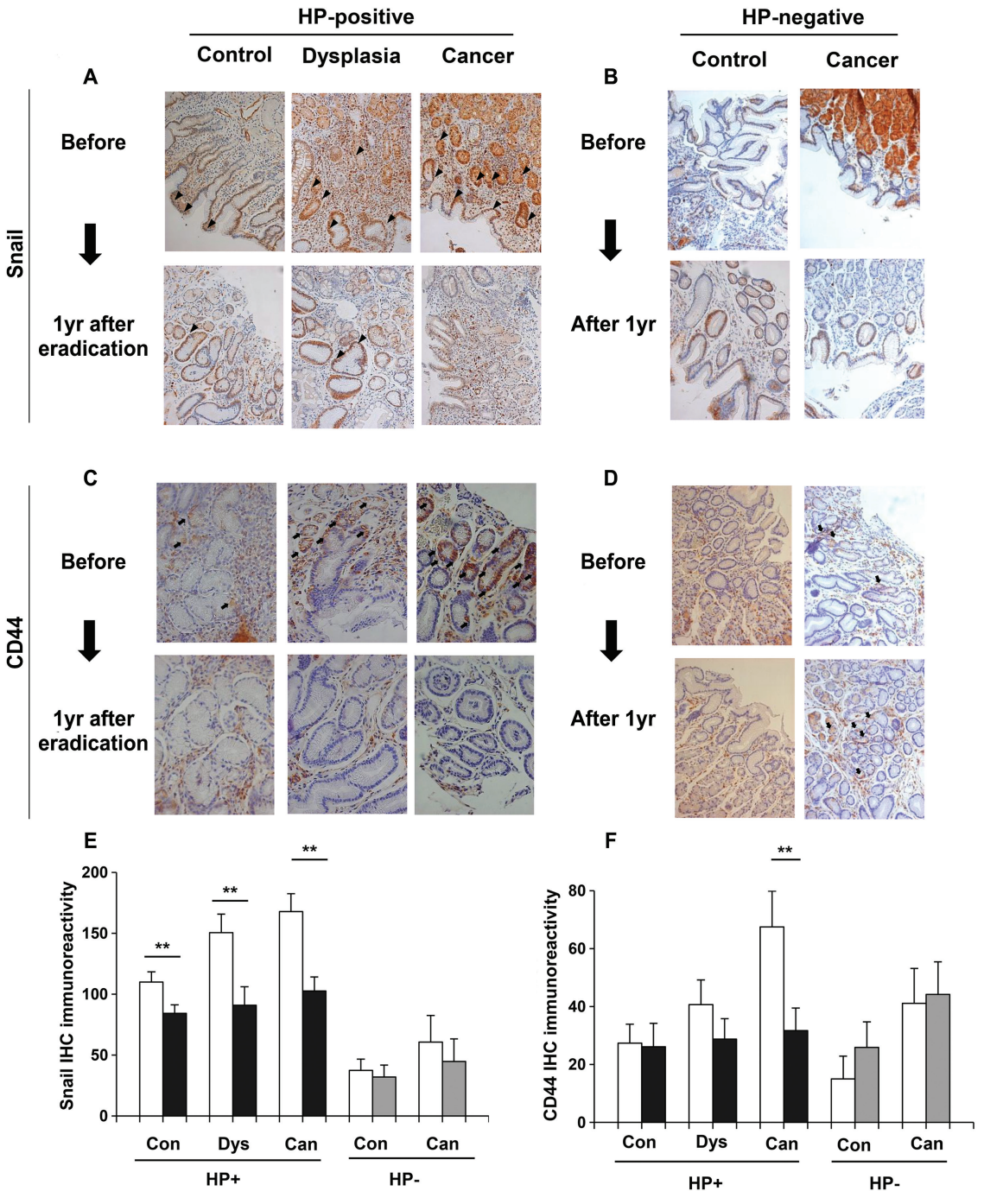

Figure 2. Representative photomicrographs of immunohistochemical staining for Snail and CD44 in non-cancerous gastric mucosal tissue (original magnification A, B, D ×200; C ×400). Staining of Snail (A) and CD44 (C) in representative control, dysplasia and cancer samples at baseline and at 1 year after eradication, as indicated in *Helicobacter 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, five patients were lost during follow-up. The white bars indicate the initial score of immunostaining, whereas 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. Can, cancer; Con, control; Dys, dysplasia; HP, *Helicobacter pylori*.

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 2). 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

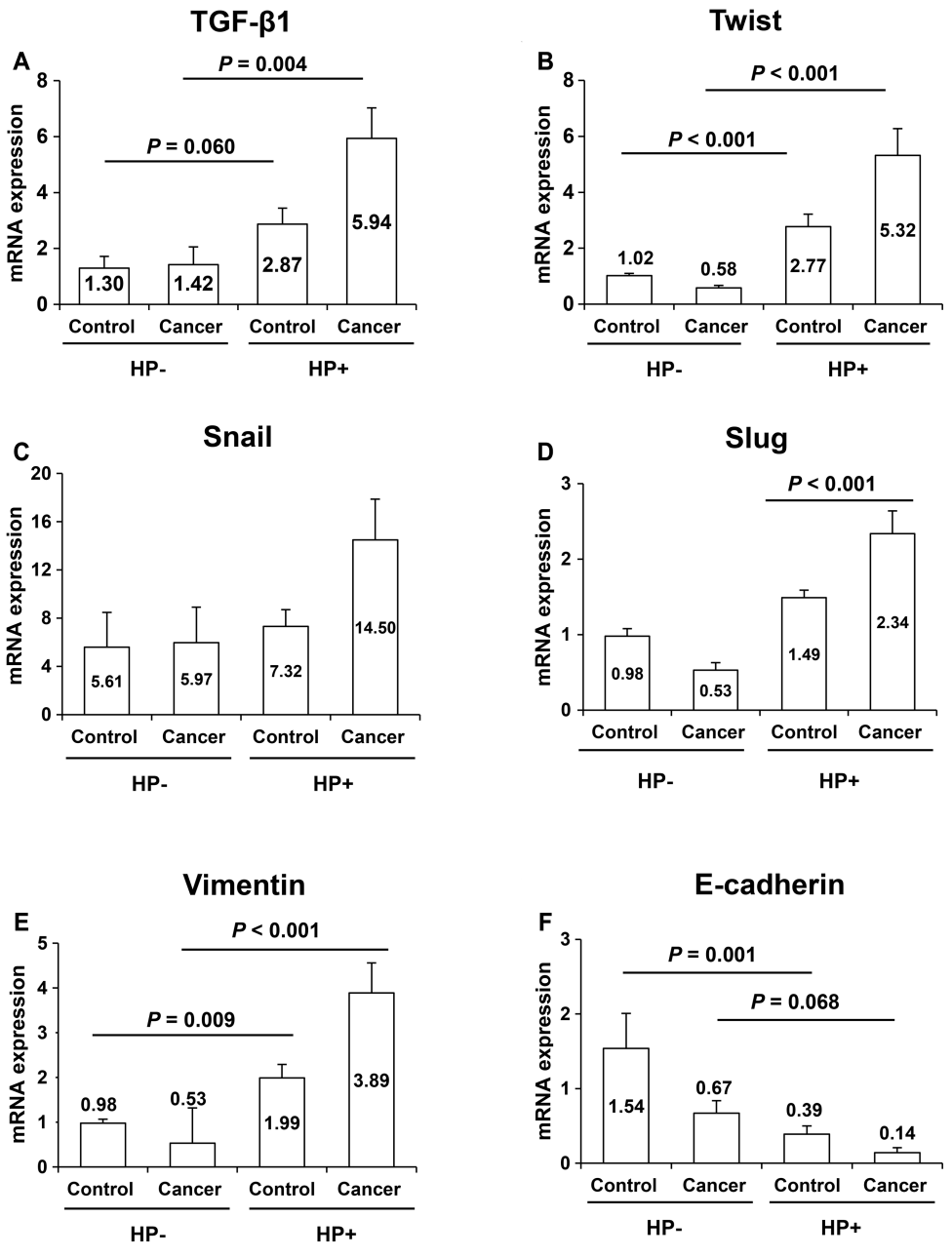

Figure 3. Relative mRNA expression of TGF-β1 and five EMT markers in controls and patients with cancer according to *Helicobacter pylori* infection status. *H.pylori*-negative: 14 patients with early gastric cancer and 20 control subjects were included. In each control and cancer group, the *H.pylori*-infected group showed elevated expression of TGF-β1 (A), Twist (B), Snail (C), Slug (D) and vimentin (E) mRNAs compared with the *H.pylori*-negative group, whereas the expression of the E-cadherin (F) gene was more suppressed in the *H.pylori*-positive group than in the *H.pylori*-negative group. Each point represents the average of duplicates ± standard errors of the means (SEM). **$P < 0.01$ by analysis of variance and post-hoc analysis with Tamhane's test was used. HP, *Helicobacter pylori*.

antrum ($P = 0.069$), but did not show any significant changes in the body (Table 2). 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 $P = 0.028$).

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 five 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 2). Moreover, the expression of these genes decreased continuously after *H.pylori* eradication for several years (P-value for slope <0.001, Figure 4A–E). 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 4F).

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 three 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

Table 2. Histology and expression of EMT-related genes between baseline and after *Helicobacter pylori* eradication

	Before	After[a]	P[b]
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[c]			
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[c](*N* = 237)			
TGF-β1 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**

PG, Pepsinogen; SE, standard error.

[a]First follow-up after *H.pylori* was eradicated. Mean follow-up duration was 13.3 months after completion of *H.pylori* eradication.

[b]Paired *t*-test was done.

[c]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. β-Actin was used as the endogenous RNA control to normalize for differences in the amount of total RNA. Bold style indicates clinical significance.

<0.05) (Supplementary Figure 1A–O is available at *Carcinogenesis* Online). The increased expression of E-cadherin mRNA was statistically significant in the dysplasia and cancer groups (Supplementary Figure 1Q and R is available at *Carcinogenesis* Online), but not in the control group (Supplementary Figure 1P, available at *Carcinogenesis* Online).

The immunohistochemical results for CD44, Snail and E-cadherin were compared before and after *H.pylori* eradication (Figure 2A and F). After eradication, all three groups showed a significant reduction in the expression of Snail (Figure 2A; upper to bottom row). For CD44, the cancer group showed a marked reduction in expression after eradication and resection, whereas the differences in the dysplasia and control groups did not reach statistical significance (Figure 2C; 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 2B and D; upper to bottom row).

Relationship between TGF-β1/EMT markers and IM

To further investigate whether IM might correlate with EMT, the initial mRNA levels of TGF-β1 and five 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 (Supplementary Figure 2A–E is available at *Carcinogenesis* Online) (*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 three 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 (Supplementary Figure 2G, available at *Carcinogenesis* Online).

Additionally, we assessed whether improvement of IM in the body was associated with a reduction in mRNA expression of TGF-β1 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 $P < 0.05$) (Supplementary Figure 3, available at *Carcinogenesis* Online, describes Snail (A–C) and E-cadherin mRNA (D–F) expression).

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 posteradication 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 progressively increased in sequence, from normal

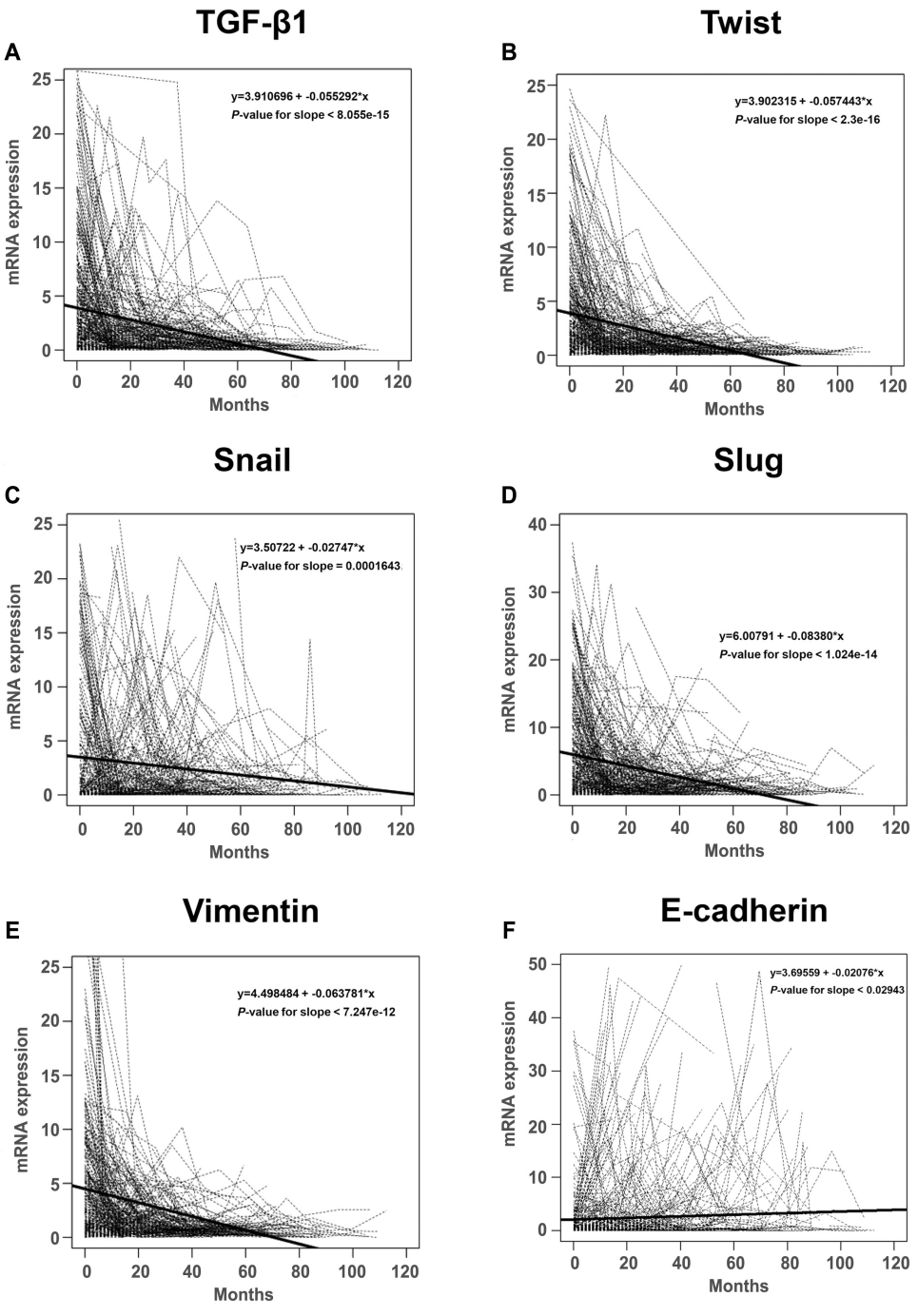

Figure 4. Effects of *Helicobacter pylori* eradication on mRNA expression of TGF-β1 and five EMT markers during long-term follow-up. *H.pylori* eradication decreased the mRNA levels of TGF-β1 (A), Twist (B), Snail (C), Slug (D) and vimentin (E). Expression of E-cadherin mRNA (F) increased following *H.pylori* eradication.

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 CD44, a significant reduction in its expression was detected by IHC staining in the cancer group when *H.pylori* was eradicated after endoscopic resection of cancer. Furthermore, 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, cytotoxin-associated gene A (CagA)-positive *H.pylori* stabilizes Snail (26) and Yu *et al.* (27) reported that CagA induces both TWIST1 and vimentin and inhibits E-cadherin by down-regulation of programmed cell death protein 4. In agreement with these studies, we observed enhanced mRNA expression of four 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 (28), further analysis according to the presence or absence of the toxin was not performed.

Bessède *et al.* (29) 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 (29).

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 (30). 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 quantitative real-time polymerase chain reaction. 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 (22,31). Other study reported that different CSC markers such as ALDH1, LGR5 and CD166 are increased in *H.pylori*-associated gastritis and gastric adenocarcinomas (32). Furthermore, expression of CD44 is known to be closely linked with the Wnt/β-catenin pathway (33). True markers for gastric CSCs still need to be identified.

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-2 and matrix metalloproteinase-9, which are related to malignancy and invasion, in patients with distal gastric adenocarcinoma (34). While TGF-β1 induced expression of Twist and Smad interacting protein 1, Snail enhanced the secretion of interleukin-1, interleukin-6 and interleukin-8 (35). Upregulation of EMT-acquired markers with gaining of migratory properties by *H.pylori* infection was recently demonstrated (36). 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 (37–40), 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 (22). 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/iryouhoken).

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 (41). 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 (42). 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 (43). On the other hand, an indirect association between EMT and IM through bone morphogenetic protein has been reported in previous studies. Bone morphogenetic protein pathway induces the expression of Cdx2 (44), and EMT is also activated by the bone morphogenetic protein family (45,46).

In the present study, the expression of mRNAs encoding EMT-inducing transcription factors or mesenchymal markers and IHC staining of CD44 were both increased in proportion to the grade of 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-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 probably associated with gastric CSCs and *H.pylori* at an early stage of tumorigenesis. Moreover, the observed steady reduction of EMT-inducing transcription factors, mesenchymal markers and IHC expression of CD44 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.

Supplementary material

Supplementary Tables 1–5 and Figures 1–3 can be found at http://carcin.oxfordjournals.org/

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References

1. Jung, K.W. et al. (2013) Cancer statistics in Korea: incidence, mortality, survival and prevalence in 2010. Cancer Res. Treat., 45, 1–14.
2. Suzuki, H. et al. (2009) *Helicobacter pylori* and gastric cancer. Gastric Cancer, 12, 79–87.
3. Ushijima, T. et al. (2012) Molecular pathways: involvement of *Helicobacter pylori*-triggered inflammation in the formation of an epigenetic field defect, and its usefulness as cancer risk and exposure markers. Clin. Cancer Res., 18, 923–929.
4. Lauren, P. (1965) The two histological main types of gastric carcinoma, an attempt at a histoclinical classification. Acta. Pathol. Microbiol. Scand., 64, 31–49.
5. Correa, P. (1994) *Helicobacter pylori* and gastric carcinogenesis. Am. J. Surg. Pathol., 19, S37–43.
6. Takaishi, S. et al. (2008) Gastric cancer stem cells. J. Clin. Oncol., 26, 2876–2882.
7. Takaishi, S. et al. (2009) Identification of gastric cancer stem cells using the cell surface marker CD44. Stem Cells, 27, 1006–1020.
8. Thiery, J.P. (2002) Epithelial-mesenchymal transitions in tumour progression. Nat. Rev. Cancer, 2, 442–454.
9. Kalluri, R. et al. (2009) The basics of epithelial-mesenchymal transition. J. Clin. Invest., 119, 1420.
10. Morel, A.P. et al. (2008) Generation of breast cancer stem cells through epithelial-mesenchymal transition. PLoS One, 3, e2888.
11. Mani, S.A. et al. (2008) The epithelial-mesenchymal transition generates cells with properties of stem cells. Cell, 133, 704–715.
12. Creighton, C.J. et al. (2010) Epithelial-mesenchymal transition (EMT) in tumor-initiating cells and its clinical implications in breast cancer. J. Mammary Gland Biol. Neoplasia, 15, 253–260.
13. Xu, Q. et al. (2013) Downregulation of miR-153 contributes to epithelial-mesenchymal transition and tumor metastasis in human epithelial cancer. Carcinogenesis, 34, 539–549.
14. Beswick, E.J. et al. (2011) Role of gastric epithelial cell-derived transforming growth factor beta in reduced CD4+ T cell proliferation and development of regulatory T cells during *Helicobacter pylori* infection. Infect. Immun., 79, 2737–2745.
15. Smythies, L.E. et al. (2005) Human intestinal macrophages display profound inflammatory anergy despite avid phagocytic and bacteriocidal activity. J. Clin. Invest., 115, 66–75.
16. Lindholm, C. et al. (1998) Local cytokine response in *Helicobacter pylori*-infected subjects. Infect. Immun., 66, 5964–5971.
17. Kim, N. et al. (2008) Prevalence and risk factors of atrophic gastritis and intestinal metaplasia in a Korean population without significant gastroduodenal disease. Helicobacter, 13, 245–255.
18. Kang, J.M. et al. (2011) CDX1 and CDX2 expression in intestinal metaplasia, dysplasia and gastric cancer. J. Korean Med. Sci., 26, 647–653.
19. Dixon, M.F. et al. (1996) The participants in the International Workshop on the Histopathology of Gastritis, Houston 1994. Classification and grading of gastritis: the updated Sydney system. Am. J. Surg. Pathol., 20,1161–1181.
20. Kato, S. et al. (2007) *Helicobacter pylori* infection-negative gastric cancer in Japanese hospital patients: incidence and pathological characteristics. Cancer Sci., 98, 790–794.
21. Schmittgen, T.D. et al. (2008) Analyzing real-time PCR data by the comparative C(T) method. Nat. Protoc., 3, 1101–1108.
22. Ryu, H.S. et al. (2012) Combination of epithelial-mesenchymal transition and cancer stem cell-like phenotypes has independent prognostic value in gastric cancer. Hum. Pathol., 43, 520–528.
23. van Diest, P.J. et al. (1997) A scoring system for immunohistochemical staining: consensus report of the task force for basic research of the EORTC-GCCG. European Organization for Research and Treatment of Cancer-Gynaecological Cancer Cooperative Group. J. Clin. Pathol., 50, 801–804.
24. Wood, S. (2006) Generalized additive models: an introduction with R. CRC press, London, UK.
25. Gelman, A. (2007) Data analysis using regression and multilevel/hierarchical models. Cambridge University Press, Cambridge, UK.
26. Lee, D.G. et al. (2014) *Helicobacter pylori* CagA promotes Snail-mediated epithelial–mesenchymal transition by reducing GSK-3 activity. Nat. Commun., 5:4423.
27. Yu, H. et al. (2014) *Helicobacter pylori* promotes epithelial-mesenchymal transition in gastric cancer by downregulating programmed cell death protein 4 (PDCD4). PLoS One, 9, e105306.
28. Kim, J.Y. et al. (2014) Association of polymorphisms in virulence factor of *Helicobacter pylori* and gastroduodenal diseases in South Korea. J. Gastroenterol. Hepatol., 29, 984–991.
29. Bessède, E. et al. (2014) *Helicobacter pylori* generates cells with cancer stem cell properties via epithelial-mesenchymal transition-like changes. Oncogene, 33, 4123–4131.
30. Collado, A. et al. (1991) Characterization of CD44 antigen during lymphoid ontogeny. Immunobiology, 183, 1–11.
31. Lau, W.M. et al. (2014) CD44v8-10 is a cancer-specific marker for gastric cancer stem cells. Cancer Res., 74, 2630–2641.
32. Levi, E. et al. (2014) Cancer stem cells in *Helicobacter pylori* infection and aging: Implications for gastric carcinogenesis. World J. Gastrointest. Pathophysiol., 5, 366–372.
33. Cabarcas, S.M. et al. (2011) The cancer stem cell niche—there goes the neighborhood? Int. J. Cancer, 129, 2315–2327.
34. Amedei, A. et al. (2014) *Helicobacter pylori* secreted peptidyl prolyl cis, trans-isomerase drives Th17 inflammation in gastric adenocarcinoma. Intern. Emerg. Med., 9, 303–309.
35. Zhou, C. et al. (2012) Inflammation linking EMT and cancer stem cells. Oral Oncol., 48, 1068–1075.
36. Chang, H. et al. (2015) *Helicobacter pylori* might induce TGF-β1-mediated EMT by means of *cagE*. Helicobacter, [Epub ahead of press].
37. Lee, Y.C. et al. (2013) The benefit of mass eradication of *Helicobacter pylori* infection: a community-based study of gastric cancer prevention. Gut, 62, 676–682.
38. Fukase, K. et al. (2008) Effect of eradication of *Helicobacter pylori* on incidence of metachronous gastric carcinoma after endoscopic resection of early gastric cancer: an open-label, randomised controlled trial. Lancet, 372, 392–397.
39. Wong, B.Y. et al. (2004) *Helicobacter pylori* eradication to prevent gastric cancer in a high-risk region of China. J. Am. Med. Assoc., 291, 187–194.
40. Ford, A.C. et al. (2014) *Helicobacter pylori* eradication therapy to prevent gastric cancer in healthy asymptomatic infected individuals: systematic review and meta-analysis of randomised controlled trials. BMJ, 348, g3174.
41. Shin, C.M. et al. (2013) Changes in aberrant DNA methylation after *Helicobacter pylori* eradication: a long-term follow-up study. Int. J. Cancer, 133, 2034–2042.
42. Stairs, D.B. et al. (2010) Cdx genes, inflammation, and the pathogenesis of intestinal metaplasia. Prog. Mol. Biol. Transl. Sci., 96, 231–270.
43. Mills, J.C. et al. (2011) Gastric epithelial stem cells. Gastroenterology, 140, 412–424.
44. Camilo, V. et al. (2012) *Helicobacter pylori* and the BMP pathway regulate CDX2 and SOX2 expression in gastric cells. Carcinogenesis, 33, 1985–1992.
45. Acloque, H. et al. (2009) Epithelial-mesenchymal transitions: the importance of changing cell state in development and disease. J. Clin. Invest., 119, 1438–1449.
46. Hamada, S. et al. (2007) Bone morphogenetic protein 4 induces epithelial-mesenchymal transition through MSX2 induction on pancreatic cancer cell line. J. Cell. Physiol., 213, 768–774.