Ph.D. Dissertation of Medicine

Effect of *Helicobacter pylori* infection and its eradication on the expression of tight junction proteins in the gastric epithelium in relation to gastric carcinogenesis

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Abstract

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Background: *Helicobacter pylori* (Hp) is classified as a human gastric cancer carcinogen causing atrophic gastritis, and intestinal metaplasia. Tight junction proteins (TJPs) play a role in the defense mechanisms of the epithelium. Hp infection disrupts TJPs by virulence factors that are secreted from Hp for colonization on gastric epithelium. This study aimed to assess the role of TJPs in gastric carcinogenesis by Hp infection. Moreover, the effect of Hp eradication on TJPs was investigated in terms of gastric tumor environments.

Methods: A total of 510 subjects (284 controls and 226 patients with gastric cancer [GC]) were prospectively enrolled in the study. The gene expression of claudin-1, 2 (CLDN-1, 2), occludin (OCLN), and zonula occludens-1 (ZO-1) was measured in normal corpus mucosa using quantitative RT-PCR and immunohistochemistry (IHC) and the expression in the controls was compared to that in patients with GC based on their Hp infection status. Changes in gene expression and IHC results were also evaluated following Hp eradication.

Results: The gene expression of ZO-1 in the Hp+ control group was

significantly lower than that in the Hp- control group (P = 0.006), whereas it was higher in the Hp+ cancer group than in the Hp-negative cancer group (P = 0.001). Moreover, the increased expression of the ZO-1 gene in the Hp+ cancer group was reduced to the levels in Hp- cancer group within a year after Hp eradication and was maintained for more than 5 years. In addition, the IHC results for ZO-1 were similar to the gene expression results. In particular, the higher IHC staining intensity of ZO-1 in the cytosol of patients with cancer (P = 0.019) was decreased following Hp eradication (P = 0.040). The difference in ZO-1 IHC intensity in each cellular compartments was also confirmed through *in vitro* Hp infection to gastric adenocarcinoma cell line.

Conclusion: Hp infection affects the expression of TJPs. Especially, ZO-1 showed high expression in Hp+ GC, and it was restored to control levels after Hp eradication, suggesting that ZO-1 seemed to be related to gastric carcinogenesis caused by Hp infection.

Keywords: Gastric cancers, Tight junction proteins, *Helicobacter pylori*, ZO-1, Carcinogenesis

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1. Introduction

1.1. Gastric cancer and *Helicobacter pylori*

Gastric cancer (GC) is the fifth most common cancer and the third most common cause of cancer-related deaths worldwide [1]. In South Korea, the incidence and mortality of GC are ranked in the top five [2]. *Helicobacter pylori* (Hp) infection of the gastric epithelium is a major risk factor for GC. Colonization of the gastric mucosa by Hp causes acute diffuse inflammation followed by chronic inflammation. Bacterial virulence and host genetic factors determine the severity and pathological sequelae of inflammation [4]. Once Hp successfully colonizes the gastric epithelium, the bacterial toxin CagA is injected into the host epithelial cells through the type IV secretion apparatus causing the 'hummingbird phenotype' [5, 6]. The morphological changes characteristic of this phenotype is associated with the loss of integrity of the gastric epithelial barrier [7, 8]. Many studies have demonstrated the beneficial effects of Hp eradication on reducing the risk of GC incidence in patients [9]. The reduction in risk of occurrence of primary GC in terms of overall risk ratio (RR) and risk difference (RD) were 0.67 [95% confidence interval (CI): 0.48–0.95] and -0.00 (95% CI: -0.01–0.00), respectively, in a meta-analysis [10]. In addition, the effect of Hp eradication on the prevention of metachronous GC was reported with a hazard ratio of 0.32 (95% CI: 0.15–0.66; P = 0.002) [11]; thus, Hp eradication is now recommended in patients with early gastric cancer (EGC)

[12].

1.2. The tumor microenvironment and tight junction proteins

Tight junctions (TJs) play a critical role in gastric mucosal defense mechanisms by preserving epithelial monolayer barrier integrity via intracellular adhesions and their interactions in the gastric mucosa, which contains gastric acids, food, and gut microbiota, including Hp. TJs consist of transmembrane proteins such as occludin (OCLN), junctional adhesion molecules (JAMs), claudins (CLDNs), and cytoplasmic proteins, which bind to transmembrane proteins and act as adaptors and signaling molecules, as is the case of zonula occludens (ZO) [13]. Upon injury or death of a cell within the epithelial cell layer, the disruption of TJs in adjacent healthy cells allows growth factors to bind and activate their receptors, thereby inducing cellular proliferation and migration to repair the wound. Dysregulation of TJ proteins (TJPs) leads to the loss of cell-to-cell association, resulting in uncontrolled growth and loss of adhesion between epithelial cells [45], similar to that observed in inflammatory bowel disease [15]. In the case of GC, the expression of TJPs changes to unusual patterns, which affects prognosis or metastasis. For example, CLDN1, 3, and 4 are strongly expressed in intestinal type adenocarcinoma, and the strong expression of CLDN4 is associated with decreased survival [16, 17]. Moreover, compared to non-neoplastic mucosal tissues, CLDN7 and 8 are upregulated in GC and are related to lymphatic metastasis [18].

The tumor microenvironment (TME) consists of cancer cells and various other components, including infiltrating immune cells, blood vessels, signaling molecules, and extracellular matrix proteins [19]. Owing to their key role in regulating cell-to-cell interactions, TJPs may play a role in TME [20, 21]. Normal tissue adjacent to the tumor (NAT) is commonly used as a healthy control in cancer studies. However, recent studies on the transcriptomic profiling of NAT have revealed that it exists in a unique intermediate state between that of healthy tissues and tumors [22, 23]. Therefore, studies on NAT may help elucidate the tumorigenesis of GC, especially in relation to Hp infection.

1.3. Aim of the study

As Hp infection influences the integrity of the gastric epithelial barrier via dysregulation of TJPs, and both Hp infection and eradication are involved in the risk of GC, TJPs may have a relationship in the gastric carcinogenesis cascade resulting from Hp infection. Thus, the present study aimed to investigate the effect of Hp infection on the gene expression of TJPs, which may differ between healthy controls and GC patients. Moreover, the Hp-eradicated healthy controls and patients with GC were followed up to assess the effect of Hp eradication on gastric tumor environments in terms of TJPs.

2. Materials and Methods

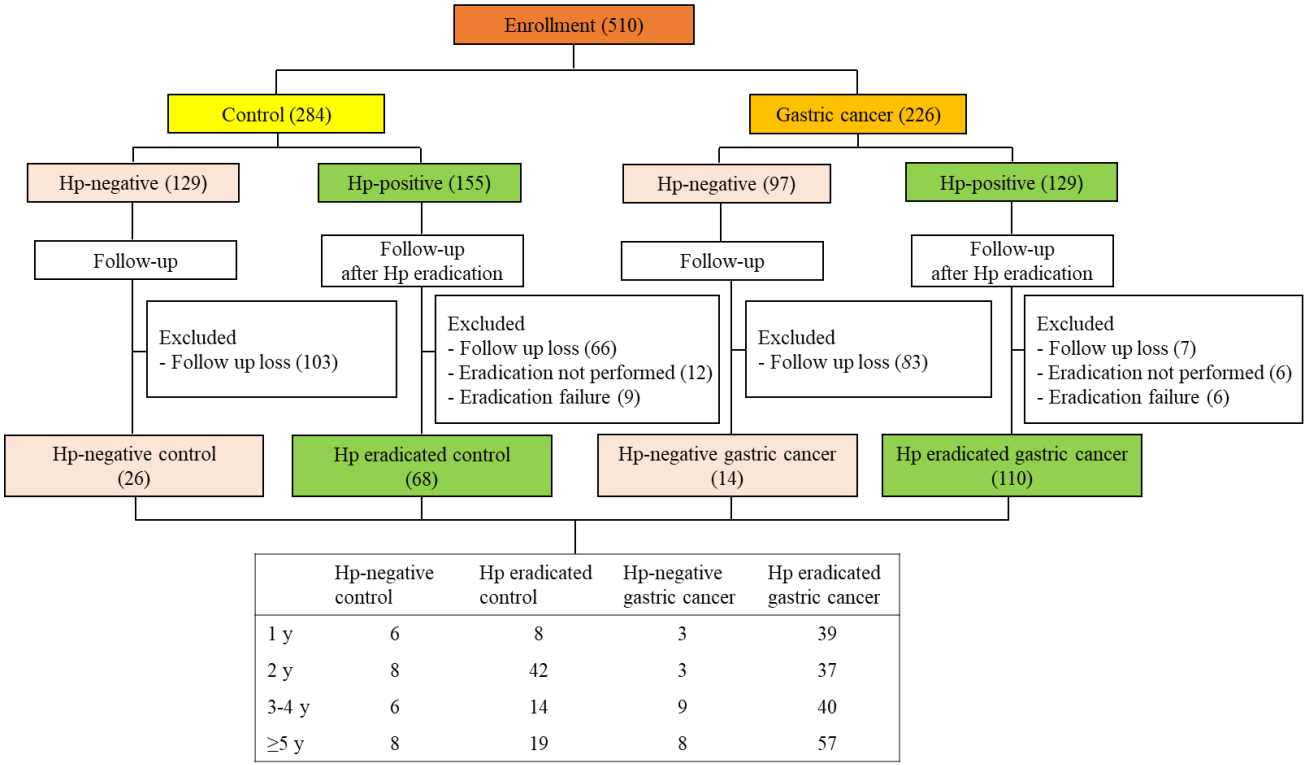
2.1. Study subjects

The study subjects who had gastrointestinal symptoms, such as epigastric discomfort, nausea, and vomiting, or who received regular check-ups for surveillance of GC at the Seoul National University Bundang Hospital between February 2006 and 2016 were consecutively enrolled. The 510 subjects enrolled included 284 healthy controls and 226 patients with GC (Fig. 1). Subjects with previous gastrectomy and Hp eradication, and patients who did not undergo Hp tests were excluded. The controls showed no evidence of GC, dysplasia, mucosa-associated lymphoid tissue lymphoma, esophageal cancer, or peptic ulcers. All subjects, who provided informed consent, were asked to complete a questionnaire requesting data on age, sex, smoking and drinking habits, and history of Hp eradication. The study protocol was approved by the Ethics Committee of the Seoul National University Bundang Hospital (IRB number: B-1907-550-302), and informed consent was obtained from all patients.

2.2. *H. pylori* testing and histology

Biopsy was performed during esophagogastroduodenoscopy (EGD) by a single gastroenterologist (N.K.) for consistency of biopsy sites. Six biopsy specimens were obtained from the lesser curvature and four from the greater curvature of the stomach (equal to the mid-antrum and mid-corpus),

Figure 1



	Hp-negative control	Hp eradicated control	Hp-negative gastric cancer	Hp eradicated gastric cancer
1 y	6	8	3	39
2 y	8	42	3	37
3-4 y	6	14	9	40
≥5 y	8	19	8	57

Figure 1. Illustration of the management of the study subjects based on their *H. pylori* (Hp) infection status. The numbers in brackets represent the number of patients in each case.

regardless of GC status [24]. An experienced gastrointestinal (GI) pathologist (H.S. Lee; blinded to subject information) examined all the specimens to ensure consistency in the histological evaluation. The grade and stages of the gastric atrophic changes were classified and scored according to the updated Sydney Scoring System (0, absent; 1, mild; 2, moderate; and 3, severe) using a visual analog scale. An enzyme-linked immunosorbent assay (ELISA) was used to determine the presence of a Hp-specific immunoglobulin (Ig) G antibody in the serum (Genedia *H. pylori* ELISA; Green Cross Medical Science Corp., Eumseong, Korea). Fasting serum samples obtained from the patients were centrifuged immediately and stored at –70 °C. Serum concentrations of pepsinogen (PG) I and II were measured using a latex-enhanced turbidimetric immunoassay (L-TIA; HBi Corp., Seoul, South Korea; imported from Shima Laboratories, Tokyo, Japan).

The Hp infection status was evaluated using five different criteria as follows: (1) histology based on the Sydney Scoring System [25], (2) CLOtest (Delta West, Bentley, Australia), (3) Hp cultures obtained from biopsy, (4) serum anti-Hp IgG antibody test (Genedia *H. pylori* ELISA; Green Cross Medical Science Corp., Eumseong, Korea), and (5) Hp eradication history. One specimen each from the lesser and greater curvatures of the stomach (i.e., from the mid-antrum and mid-corpus) was fixed in neutral buffered 10% formalin and embedded in paraffin. When any of the first three invasive tests (1, 2, or 3 described above) was positive, the

patient was diagnosed with current Hp infection; if all tests were negative, the patient was considered as uninfected. The positivity of the Hp virulence protein CagA was examined by polymerase chain reaction (PCR) (primer sequences: forward 5'-GAT AAC AGG CAA GCT TTT GAG G-3' and reverse 5'-CTG CAA AAG ATT GTT TGG CAG A-3') [26, 27] using DNA extracted from cultured Hp isolated from biopsied tissue.

2.3. RNA extraction, reverse-transcription PCR, and quantitative real-time PCR (qPCR)

Gastric biopsy specimens were obtained from a noncancerous corpus during endoscopy. These biopsy specimens were used to measure the messenger RNA (mRNA) expression levels of CLDN1, CLDN2, OCLN, and ZO-1. Total RNA was extracted from mucosal biopsy specimens using TRIzol® reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol, and the isolated RNA was purified using RNeasy mini kits (Qiagen, Valencia, CA, USA). Complementary DNA (cDNA) synthesis was performed using total RNA and a high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's instructions. The qPCR was performed in triplicate using a StepOnePlus™ Real-time PCR system (Applied Biosystems) with SYBR Premix Ex TaqTM (Takara Bio, Shiga, Japan) according to the manufacturer's protocol. The primers used were described in a previous study [28]. The mRNA expression levels of the target genes were compared

to those of the endogenous control β-actin using the 2−ΔΔCt method.

2.4. *H. pylori* eradication therapy and follow-up

The subjects with current Hp infection were treated with initial eradication therapy, which consisted of triple therapy prior to 2012 [29] and a 10-day sequential therapy thereafter [30]. The triple therapy regimen consisted of a combination of standard doses of esomeprazole [40 mg twice a day (bid)], amoxicillin (1000 mg bid), and clarithromycin (500 mg bid) for one week. The 10-day sequential therapy included esomeprazole (40 mg) and amoxicillin (1000 mg) bid for 5 days, followed by esomeprazole (40 mg), clarithromycin (500 mg), and metronidazole (500 mg) bid for the next 5 days. Hp eradication was assessed using the 13C-urea breath test 4 weeks after the eradication therapy [11, 29]. In case of failure of the first-line regimen, the patients were administered another eradication therapy consisting of either a 14-day quadruple regimen containing bismuth (esomeprazole [40 mg bid], tri-potassium dicitrate bismuthate [300 mg four times a day (qid); Denol; Green Cross Medical Science Corp ., Eumseong, Korea], metronidazole [500 mg three times a day (tid)], and tetracycline [500 mg qid]) or a 14-day moxifloxacin-based triple therapy (moxifloxacin [400 mg qid; Avelox; Bayer Health Care, AG, Wuppertal, Germany], esomeprazole [40 mg bid], and amoxicillin [1000 mg bid]).

At each elective follow-up visit at 1, 2, 3–4, and ≥ 5 years (maximum 134 months) for endoscopic surveillance following Hp eradication, the

number of Hp eradicated control group was 68, and Hp eradicated gastric cancer group was 110. All of the cancer patients who were included for follow-up were treated by endocopic therapy such as endoscopic mucosal resection or endoscopic submucosal dissection but not by surgical operation. Hp status and levels of OCLN and ZO-1 (qPCR) were evaluated in each follow-up in the control and in the GC patients who underwent Hp eradication.

2.5. Immunohistochemistry (IHC) and ZO-1

For IHC, tissue sections were treated with 3% hydrogen peroxide and non-specific binding sites were blocked. The sections were then incubated with anti-ZO-1 (ZO-1) antibodies (61-7300, Thermo Fisher Scientific, Waltham, MA, USA). An automatic immunostainer (BenchMark XT; Ventana Medical Systems Inc., Tucson, AZ, USA) and an ultraView Universal DAB Detection Kit (Ventana Medical Systems) were used for immunostaining [31]. IHC for ZO-1 was performed in human renal glomeruli, gastric mucosa, and colonic mucosa tissues as positive controls. IHC for the negative control was performed by substituting the primary anti-ZO-1 (ZO-1) antibody in the same tissues. Normal gastric mucosa epithelium served as an external positive control for all antibodies and showed expression of all proteins in a particulate pattern confined to the apical portion of the lateral epithelial cell membrane. Fig. 4a illustrates the normal cellular localization of various TFPs in polarized glandular epithelial

cells. Negative controls consisted of substituting a buffer for the primary antibody. ZO-1 expression was quantified only in the gastric epithelium cell region, depending on the cellular compartment (membrane, cytosol, or nucleus). For expression in the membrane and nucleus, the total number of stomach epithelial cells in slides and the positively stained ZO-1 in the membrane and nucleus among the total cells were counted using cell counter plugins included in ImageJ 1.50i software [32]. Next, positivity was quantified as the ratio of the number of cells stained with ZO-1 to the total number of cells counted in a section. This quantification was performed by pathologist H.S. Lee, without prior knowledge of the clinical details of individual patients. Evaluation of ZO-1 expression in the cytosol was performed by multiplying the intensity of staining by the area (%) of the epithelial region (0–300). The intensity was scored based on the staining intensity of the epithelial peripheral region scored as 2 for 'moderate' expression using Image-Pro Plus (Media Cybernetics Inc., Rockville, MD, USA). Expression was scored as follows: 0, not stained; 1, weak; 2, moderate; and 3, marked. Each sample was scored in a blinded manner.

2.6. Cell culture and Western blotting

Helicobacter pylori (ATCC 43504; CagA+) was purchased from the American Type Culture Collection (Rockville, MD, USA). Bacteria were cultured under microaerophilic conditions (5% O_2, 10% CO_2, and 85% N_2) at 37 °C. AGS (human GC cell line) cells were grown in Roswell Park

Memorial Institute Medium 1640 (Gibco, Grand Island, NY, USA) and HFE-145 (human normal gastric epithelial cell line) cells were grown in Dulbecco's Modified Eagle's Medium (Gibco, Grand Island, NY, USA) both supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, USA).

2.7. *H. pylori* infection

Cells were grown in a humidified CO_2 atmosphere at 37 °C. Before co-culturing with Hp, 1×106/mL AGS cells were disseminated in new plates, incubated for 24 h to reach 80-90% confluence, and then starved for another 24 h. Bacterial cells that were cultured on chocolate agar plates for 48 h were resuspended in Brucella broth (Sigma-Aldrich, St Louis, MO, USA). The optical density at 600 nm (OD600) of the resuspended bacterial suspension was measured. OD1 represented 1×10^9 colony-forming units (CFU)/mL of bacteria and it was confirmed by counting bacterial colonies on the plate cultured from diluted resuspended bacteria. From the measured OD value of the resuspended bacteria, a multiplicity of infection (MOI) of 100 was used (bacteria CFU/AGS cells).

2.8. Western blotting

After 0, 3, 6, 9, and 24 h of co-incubation of AGS cells with Hp, nuclear and cytosolic proteins were extracted using NE-PER™ Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific, Waltham, MA,

USA), and membrane proteins were extracted using the Mem-PER Plus Membrane Protein Extraction Kit (Thermo Fisher Scientific, Waltham, MA, USA). For western blot analysis, proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. Membranes were blocked with dried milk (5% w/v) and blotted with a primary antibody followed by a secondary antibody. Anti-ZO-1 (61-7300, Invitrogen, Waltham, MA, USA), Na+/K+-ATPase α1 (sc-514614), Hsp90 (sc-13119), and lamin B1 (sc-6216) (Santa Cruz Biotechnology, Dallas, TX, USA) antibodies were used as the primary antibodies.

2.9. Immunofluorescence for confocal imaging

After 4 hours of co-incubation of HFE-145 cells with Hp, 4% paraformaldehyde (Biosesang, Sungnam, Korea) was treated for 15 minutes at room temperature for fixing the cell samples. After fixing the cell samples, 0.2% triton-X-100 was treated to permeabilize the cell samples, and 2% bovine serum albumin was treated for blocking. Anti-ZO-1 (C-terminal) (ab190085, Abcam, Cambridge, UK) and Donkey Anti-Goat IgG (Alexa Fluor® 555) (ab150130, Abcam, Cambridge, UK) were used for primary and secondary antibodies, and DAPI staining solution (ab228549, Abcam, Cambridge, UK) was used for staining cell nuclei. Immunofluorescence images were taken using Laser Scanning Confocal Microscope (LSM800, Berlin, Germany).

2.10. Statistical analysis

SPSS for Windows (version 18.0; SPSS, Chicago, IL, USA) was used for all statistical analyses. Data were expressed as mean ± standard error of the mean. Continuous variables were analyzed using a one-way analysis of variance or a t-test when the sample size was >30 in each group, or the Kruskal-Wallis or Mann-Whitney tests. Student's t-test and Mann-Whitney U test were performed as post hoc tests. The chi-square test was used to analyze categorical variables. Longitudinal data were analyzed using a linear mixed regression analysis with a random intercept model. Statistical significance was set at $P < 0.05$.

3. Results

3.1. Characteristics of the subjects

Five hundred ten subjects, including 284 healthy controls and 226 patients with GC were enrolled (Fig. 1). Among the 284 subjects in the control group 129 subjects were Hp-negative and 155 subjects were Hp-positive. Among the 226 patients with GC 97 patients were Hp-negative and 129 patients were Hp-positive. There were 26 subjects in Hp-negative control and 68 subjects who were successfully Hp-eradicated control. Also, there were 14 Hp-negative patients with GC and 110 patients with GC who were successfully Hp-eradicated. The baseline characteristics of the 510 subjects enrolled in this study are shown in Table 1. Compared with the

control group, subjects in the GC group were older ($P < 0.001$) and predominantly male ($P < 0.001$). Approximately 55% of the subjects in both the control and GC groups were Hp-positive. Among these, 82.5% were infected with CagA+ Hp but the difference between the control (85.9%) and GC (79.1%) groups was not statistically significant. Atrophic gastritis was significantly more frequent in the Hp-positive group than in the Hp-negative group in both the control and GC groups ($P < 0.001$). The incidence of intestinal metaplasia was higher in the Hp-positive group than in the Hp-negative control group ($P < 0.001$). The PG I/II ratio was significantly lower in the Hp-positive group than in the Hp-negative group in both the control and GC groups ($P < 0.001$). Among the 226 patients with GC, 152 (67.3%) had EGC and 74 (32.7%) had advanced gastric cancer (AGC), categorized based on the invasiveness of cancer. In addition, 65.9% of the patients were classified as the intestinal type according to the Lauren classification (Table 1).

3.2. mRNA expression levels of ZO-1 in the GC and control groups depended on *H. pylori* infection status

The mRNA expression levels of the four TJPs were compared between normal-appearing corpus mucosa from patients with GC and the control group based on their Hp infection status (Fig. 2a-d). The mRNA expression of CLDN1 was not influenced by Hp infection status in control patients, whereas the Hp-negative GC group showed significantly higher expression

of CLDN1 mRNA than the Hp-positive GC group (Fig. 2a, $P = 0.016$). CLDN2 mRNA expression was lower in the GC group than in the control group, although the difference was not statistically significant (Fig. 2b). The expression of OCLN mRNA was significantly decreased in Hp-infected patients, regardless of GC status (Fig. 2c; control Hp-negative vs. control Hp-positive, $P = 0.001$; Hp-negative GC vs. Hp-positive GC, $P = 0.001$). In the case of ZO-1, the expression pattern was reversed in the control and GC groups (Fig. 2d). The expression of ZO-1 was significantly lower in the Hp-positive control group than in the Hp-negative control group ($P = 0.006$), whereas in the GC group Hp-positive patients showed higher ZO-1 expression than Hp-negative patients ($P = 0.001$). To further investigate the pattern of expression of these TJPs in GC, subgroup analysis was performed for EGC and AGC to examine whether the expression of these TJPs differed depending on the invasiveness of cancer (Fig. 2e-h). The decrease in CLDN1 gene expression in the Hp-positive GC group was only observed in EGC, but not in AGC (Fig. 2e). The decrease in CLDN2 mRNA expression in the GC group, compared to that in the controls, was more severe in AGC than in EGC, regardless of Hp infection status, but the difference was not statistically significant (Fig. 2f). In contrast, OCLN and ZO-1 mRNA expression in EGC and AGC showed similar patterns regardless of cancer invasiveness. In the case of OCLN, the Hp-positive group showed decreased mRNA expression compared with the Hp-negative group in both EGC ($P = 0.027$) and AGC (Fig. 2g, $P < 0.001$). In addition, in the case of ZO-1, the

Table 1. Baseline characteristics of study subjects

	Controls (*n* = 284)			Gastric cancer (*n* = 226)		
	Hp-negative (*n* = 129)	Hp-positive (*n* = 155)	*P*-value	Hp-negative (*n* = 97)	Hp-positive (*n* = 129)	*P*-value
Age (mean±SD, years)	49.42±12.97	52.96±11.55	**0.005**	63.54±12.07	57.18±10.86	**<0.001**
Sex, n (%)			0.334			0.498
Male	42 (32.6)	59 (38.1)		62 (63.9)	88 (68.2)	
Female	87 (67.4)	96 (61.3)		35 (36.1)	41 (31.8)	
Current/ex-smoker, n (%)	43 (33.3)	52 (33.5)	0.969	61 (62.9)	76 (58.9)	0.545
Alcohol drinker, n (%)	85 (65.9)	96 (61.9)	0.490	67 (69.1)	95 (73.6)	0.450
Family history of GC, n (%)	60 (46.5)	72 (46.5)	0.992	19 (19.6)	38 (29.5)	0.091
[a]Atrophic gastritis, n (%)	10 (8.8)	33 (25.4)	**<0.001**	46 (47.4)	79 (61.2)	**0.039**
[b]Intestinal metaplasia, n (%)	9 (7.0)	31 (20.0)	**<0.001**	69 (71.1)	96 (74.4)	0.582
Serum pepsinogen						
I	48.61±23.62	66.92±47.81	**<0.001**	56.63±37.52	64.91±42.40	0.133
II	13.49±15.53	27.88±29.67	**<0.001**	16.22±27.99	22.78±13.44	**0.021**
I/II ratio	5.64±7.79	3.32±1.86	**<0.001**	5.01±2.20	3.03±1.55	**<0.001**
EGC, n (%)				58 (59.8)	120 (93.0)	**<0.001**
Lauren classification, n (%)						0.144
Intestinal				58 (59.8)	91 (70.5)	
Diffuse				36 (37.1)	37 (28.7)	
Mixed				3 (3.1)	1 (0.8)	
[c]Stage, n (%)						**<0.001**
I				51 (62.2)	96 (92.3)	
II				14 (17.1)	6 (5.8)	
III				14 (17.1)	2 (1.9)	
IV				3 (3.6)	0 (0)	

The data are presented as number (%) or mean ± standard deviation.

[a, b] Atrophic gastritis and intestinal metaplasia were measured histologically based on Sydney Classification at least grade I in the body.

P-value was calculated using the student's T test for continuous variables; Chi-square test for categorical variables

Values presented in bold indicate statistically significant differences.

Hp, *Helicobacter pylori*; GC, gastric cancer; EGC, early gastric cancer.

[c] Some data were missing.

Hp-positive EGC group showed increased mRNA expression compared with the Hp-negative EGC group (P = 0.005), whereas in AGC, the difference was not statistically significant (Fig. 2h). Taken together, the mRNA expression levels of CLDN1 and OCLN were lower in Hp-positive GC patients than in Hp-negative GC patients, whereas ZO-1 mRNA expression increased in Hp-positive GC patients. Moreover, the increased expression of ZO-1 mRNA was maintained in the EGC and AGC subgroups of patients with GC.

3.3. Changes in the ZO-1 and OCLN mRNA expression level following *H. pylori* eradication

As OCLN and ZO-1 mRNA expression showed a consistent pattern depending on Hp infection status, changes in their mRNA expression were investigated following Hp eradication in the control and GC groups (Fig. 3). The control and GC groups were recommended for the regular follow-up but it became variable due to several situations. At each elective follow-up visit at 1, 2, 3–4, and ≥ 5 years (maximum 134 months) Hp status and levels of OCLN and ZO-1 (qPCR) were evaluated in control and GC patients who were Hp eradicated. From the visitors, gastric mucosal tissue was taken from the non-tumorous (normal) during follow-up for the qPCR to measure the TJPs gene expression. Among the follow-up cases, there was missing data because of a lack of specimens or not detected in qPCR, the number of final patients for analysis was 45 in the control Hp eradicated

Figure 2

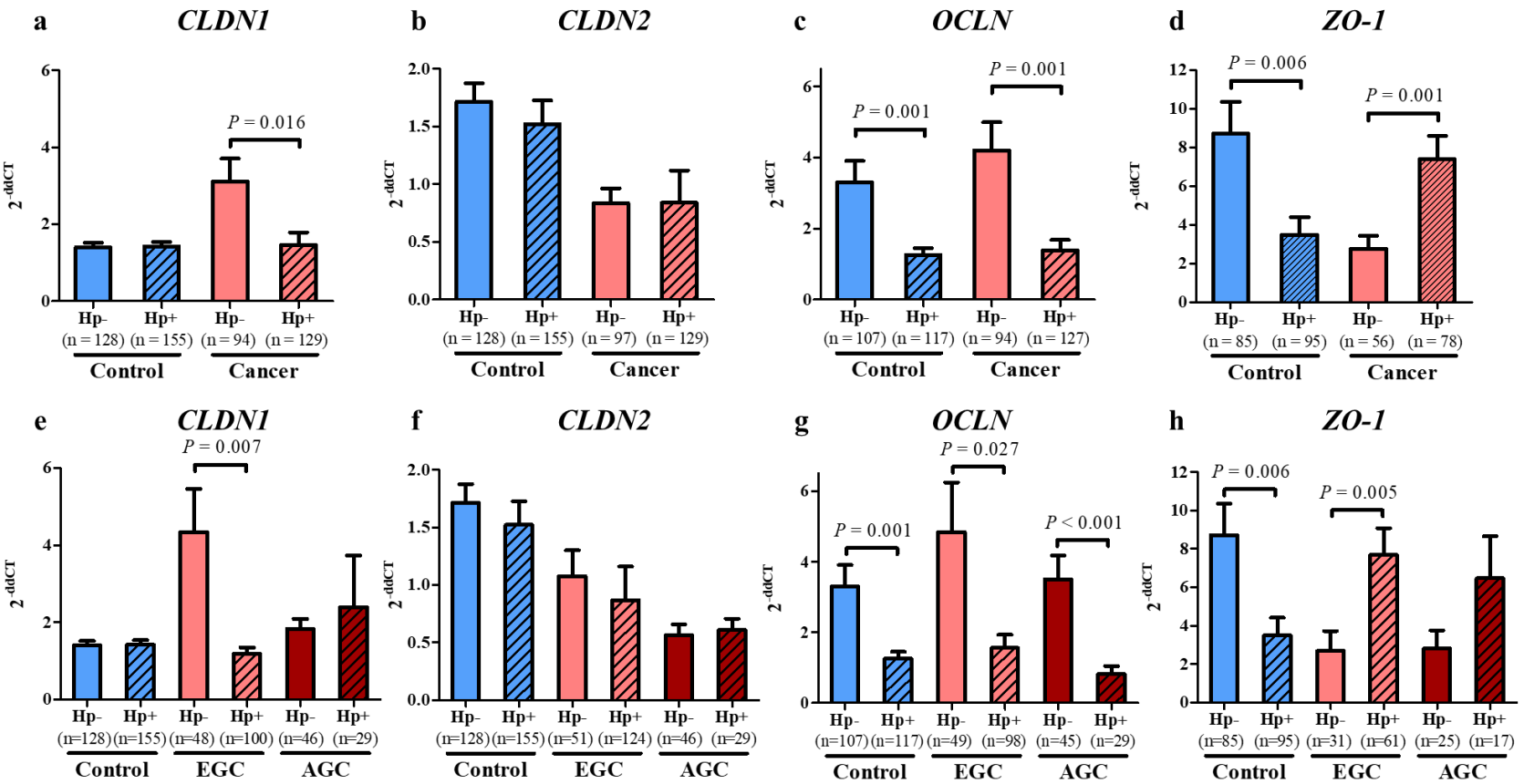

a
CLDN1
P = 0.016
2-ddCT
Hp- (n = 128)
Hp+ (n = 155)
Hp- (n = 94)
Hp+ (n = 129)
Control
Cancer
b
CLDN2
Hp- (n = 128)
Hp+ (n = 155)
Hp- (n = 97)
Hp+ (n = 129)
Control
Cancer
c
OCLN
P = 0.001
P = 0.001
Hp- (n = 107)
Hp+ (n = 117)
Hp- (n = 94)
Hp+ (n = 127)
Control
Cancer
d
ZO-1
P = 0.006
P = 0.001
Hp- (n = 85)
Hp+ (n = 95)
Hp- (n = 56)
Hp+ (n = 78)
Control
Cancer
e
CLDN1
P = 0.007
Hp- (n=128)
Hp+ (n=155)
Hp- (n=48)
Hp+ (n=100)
Hp- (n=46)
Hp+ (n=29)
Control
EGC
AGC
f
CLDN2
Hp- (n=128)
Hp+ (n=155)
Hp- (n=51)
Hp+ (n=124)
Hp- (n=46)
Hp+ (n=29)
Control
EGC
AGC
g
OCLN
P = 0.001
P = 0.027
P < 0.001
Hp- (n=107)
Hp+ (n=117)
Hp- (n=49)
Hp+ (n=98)
Hp- (n=45)
Hp+ (n=29)
Control
EGC
AGC
h
ZO-1
P = 0.006
P = 0.005
Hp- (n=85)
Hp+ (n=95)
Hp- (n=31)
Hp+ (n=61)
Hp- (n=25)
Hp+ (n=17)
Control
EGC
AGC

Figure 2. Comparison of mRNA expression of tight junction proteins (a, e) claudin1 (*cldn1*), (b, f) claudin2 (*cldn2*), (c, g) occludin (*ocln*), and (d, h) zona occludens-1 (*zo-1*) between patients with gastric cancer and controls based on *H. pylori* (Hp) infection status (a-d) and cancer invasiveness (e-h). The expression of *ocln* decreased significantly not only in the Hp^+ control group ($P = 0.001$) (c), but also in the Hp^+ cancer group regardless of cancer invasiveness ($P = 0.027$ in EGC; $P < 0.001$ in AGC) (g). The Hp^+ control group showed decreased expression ($P = 0.006$) of *zo-1*, whereas the Hp^+ cancer group showed increased expression ($P = 0.001$) (d). *cldn1* expression increased significantly only in EGC Hp^- patients ($P = 0.007$) (e). There was no significant difference in *cldn2* expression (b, f). The mRNA expression level of each tight junction protein was measured in non-cancerous gastric biopsy (corpus) specimens using RT-qPCR. The *P*-values were calculated using the student's *t*-test or Mann-Whitney test. EGC, early gastric cancer; AGC, advanced gastric cancer.

group, and 62 in GC Hp eradicated group. Longitudinal data were analyzed using linear mixed regression analysis with a random intercept model. There was no significant change in the mRNA expression of OCLN during the follow-up period in the Hp-eradicated control (Fig. 3a) and GC groups (Fig. 3b). However, in the case of ZO-1, a marked difference was observed between the control and GC groups. Although there was no significant change in ZO-1 mRNA expression in the control group (Fig. 3c), it decreased significantly following Hp eradication in the GC group (P = 0.047) (Fig. 3d).

3.4. ZO-1 protein levels in the various cellular compartments

Next, IHC was performed to investigate the expression pattern of the ZO-1 protein in the gastric mucosa of the gastric corpus. Before staining patient tissue sections, control staining was performed on the gastric mucosa using a human tissue microarray test block (Fig. 4). The antibody did not cause non-specific staining and the gastric mucosa was targeted for antibody activity. The antibody specificity was tested again on actual patient tissue sections (Fig. 5a, left panel), and there was no non-specific staining. Usually, implied from the name, ZO-1 is known to localize at the cellular membrane. However, some reports have mentioned the translocation of ZO-1 not only to the membrane but also to the cytosol and nucleus [33-35]. In addition, the IHC results evidenced ZO-1 staining in various cellular compartments,

Figure 3

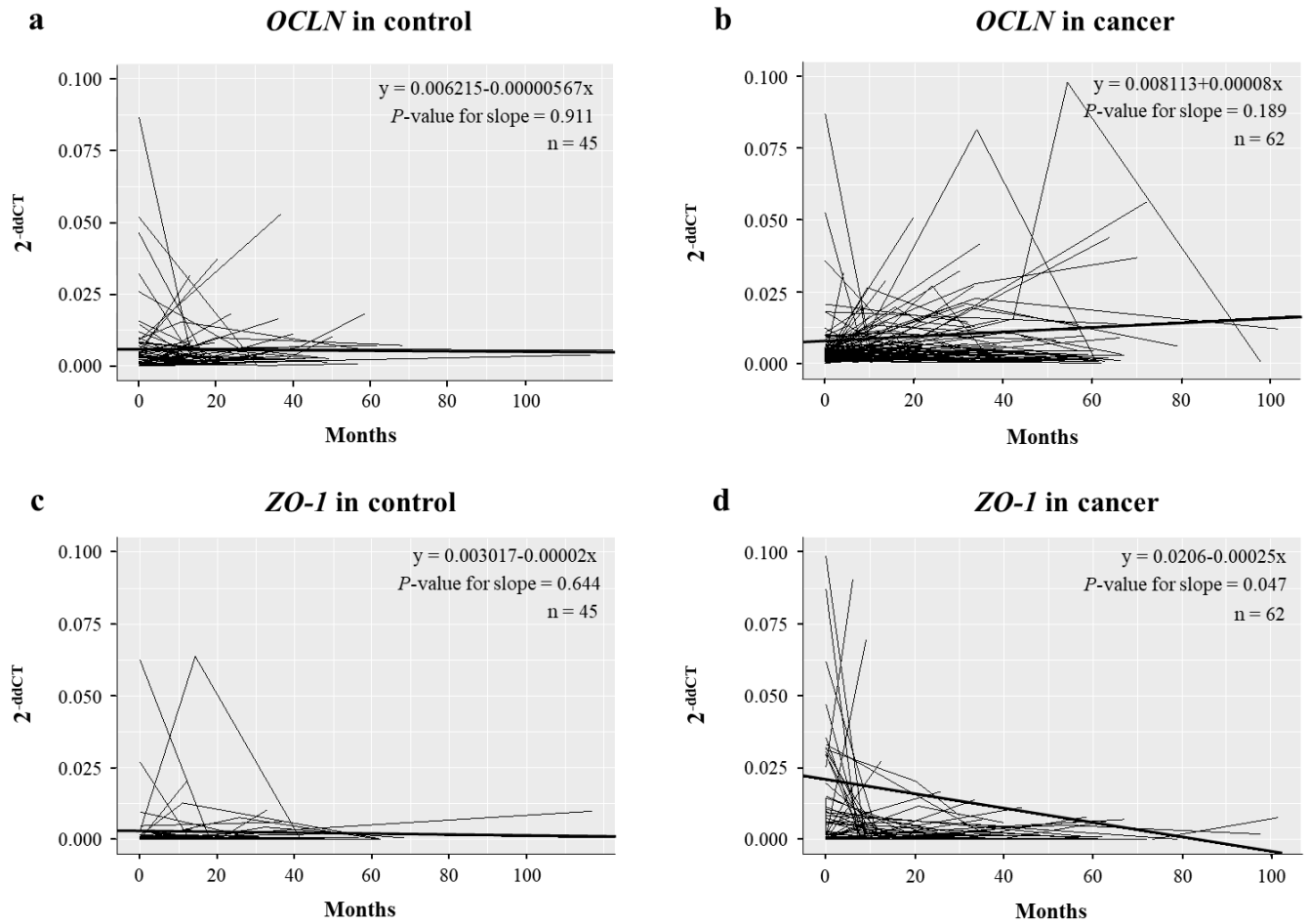

a
OCLN in control
y = 0.006215-0.00000567x
P-value for slope = 0.911
n = 45
b
OCLN in cancer
y = 0.008113+0.00008x
P-value for slope = 0.189
n = 62
c
ZO-1 in control
y = 0.003017-0.00002x
P-value for slope = 0.644
n = 45
d
ZO-1 in cancer
y = 0.0206-0.00025x
P-value for slope = 0.047
n = 62
2 -ddCT
Months
0.100
0.075
0.050
0.025
0.000
0
20
40
60
80
100

Figure 3. Changes in mRNA expression of occludin (*ocln*) and zona occludens-1 (*zo-1*) during follow-up following successful eradication of *H. pylori* (Hp) infection. Changes in mRNA expression of *ocln* in Hp-eradicated control (a), and Hp-eradicated cancer patients (b). Changes in mRNA expression of *zo-1* in Hp-eradicated control (c), and Hp-eradicated cancer patients (d). There was no significant difference in the mRNA expression level of *ocln* during follow-up periods in the eradicated control (a) vs. GC group (b). There was no significant change in *zo-1* mRNA expression in the control group after Hp eradication (c), whereas it decreased significantly after Hp eradication in the GC group ($P = 0.047$) (d).

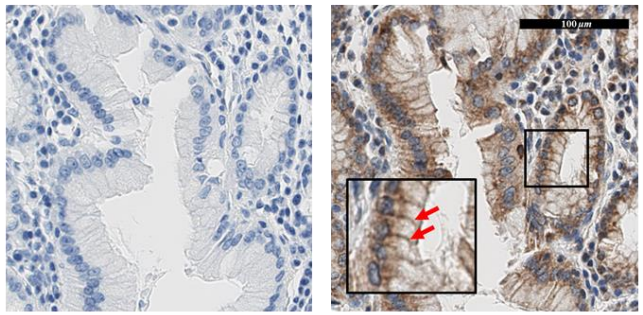
including the membrane, cytosol, and nucleus (Fig. 5a). Thus the membrane (Fig. 5b), cytosol (Fig. 5c), and nucleus (Fig. 5d), was analyzed separately in the Hp-negative, positive, and eradicated groups. In the control Hp-negative group, ZO-1 was expressed in all cellular compartments (Fig. 5b-d), whereas in the control Hp-positive group, ZO-1 expression significantly decreased in the membrane and cytosol (Fig. 5c). In contrast, the Hp-positive GC group showed increased cytosolic expression of ZO-1 compared with the Hp-negative GC group (P = 0.019; Fig. 5c). This increase in cytosolic ZO-1 levels in IHC was similar to the increase in ZO-1 mRNA expression observed by qPCR. Moreover, IHC revealed that ZO-1 levels decreased (Fig. 5a) following treatment for Hp eradication, which was similar to the qPCR results. In the control group, following Hp eradication, the decreased expression of ZO-1 in the membrane and cytosol slightly increased to a level similar to that in the Hp-negative group, although the difference was not statistically significant (Fig. 5c). In contrast, in the GC group and following Hp eradication, the increased expression of ZO-1 in the Hp-positive group decreased significantly in the cytosol (P = 0.040) (Fig. 5c) but did not reach statistical significance in the membrane (Fig. 5b) and nucleus (Fig. 5d). IHC results indicated that ZO-1 protein expression was differentially regulated depending on Hp status in control subjects and GC patients. Interestingly, in patients with GC, ZO-1 expression in Hp-positive patients was significantly higher than that in Hp-negative patients, and was restored significantly after Hp eradication.

Figure 4

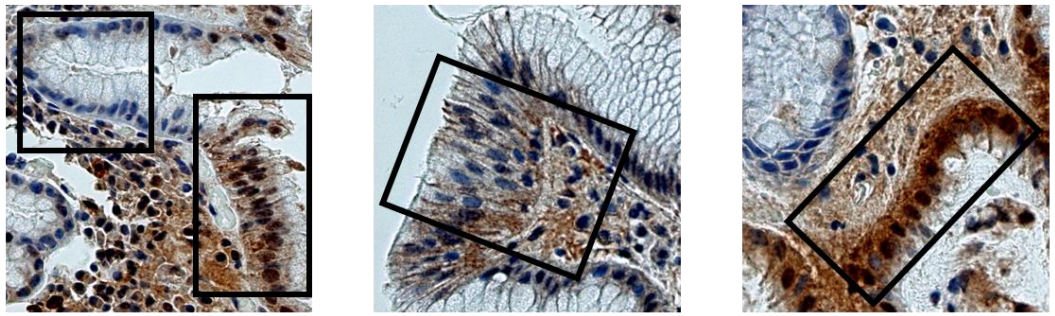
a

Negative control **Actual staining**

Human tissue microarray test (gastric mucosa) as positive control



b **Not stained (0)**



Moderate (2) **Weak (1)** **Marked (3)**

Figure 4. (a) The Control staining for Anti-ZO-1 antibody for IHC. IHC for control was performed in human tissue microarray test block. Negative control was confirmed with the substitution of buffer for the primary anti-ZO-1 antibody (left panel). Normal gastric mucosa epithelium served as an external positive control for all antibodies, and showed expression of all proteins in a particulate pattern, confined to the apical portion of the lateral epithelial cell membrane (right panel, red arrows for membrane staining). (b) The ZO-1 staining patterns of cytosol. The intensity was scored based on the staining intensity of the epithelial peripheral region scored as 2 for 'moderate' expression. The staining intensity was scored as follows: 0, not stained; 1, weak; 2, moderate; and 3, marked

Figure 5

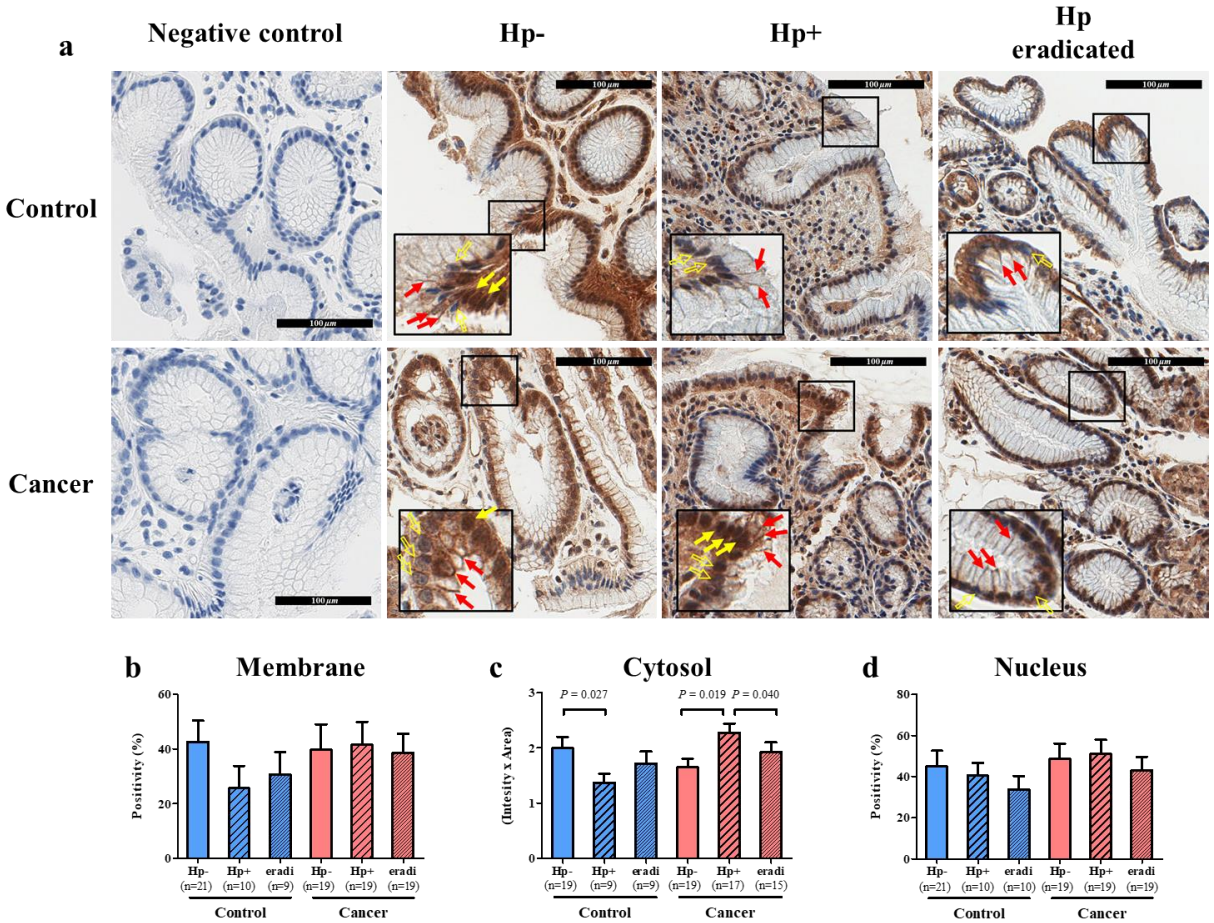

a
Negative control
Hp-
Hp+
Hp eradicated
Control
Cancer
100 μm
b
Membrane
Positivity (%)
c
Cytosol
(Intesity x Area)
P = 0.027
P = 0.019
P = 0.040
d
Nucleus
Positivity (%)
Hp- (n=21)
Hp+ (n=10)
eradi (n=9)
Hp- (n=19)
Hp+ (n=19)
eradi (n=19)
Hp- (n=19)
Hp+ (n=9)
eradi (n=9)
Hp- (n=19)
Hp+ (n=17)
eradi (n=15)
Hp- (n=21)
Hp+ (n=10)
eradi (n=10)
Hp- (n=19)
Hp+ (n=19)
eradi (n=19)
Control
Cancer

Figure 5. Immunohistochemistry (IHC) images and analysis of ZO-1 staining in non-cancerous gastric biopsy specimens. The antibody specificity was tested on actual patient tissue sections (Fig. 5a left panel) and there was no non-specific staining. IHC images were analyzed based on ZO-1 staining in various cell compartments marked as the membrane (red arrows; b), cytosol (c), and nucleus (yellow arrows: colored, positive; empty, negative; d). IHC analysis in the same patients before (Hp-positive) and after Hp eradication (Hp-eradicated). There were no significant changes in the membrane (b) and nucleus (d) whereas, in the cytosol, control Hp^+ patients showed decreased expression ($P = 0.027$), and the expression was restored following Hp eradication although the difference was not statistically significant. The Hp^+ cancer group showed increased expression compared to Hp^- ($P = 0.019$) group and the decrease in the Hp-eradicated group was significant ($P = 0.040$) (c). *P*-values were calculated using the Mann-Whitney test.

3.5. Dynamic changes in ZO-1 localized to the cytosol and nucleus in the early stages of *H. pylori* infection detected by the western blotting and immunofluorescence

As the IHC results showed changes in ZO-1 localization in the gastric epithelia depending on Hp infection status, such changes were further studied in gastric epithelial cells using western blotting (Fig. 6). AGS cells were co-cultured with Hp (MOI = 100), and ZO-1 protein levels in the nucleus and cytosol were measured at 3, 6, 9, and 24 h after Hp infection. Dynamic changes in ZO-1 localization in the early stages of Hp infection were evident when ZO-1 expression was separately evaluated in the membrane, cytosol, and nucleus. The purity of the membrane, cytoplasmic, and nuclear fractions was confirmed using Na+/K+-ATPase (membrane marker), Hsp90 (cytoplasmic marker), and lamin B1 (nucleus marker). In the membrane fraction, ZO-1 increased slightly at 3 h, decreased until 9 h, and finally increased at 24 h compared to that at 0 h; however, the difference was not statistically significant (Fig. 6a). In the cytosolic fraction, ZO-1 increased 6 h after Hp infection (P = 0.057 compared with 3 h) and decreased significantly 9 h after Hp infection (P = 0.029 compared with 6 h) (Fig. 5b). The decrease in ZO-1 protein levels was obvious 24 h after Hp infection (P = 0.029 compared to that at 3, 6, and 9 h) (Fig. 6b). In the nuclear fraction, the amount of ZO-1 appeared to increase 3 and 6 h after Hp infection, decreased at 9h to a level similar to that at 3h, and remained at this level until 24 h, although the difference was not statistically significant

(Fig. 6c).

Moreover, to visualize the ZO-1 movement into the cell, the immunofluorescence (IF) was performed using co-cultured gastric normal epithelial HFE-145 cells with Hp (MOI = 100) (Fig. 7). Before the infection of Hp (Fig. 7c), nuclei appeared to be surrounded by ZO-1 fuzzily, while after Hp infection (Fig. 7f), the ZO-1 seemed to penetrated nuclei guessed from the disappeared ZO-1 surrounding nuclei fuzzily. We suggested the change of localization of ZO-1 from the disappeared ZO-1 after Hp infection.

Figure 6

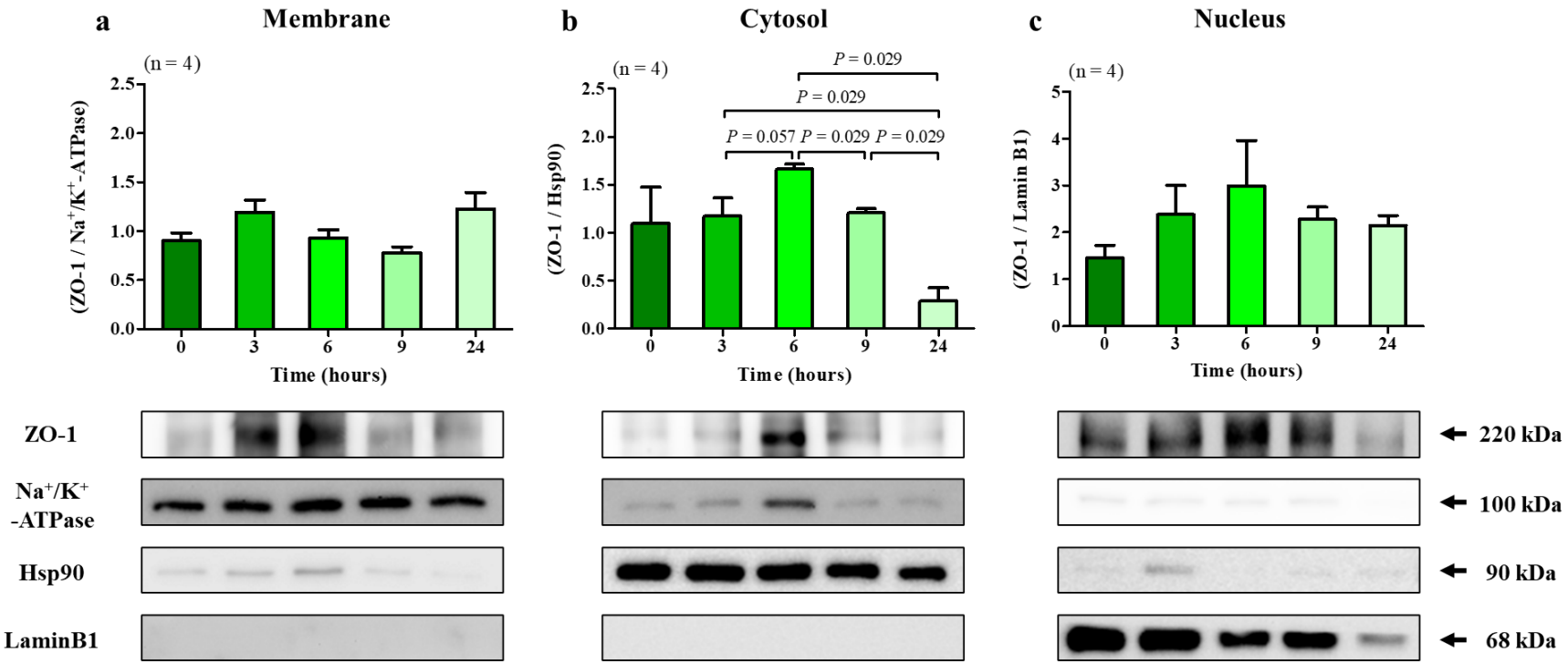

a
Membrane
(n = 4)
(ZO-1 / Na+/K+-ATPase)
Time (hours)
b
Cytosol
(n = 4)
P = 0.029
P = 0.029
P = 0.057 P = 0.029 P = 0.029
(ZO-1 / Hsp90)
Time (hours)
c
Nucleus
(n = 4)
(ZO-1 / Lamin B1)
Time (hours)
ZO-1
Na+/K+ -ATPase
Hsp90
LaminB1
220 kDa
100 kDa
90 kDa
68 kDa

Figure 6. Dynamic changes in the localization of ZO-1 proteins in the cytosol and nucleus in the early stages of *H. pylori* (Hp) infection were detected by western blotting. Normal AGS cells were infected with Hp, and cellular proteins were isolated 3, 6, 9, and 24 h following Hp infection and fractionated into (a) membrane, (b) cytosolic, and (c) nuclear fractions. In the membrane, the ZO-1 increased at 3 h slightly and decreased until 9 h, and finally showed an increased level at 24 h compared to 0 h (not significant). In the cytosol, ZO-1 protein level increased at 6 h following Hp infection (P = 0.057 compared to 3 h), but disappeared at 24 h following Hp infection (P = 0.029 compared to 3, 6, and 9 h). In the nucleus, ZO-1 protein increased until 6 h after Hp infection and decreased after 9 h (not significant). Na^+/K^+-ATPase (membrane), Hsp90 (cytosolic), and Lamin B1 (nuclear) were used as markers to confirm the purity of the corresponding fractions. P-values were calculated using the Mann-Whitney test.

Figure 7

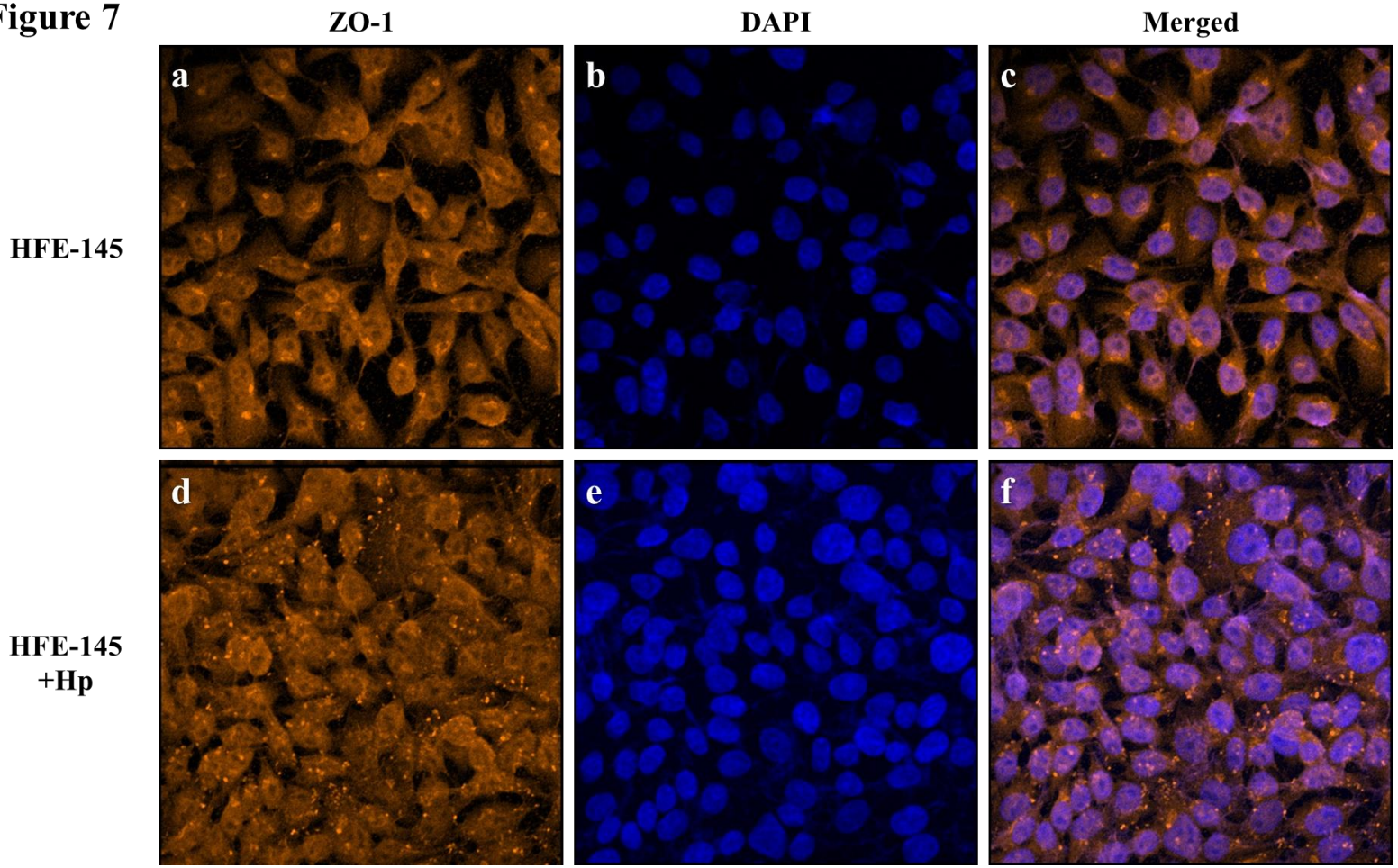


Figure 7. The changes in the localization of ZO-1 proteins after *H. pylori* (Hp) infection were detected by immunofluorescence (IF). The gastric normal epithelial cell line HFE-45 was infected with Hp, and the confocal images were taken with IF staining. Immunofluorescence images were taken using Laser Scanning Confocal Microscope (LSM800, Berlin, Germany). ZO-1 (a, d) was stained using the C-terminal binding antibody (orange), and the cellular nuclei (b, e) was stained using DAPI (blue). it revealed that Hp infection formed grains of ZO-1 overlapped with cell nuclei (blue, middle panel). The images were taken with 200X magnification.

4. Discussion

Our results showed that mRNA expression of TJPs (CLDN-1,2, OCLN, and ZO-1) in the non-tumorous gastric mucosa was different in response to Hp infection, and differences between the control and GC groups. In particular, the expression of ZO-1 in the Hp-positive GC group was significantly higher than that in the Hp-negative GC group (P = 0.001), while, the expression pattern in the control group showed the expected result: lower expression in the Hp-positive control group compared to Hp-negative control group. Interestingly, the increased expression of ZO-1 in the Hp-positive GC group significantly decreased following Hp eradication to Hp-negative GC group levels, which were maintained for >5 years. Such expression level change in terms of Hp infection status may result from the localization change of ZO-1 in each cellular compartment as shown in IHC and IF results.

The levels of TJPs in GC tissues vary depending on the tissue type and tumorigenesis stage. For example, CLDN-4 expression decreased in GC tissues and was associated with tumor aggressiveness and survival [17, 36]. In addition, the downregulation of CLDN-18 was associated with the proliferative and invasive potential of GC [37]. In contrast, CLDN-7 was overexpressed in dysplasia and adenocarcinoma tissues but not in the surrounding epithelial cells [38]. However, most of these reports regarding TJPs in GC did not consider Hp infection, even though Hp is one of the

most important causes of GC. When Hp colonizes the surface of gastric epithelial cells, it starts to disrupt TJs [8] and gains essential nutrients that are released from the disruption of gastric epithelial cell junctions [7,39, 40]. CagA, an important virulence factor of Hp, is known to play a role in its interaction with ZO-1 at the site of bacterial attachment. Several studies have shown that long-term exposure of epithelial cells to CagA disrupts epithelial barrier function [7, 41, 42]. For instance, CagA+ Hp P1 infection of primary gastric epithelial cells altered the cellular localization of ZO-1 in the presence of CagA [41]. Moreover, infection of MKN28 human gastric epithelial cells with the CagA+ Hp 60190 strain resulted in the mislocalization of OCLN (originally located in the membrane but moved to the cytosol), indicating the ability of Hp to induce injury to the gastric wall [42].

Previously, we suggested that the gene expression of TJPs differed in patients with irritable bowel syndrome [43] and functional dyspepsia [44] according to Hp infection, sex, and disease subtype. Based on these observations, we hypothesized that TJPs play a role in Hp-associated gastric carcinogenesis. In addition, improving the TME, such as modifying the levels of TJPs, by Hp eradication [9] might contribute to the prevention of GC and metachronous GC [11, 45]. Thus, if TJPs play a role in Hp-associated gastric carcinogenesis, TJP expression might show reversibility after the successful eradication of Hp. As the above studies reported that the relationship between TJs and CagA positivity is important, we also analyzed

whether TJPs gene expression depends on CagA positivity. The CagA+ GC group showed increased ZO-1 expression compared to CagA- GC group but the difference was not statistically significant (data not shown). Therefore, the reason why there was no significant difference between TJPs and CagA might be that the reported studies on TJPs have been mainly conducted on cell lines, which represent a tightly controlled environment. Moreover, the small number of Hp-positive and CagA-negative patients, which represented less than 20% of our cohort of Hp-positive patients, made it difficult to reproduce the results of the cell line experiments. In fact, CagA positivity is >85% in Korea [27, 46].

ZO-1 was the first TJ-associated protein to be isolated in 1986 from the TJ-enriched membrane fraction [47]. It not only serves as a scaffold protein that links TJPs within the lipid bilayer to the actin cytoskeleton, but also acts as a signaling pathway in cell-to-cell communication [48]. In particular, ZO-1 translocates from TJs into the nucleus, resulting in a change in cell-to-cell contacts in sub-confluent or migrating epithelial cells. However, in confluent epithelial cells, ZO-1 binds to the nuclear transcription factor ZONAB (zonula occludens 1(ZO-1; ZO-1)-associated nucleic-acid binding protein) to block growth-stimulatory activity [33]. CagA+ Hp infection is associated with the induction of the pro-inflammatory cytokine interleukin (IL)-8 and morphological changes in human GC AGS cells [49]. As CagA is known to interact with ZO-1 at the bacterial attachment site, we sought to determine whether ZO-1 expression changed following CagA+ Hp infection.

In South Korea, unlike in Turkey, more than 80% of Hp isolated from patients with the gastroduodenal disease was CagA+ [27]. In the present study, 82.5% of patients were also infected with CagA+ Hp. Therefore, to demonstrate the correlation between the change in ZO-1 expression and GC in terms of Hp infection more clearly, we co-cultured Hp with the human GC cell line AGS (Fig. 6). As the IHC analysis showed that the ZO-1 expression pattern changed significantly in the cytosol, but not in the membrane or nucleus, we measured ZO-1 levels in the membrane, cytosol, and nucleus separately (Fig. 6a-c). During early infection with Hp, ZO-1 expression in the membrane showed no significant changes but increased significantly within 6 h and decreased significantly after 24 h in the cytosol. Although there was a similar pattern of increase within 6 h of infection in the nucleus, ZO-1 levels continued to increase even after 24 h compared to its initial amount (without any statistically significant difference), which was different from the pattern of ZO-1 expression in the cytosol.

A previous study showed that ZO-1 acts as a signaling molecule to regulate IL-8 expression via activation of the NF-κB (nuclear factor kappa B) pathway [50]. Furthermore, CagA+ Hp infection causes inflammation through many pathways, including IL-8 overexpression [51]. Therefore, based on current and prior data, we propose the following hypothesis: Hp-induced CagA stimuli may play a role in translocating ZO-1 from the membrane to the nucleus (Fig. 8). In the normal gastric epithelium, Hp-derived CagA disrupts the TJ in the membrane, causing the release of ZO-1

into the cytosol, followed by its degradation (Fig. 8a). In contrast, in the gastric epithelium of patients with GC, CagA stimulation increases ZO-1 expression and its translocation into the cytoplasm and nucleus, causing hyperactivation of the NF-κB pathway (Fig. 8b). Altogether, this action of ZO-1 might contribute to the GC microenvironment in NAT.

Our study had several limitations. First, to confirm the ZO-1 cascade in response to Hp infection, we used AGS cells, a human gastric adenocarcinoma cell line, instead of normal gastric cell lines. However, the IHC pattern of movement of the ZO-1 protein from the membrane to the cytosol was found in patients with GC but not in controls. Second, the number of patients with a long-term follow-up was relatively small. We attempted to reduce follow-up loss by calling the subjects and emphasizing the need for annual follow-up in EGD. However, the strategy was ineffective, mainly because the Korean government provides GC screening every two years by EGD or gastrography for all people aged over 40 years for free.

In conclusion, Hp infection affected each TJPs differently in the control subjects and patients with GC. Particularly, ZO-1 showed a higher expression in the Hp-positive GC group than in the Hp-negative GC group, which was restored following Hp eradication, suggesting that ZO-1 seemed to be related to gastric carcinogenesis caused by *H. pylori* infection. Moreover, the ZO-1 expression difference in each cellular compartments may imply the delivering function of ZO-1 in the gastric carcinogenesis

process caused by Hp infection.

Figure 8

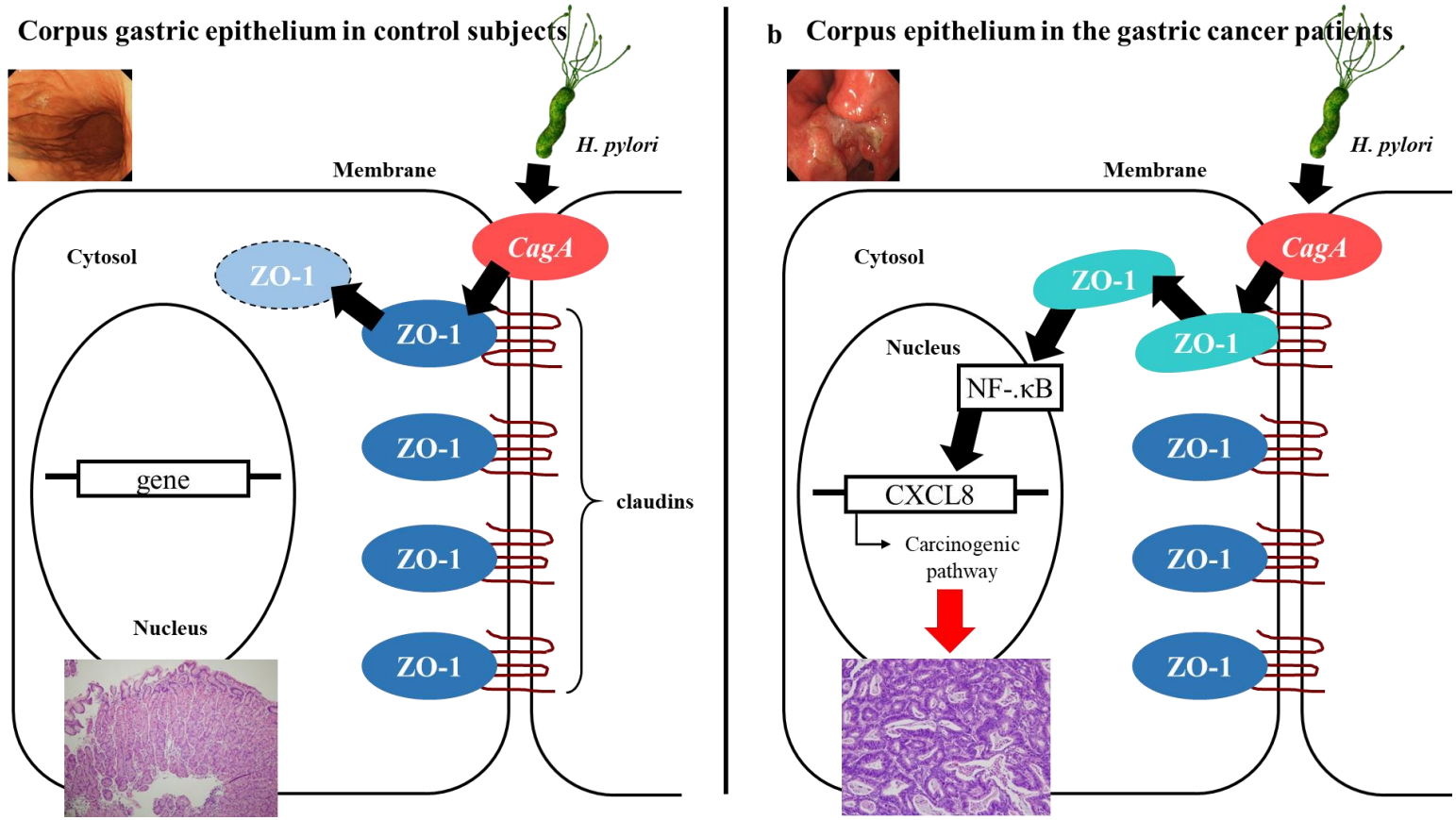

a Corpus gastric epithelium in control subjects
H. pylori
Membrane
Cytosol
CagA
ZO-1
ZO-1
ZO-1
ZO-1
ZO-1
claudins
gene
Nucleus
b Corpus epithelium in the gastric cancer patients
H. pylori
Membrane
Cytosol
CagA
ZO-1
ZO-1
Nucleus
NF-.κB
CXCL8
Carcinogenic pathway
ZO-1
ZO-1
ZO-1

Figure 8. Schematic of the proposed mechanism involved in *H. pylori* (Hp) infection-mediated changes in gene expression in gastric epithelial cells. ZO-1 may serve to transmit the *CagA* stimuli on the cell membrane to the nucleus through the cytoplasm in patients with GC. (a) *CagA*$^{+}$ Hp infection of normal gastric epithelium disrupts the tight junction causing the release of ZO-1 into cytosol followed by its degradation. (b) In the gastric epithelium of patients with GC, the *CagA* stimuli increase the level and translocation of ZO-1 into the cytoplasm and nucleus causing hyperactivation of the NF-κB pathway, thereby converting the cellular environment into a cancerous one. GC, gastric cancer.

5. References

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국문 초록

배경: 헬리코박터 파일로리의 감염은 위축성 위염, 장상피화생을 유발하며, 위암 발암물질로 분류된다. 밀착연접단백질은 위 상피세포의 방어 메커니즘에서 역할을 한다. 헬리코박터 파일로리가 위 상피에 집락을 형성하기 위해 분비하는 독성 인자는 밀착연접단백질을 파괴한다. 본 연구는 헬리코박터 파일로리 감염에 의한 위암 발생과 관련하여 밀착연접단백질의 역할을 평가하는 것을 목적으로 하였다. 또한, 밀착연접단백질에 대한 헬리코박터 파일로리 제균 치료의 영향을 위 종양 환경 측면에서 조사하였다.

방법: 총 510명의 피험자(대조군 284명, 위암 환자 226명)를 연구에 전진적으로 등록하였다. 정상 위 점막에서 정량적인 실시간 역전사 중합효소 연쇄반응(qRT-PCR)과 면역조직화학염색(IHC)을 이용하여 claudin-1, 2(CLDN-1, 2), occludin(OCLN), zonula ocludens-1(ZO-1)의 유전자 발현을 측정하였으며, 대조군에서의 발현은 헬리코박터 파일로리 감염 상태에 기초하여 위암 환자의 발현과 비교하였다. 헬리코박터 파일로리 제균치료에 따른 유전자 발현 변화도 평가됐다.

결과: 헬리코박터 파일로리 양성 대조군에서 ZO-1의 유전자 발현은 헬리코박터 파일로리 음성 대조군 (P = 0.006)보다 유의하게 낮은 반면, 헬리코박터 파일로리 양성 위암군에서는 헬리코박터 파일로리 음성 위군(P = 0.001)보다 높았다. 또한 헬리코박터 파일로리 양성 암군에서 ZO-1 유전자의 발현 증가는 헬리코박터 파일로리 제균치료 1년 이내에 헬리코박터 파일로리 음성 위암군 수준으로 감소하여 5년 이상 유지되었다. 또한, ZO-1에 대한 면역조직화학염색 결과는 유전자 발현 결과와 유사했다. 특히 헬리코박터 파일로리 제균 치료 (P = 0.040) 후 암환자의 세포질에서 ZO-1의 높은 면역조직화학염색강도 (P = 0.019)가 감소하였다. 각 세포 구획에서 ZO-1 면역조직화학염색 강도의 차이는 위선암 세포주에 대한 헬리코박터 파일로리 감염 실험을 통해서도 확인되었다.

결론: 헬리코박터 파일로리 감염은 밀착연접단백질의 발현에 영향을 미친다. 특히 헬리코박터 파일로리 양성 위암군에서 ZO-1의 발현이 높게 나타났으며, 헬리코박터 파일로리 제균치료 후 대조군 수준으로 회복되어 ZO-1이 헬리코박터 파일로리 감염에 의한 위암 발생과 관련이 있을 것으로 추측된다.