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ORIGINAL ARTICLE

Increased numbers of Foxp3-positive regulatory T cells in gastritis, peptic ulcer and gastric adenocarcinoma

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Abstract

AIM: To determine the number of regulatory T cells (Tregs) in gastric mucosa of patients with gastritis, peptic ulcers and gastric cancer.

METHODS: This study was a retrospective analysis of gastric antrum biopsy specimens from healthy controls (n = 22) and patients with gastritis (n = 30), peptic ulcer (n = 83), or gastric cancer (n = 32). Expression of CD4, CD25 and Foxp3 was determined by immunohistochemistry in three consecutive sections per sample.

RESULTS: Compared with healthy controls, there was an increased number of $CD25^+$ and $Foxp3^+$ cells in patients with gastritis (P = 0.004 and P = 0.008), peptic ulcer (P < 0.001 and P < 0.001), and gastric cancer (P < 0.001 and P < 0.001). The ratio of $CD25^+/CD4^+$ or $Foxp3^+/CD4^+$ cells was also significantly higher in all disease groups (P < 0.001, respectively). The number of $CD4^+$, $CD25^+$, and $Foxp3^+$ cells, and the ratio of $CD25^+/CD4^+$ and $Foxp3^+/CD4^+$ cells, were associated with the histological grade of the specimens, including acute inflammation, chronic inflammation, lymphoid follicle number, and *Helicobacter pylori* infection. The number of $CD4^+$, $CD25^+$ and $Foxp3^+$ cells, and the ratio of $CD25^+/CD4^+$ and $Foxp3^+/CD4^+$ cells, were negatively associated with intestinal metaplasia among gastritis (P < 0.001, P < 0.001, P < 0.001, P = 0.002 and P = 0.002) and peptic ulcer groups (P = 0.013, P = 0.004, P < 0.001, P = 0.040 and P = 0.003).

CONCLUSION: Tregs are positively associated with endoscopic findings of gastroduodenal diseases and histological grade but negatively associated with intestinal metaplasia in gastritis and peptic ulcer groups.

Key words: T regulatory cells; *Helicobacter pylori*; Gastroduodenal diseases; Intestinal metaplasia; Immunohistochemistry

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INTRODUCTION

In 1988, Correa delineated a multistep pathway from active chronic gastritis to atrophic gastritis, intestinal metaplasia (IM), dysplasia and finally gastric adenocarcinoma[1]. The presence of severe gastric atrophy, corpus-predominant gastritis, or IM is suggested to have an increased risk of cancer development[2]. The progression from IM to gastric cancer is supported by studies from animal models[3-5]. Hence, finding markers associated with these early histological changes could improve the prognosis and treatment of the disease. A common pathogen, *Helicobacter pylori* (*H. pylori*), is recognized as a primary etiologic agent in chronic gastritis. Persistent infection by *H. pylori* is associated with peptic ulceration and/or gastric malignancy[6,7]. In 1994, *H. pylori* was categorized as a group I carcinogen by the World Health Organization's International Agency for Research on Cancer owing to its epidemiologic association with gastric adenocarcinoma and gastric mucosa-associated lymphoid tissue lymphoma.

Regulatory T cells are a small population of T lymphocytes that may induce and maintain immunologic self-tolerance to prevent the development of autoimmune diseases[8-10]. Naturally occurring $CD4^+CD25^+$ Tregs frequently co-express cytotoxic T lymphocyte antigen 4 (CTLA-4), glucocorticoid-induced tumor necrosis factor receptor family-related gene (GITR), transforming growth factor β (TGF-β), and forkhead box p3 (Foxp3)[11]. Using a mouse model and other methods, Foxp3 has been identified as a crucial transcription factor that regulates the development of $CD4^+CD25^+$ Tregs function[12,13] and thus may serve as a reliable marker for $CD4^+CD25^+$ Tregs.

The roles of $CD4^+CD25^+$ Tregs in suppressing the immune response to *H. pylori* have been reported recently in several studies[14-17]. $CD4^+CD25^+$ Tregs reduce proinflammatory cytokine production and *H. pylori*-induced gastritis in mice[17-19]. Depletion of Tregs in infected mice results in increased gastric inflammation and reduced colonization by *H. pylori*[16]. Furthermore, there is a higher proportion of $Foxp3^+$ Tregs in tumor-infiltrating lymphocytes in the gastric mucosa of cancers as compared with normal gastric mucosa[15,20,21]. Apart from its positive correlation with tumor specimens, the number of $Foxp3^+$ Tregs is also elevated in *H. pylori*-associated gastritis[21].

These results suggest that Tregs play a pivotal role in persistent *H. pylori* colonization in gastric mucosa, which may then lead to development of gastric cancers. However, the relationship between $CD4^+CD25^+$ Tregs and precancerous lesions of the stomach remain unclear. In the present study, we investigated the association between Tregs, histological grade and clinical sequelae in the context of the multistep progression to gastric cancer by examining histological profiles in healthy controls and patients with chronic gastritis, peptic ulcers or adenocarcinoma. We retrospectively analyzed the number of $CD4^+$, $CD25^+$, or $Foxp3^+$ T cells by immunohistochemistry in the antrum mucosa and examined the relationship between marker expression and precancerous lesions.

MATERIALS AND METHODS

Study subjects

Between January 2008 and June 2009, a total of 4563 patients were examined by upper gastroduodenal endoscopy at Ton-Yen General Hospital, Hsinchu, Taiwan. Among them, 256 patients who had gastric antral biopsy for histological diagnosis were considered for this retrospective study. After medical chart review for these cases, we excluded patients with chronic heart, lung, liver or kidney diseases, patients with a history of prior gastric surgery or anti-*H. pylori* eradication therapy, and patients taking non-steroidal anti-inflammatory drugs within one week prior to endoscopy. This gave us a pool of 135 enrolled non-cancer subjects, including 113 symptomatic patients from our out-patient department (30 patients with gastritis and 83 patients with peptic ulcers) and 22 healthy controls (asymptomatic cases undergoing physical check up). In addition, 32 patients with histologically proven gastric adenocarcinoma diagnosed between 2004 and 2008 were enrolled as the gastric cancer group. The Institutional Review Board of Ton-Yen General Hospital approved this study.

Demographic factors reported for the 167 patients included age and gender. Endoscopic and histological data were reviewed exclusively by Guan-Ying Tseng and Hsiao-Bai Yang, respectively. We defined an ulcer as a circumscribed mucosal break (> 5 mm in diameter, with apparent depth) in the stomach or duodenum, covered with exudates. *H. pylori* status was assessed by histology with hematoxylin and eosin stain and by rapid urease test on biopsies using Pronto Dry (Medical Instruments Corp., Solothurn, Switzerland). Patient was considered *H. pylori* positive if results by one or both diagnostic methods were positive and *H. pylori* negative if results by both methods were negative.

Histological assessment

For diagnosis, the gastric antrum biopsy specimens were obtained from the non-ulcer region adjacent to the ulcer site of the non-cancer subjects, or taken from the tumor region of the cancer patients, fixed in 10% formalin buffer, and stained with hematoxylin-eosin. The histological findings were graded by Yang HB according to the updated Sydney System[22,23]. The parameters included: (1) chronic inflammatory score (CIS; range 1-3 for mild, moderate, or severe lymphocytic and plasma cell infiltration); (2) acute inflammatory score (AIS; range 0-3 for absence or degree of neutrophil infiltration in lamina propia, epithelia, crypt or gland lumens); (3) lymphoid follicle number (LF; range 0-7 for total LF numbers in one slide); (4) *H. pylori* density (HPD; range 0-5); (5) IM (0, absence or < 5% of the upper third of gastric mucosa; 1-2, goblet cells found in the upper third or upper half of gastric mucosa; 3, goblet cells and paneth cells found, also considered complete IM); and (6) atrophic change (AT; 0-3 for absence, mild, moderate or severe).

Table 1 Clinical characteristics of study subjects classified on endoscopy (n = 167)

	Healthy controls (n = 22)	Gastritis (n = 30)	Peptic ulcer (n = 83)	Gastric cancer (n = 32)
Age (yr, mean ± SD)	46.4 ± 14.3	55.4 ± 15.0[a]	61.7 ± 14.9[a]	67.9 ± 13.9[a]
Gender				
Male	13	17	53	30
Female	9	13	30	2
Helicobacter pylori infection				
Negative	22	20	33	21
Positive	0	10	50[b]	11

[a]P < 0.05, each disease *vs* healthy controls; [b]P = 0.02, peptic ulcer *vs* gastritis. Data were analyzed using the Mann-Whitney U test, χ^2 test and Fisher's exact test.

Table 2 Histological grading of non-cancer subjects (n = 135)

	Healthy controls	Gastritis	Peptic ulcer
Chronic inflammatory score (1/2/3)[a]	21/1/0	5/5/20	1/3/79
Acute inflammatory score (0/1/2/3)[a]	22/0/0/0	5/6/12/7	3/1/21/58
Lymphoid follicle number (0/1-2/≥ 3)[a]	21/1/0	17/9/4	39/28/16
Helicobacter pylori density (0/1/2/3/4/5)[a]	22/0/0/0/0/0	20/2/1/3/3/1	33/2/2/8/24/14
Intestinal metaplasia score (0/1/2/3)[a]	22/0/0/0	10/2/4/14	22/9/20/32
Atrophy score (0/1/2/3)[a]	19/1/1/1	8/9/11/2	14/40/20/9

[a]P < 0.001. Data were analyzed using Kruskal-Wallis H test.

Immunohistochemical staining

Consecutive paraffin-embedded serial sections of gastric biopsies (4 μm) were deparaffinized and rehydrated with xylene and ethanol for single staining of CD25, CD4, and Foxp3. Antigen retrieval was performed in a 95 ℃ water bath using Tris-ethylene diamine tetraacetic acid (EDTA) buffer (10 mmol/L Tris, pH 9.0, 1 mmol/L EDTA, 0.05% w/v Tween 20) for 10 min in CD4, or sodium citrate buffer (10 mmol/L sodium citrate, pH 6.0, 0.05% Tween 20) for 20 min in Foxp3 or 10 min in CD25. Sections were cooled for 20 min, and then endogenous peroxidase was blocked using 3% H_2O_2 for 10 min. Sections were incubated with blocking buffer (1% bovine serum albumin in phosphate-buffered solution) for 1 h at room temperature and then incubated with primary antibody for 2 h at room temperature. The working dilution of the primary antibody was 1:200 for mouse anti-human CD4 (clone 4B12; Novocastra, Newcastle, United Kingdom), 1:400 for mouse anti-human CD25 (clone 4C9; Novocastra), and 1:50 for mouse anti-human Foxp3 (clone 236A/E7; Santa Cruz Biotechnology, Santa Cruz, CA). Sections were stained using NovoLink Polymer Detection Systems (Novocastra) followed by 3,3'-diaminobenzidine (Sigma) for 5 min, and counterstained with hematoxylin (Sigma). Lymph node tissue was used as a positive control. Additional sections were processed without primary antibody as a negative control.

Quantification of the number of immunostained cells was conducted in three consecutive single-stained sections in order of CD25, CD4 and Foxp3. For enumeration of $CD4^+$ T cells, lymphocytes infiltrating the non-IM region were counted at least ten high-powered fields (HPF; 400 ×) from each CD4-stained section. Selected regions of each CD4-stained section were then retraced in the corresponding CD25- and Foxp3-stained sections to enumerate $CD25^+$ and $Foxp3^+$ cells. For each sample, the mean ratio of $CD25^+/CD4^+$ and $Foxp3^+/CD4^+$ cells was also calculated. Results were expressed as the median value and inter-quartile range of all tested patients in each group.

Statistical analysis

χ^2 test or Fisher's exact test was performed to compare groups with categorical variables. Continuous variables were compared between groups using a Mann-Whitney U test or Kruskal-Wallis H test. Correlations between the number of $CD25^+$ cells and $Foxp3^+$ cells were established based on Spearman's rank correlation analysis, and the ratio of $CD25^+/CD4^+$ cells and of $Foxp3^+/CD4^+$ cells was calculated. Statistical tests were two-sided, with $P < 0.05$ considered statistically significant. Statistical analysis was performed using SPSS version 12.0 (SPSS Inc., Chicago, IL).

RESULTS

Patients

The demographic and clinical characteristics of the 167 study subjects are shown in Table 1. On endoscopy, there were 22 normal, 30 gastritis, 83 peptic ulcer and 32 gastric cancer cases. Subjects ranged in age from 27 years to 95 years (59.7 ± 15.9 years). The mean age of the healthy controls (46.4 ± 14.3 years) was substantially lower than that of disease groups. A much higher male ratio was found in the gastric cancer group (93.8%) than in the healthy control group (59.1%, P = 0.004). The prevalence of *H. pylori* infection was significantly higher in the peptic ulcer group (60.2%) than in the gastritis group (33.3%, P = 0.020).

The histological grading of non-cancer subjects (n = 135) is shown in Table 2. The scores of the six parameters, including AIS, CIS, LF, HPD, IM and AT, were significantly associated with the severity of gastroduodenal diseases as determined endoscopically (AIS, $P < 0.001$; CIS, $P < 0.001$; LF: $P < 0.001$; HPD: $P < 0.001$; IM: $P < 0.001$; AT: $P < 0.001$). The number of $CD25^+$, $CD4^+$, or $Foxp3^+$ cells and the ratio of $CD25^+/CD4^+$ and of $Foxp3^+/CD4^+$ had no correlation with respect to age or sex in non-cancer patients.

Number of $Foxp3^+$ Tregs and ratio of $Foxp3^+/CD4^+$ Tregs are higher in gastroduodenal diseases

To enumerate Tregs present in the gastric mucosa of gastroduodenal diseases, we determined the number of

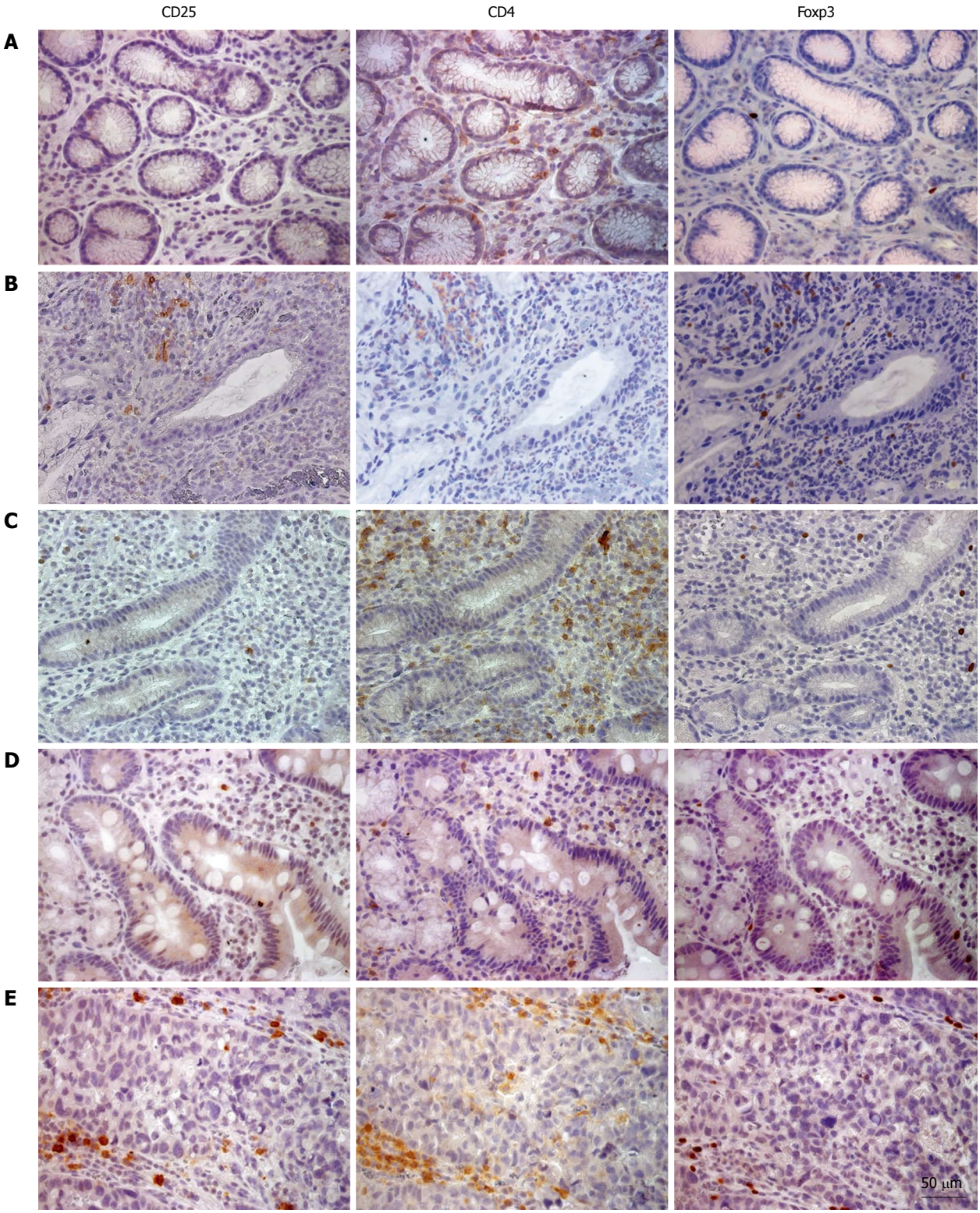

Figure 1 Immunohistochemistry of CD25, CD4, and Foxp3 in healthy controls, acute gastritis, chronic gastritis, intestinal metaplasia, and gastric cancer (original magnification, 400 ×). Immunohistochemistry of CD25 (left), CD4 (middle), and Foxp3 (right) in gastric mucosa from A: Healthy controls; B: Acute gastritis; C: Chronic gastritis; D: Intestinal metaplasia; E: Gastric cancer. CD4 and CD25 staining (brown) were found on the surface of T lymphocytes, and Foxp3 staining (brown) was located in the nucleus of T lymphocytes in lamina propria around glands.

$CD25^+$ cells, $CD4^+$ cells and $Foxp3^+$ cells in three consecutive sections. The expression of CD4 and CD25 was found on the surface of lymphocytes, whereas expression of Foxp3 was seen in the nucleus of lymphocytes (Figure 1).

The number of $CD25^+$ cells was significantly correlated with that of $Foxp3^+$ cells (Figure 2A; $r = 0.876$, $P < 0.001$). Furthermore, the ratio of $CD25^+/CD4^+$ was also significantly correlated with that of $Foxp3^+/CD4^+$ (Figure 2B; r

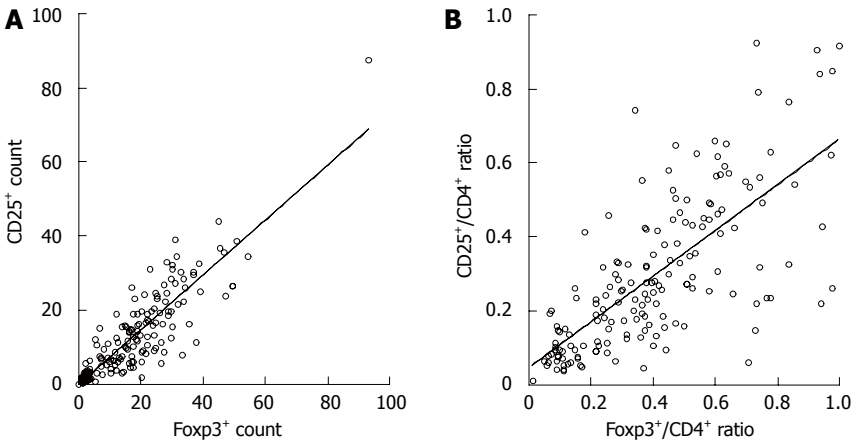

Figure 2 CD25 expression correlates with Foxp3 expression in human T lymphocytes. A: Correlation between $CD25^+$ and $Foxp3^+$ cells from 22 healthy controls and 30 gastritis, 83 peptic ulcer, and 32 gastric cancer patients; B: Correlation between the ratio of $CD25^+/CD4^+$ and of $Foxp3^+/CD4^+$ cells. Data were analyzed by Spearman's rank correlation.

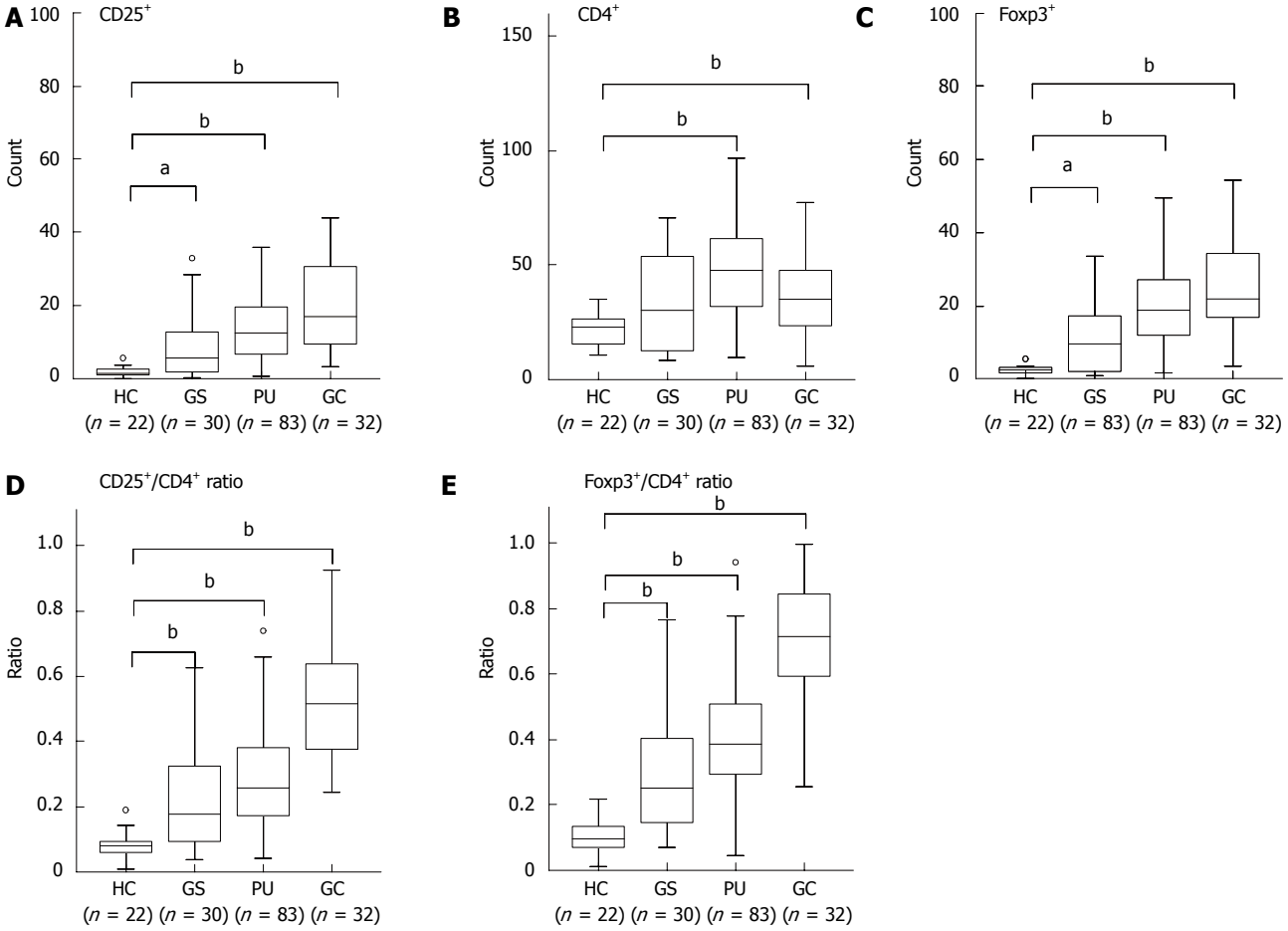

Figure 3 Box plots for the number of $CD25^+$, $CD4^+$, and $Foxp3^+$ cells, and the ratio of $CD25^+/CD4^+$ and $Foxp3^+/CD4^+$ in healthy controls, gastritis, peptic ulcer, and gastric cancer. Box plots for A: The number of $CD25^+$ cells; B: The number of $CD4^+$ cells; C: The number of $Foxp3^+$ cells; D: The ratio of $CD25^+/CD4^+$; E: The ratio of $Foxp3^+/CD4^+$ in non-intestinal metaplasia areas of antral gastric mucosa from healthy controls (HC) and patients with gastritis (GS), peptic ulcer (PU), and gastric cancer (GC). Data were analyzed by the Mann-Whitney *U* test. $^aP < 0.05$ and $^bP < 0.001$.

= 0.717, $P < 0.001$), revealing a strong positive correlation between CD25 and Foxp3.

Peptic ulcer and gastric cancer patients had a significantly higher number of $CD4^+$ cells than the healthy controls (Figure 3B; $P = 0.001$). The number of $CD25^+$ and $Foxp3^+$ cells was lowest in the healthy controls and increased progressively in the more severe disease groups (Figure 3A and 3C; gastritis *vs* healthy control: $CD25^+$, $P = 0.004$ and $Foxp3^+$, $P = 0.008$; peptic ulcer *vs* healthy control: $CD25^+$, $P < 0.001$ and $Foxp3^+$, $P < 0.001$; gastric

cancer *vs* healthy control: $CD25^+$, $P < 0.001$ and $Foxp3^+$, $P < 0.001$; peptic ulcer *vs* gastritis: $CD25^+$, $P = 0.001$ and $Foxp3^+$, $P < 0.001$; gastric cancer *vs* gastritis: $CD25^+$, $P < 0.001$ and $Foxp3^+$, $P < 0.001$; gastric cancer *vs* peptic ulcer: $CD25^+$, $P = 0.018$ and $Foxp3^+$, $P = 0.044$). In addition, the ratio of $CD25^+/CD4^+$ and of $Foxp3^+/CD4^+$ was increased progressively in the more severe disease groups (Figure 3D and 3E; gastritis *vs* healthy control: $CD25^+/CD4^+$, $P < 0.001$ and $Foxp3^+/CD4^+$, $P < 0.001$; peptic ulcer *vs* healthy control: $CD25^+/CD4^+$, $P < 0.001$ and $Foxp3^+/CD4^+$, $P < 0.001$; gastric cancer *vs* healthy control: $CD25^+/CD4^+$, $P < 0.001$ and $Foxp3^+/CD4^+$, $P < 0.001$; peptic ulcer *vs* gastritis: $CD25^+/CD4^+$, $P = 0.012$ and $Foxp3^+/CD4^+$, $P = 0.002$; gastric cancer *vs* gastritis: $CD25^+/CD4^+$, $P < 0.001$ and $Foxp3^+/CD4^+$, $P < 0.001$; gastric cancer *vs* peptic ulcer: $CD25^+/CD4^+$, $P < 0.001$ and $Foxp3^+/CD4^+$, $P < 0.001$). Together, these results suggested that Tregs were associated with gastritis, peptic ulcer and gastric cancer.

Foxp3$^+$/CD4$^+$ ratio is positively associated with degree of inflammation, number of LFs and H. pylori infection

We next analyzed the relationship between Tregs and histological findings in non-cancer subjects ($n = 135$; Figure 4). The ratios $CD25^+/CD4^+$ and $Foxp3^+/CD4^+$ were significantly higher with a higher degree of chronic and acute inflammation (CIS 3, AIS 3-4) than with a lower degree of chronic and acute inflammation (CIS 1-2, AIS 0-1) (Figure 4A and B; CIS 3 *vs* 1: $CD25^+/CD4^+$, $P < 0.001$ and $Foxp3^+/CD4^+$, $P < 0.001$; AIS 2 *vs* 0: $CD25^+/CD4^+$, $P < 0.001$ and $Foxp3^+/CD4^+$, $P < 0.001$; AIS 3 *vs* 0: $CD25^+/CD4^+$, $P < 0.001$ and $Foxp3^+/CD4^+$, $P < 0.001$). The ratios were also significantly higher in patients with higher numbers of LFs (Figure 4C; LF 1-2 *vs* 0: $CD25^+/CD4^+$, $P = 0.005$ and $Foxp3^+/CD4^+$, $P < 0.001$; LF $\geqslant$ 3 *vs* 0: $CD25^+/CD4^+$, $P = 0.013$ and $Foxp3^+/CD4^+$, $P < 0.001$). Moreover, the ratios were significantly higher in gastric mucosa of patients with *H. pylori* infection than in patients without infection (Figure 4D; Hp+ *vs* Hp-: $CD25^+/CD4^+$, $P = 0.001$ and $Foxp3^+/CD4^+$, $P < 0.001$). No significant association was found between Treg number and IM or AT. Together, the presence of Tregs was positively associated with inflammation, LFs, and *H. pylori* infection.

CD25$^+$/CD4$^+$ and Foxp3$^+$/CD4$^+$ ratios are lower in gastritis and peptic ulcer patients with IM than in those without IM

We compared Tregs in gastric mucosa of gastritis and peptic ulcer patients with and without IM (Figure 5). There were significantly fewer $CD4^+$, $CD25^+$, or $Foxp3^+$ cells in gastritis patients with IM than in those without (Figure 5A-C; IM+ *vs* IM-: $CD25^+$, $P < 0.001$; $CD4^+$, $P < 0.001$; $Foxp3^+$, $P < 0.001$). The ratios $CD25^+/CD4^+$ and $Foxp3^+/CD4^+$ were also significantly lower in gastritis patients with IM (Figure 5D and E; IM+ *vs* IM-: $CD25^+/CD4^+$, $P = 0.002$ and $Foxp3^+/CD4^+$, $P = 0.002$). A similar profile was observed in the peptic ulcer patients (Figure 5A-E; IM+ *vs* IM-: $CD25^+$, $P = 0.004$; $CD4^+$, $P = 0.013$; $Foxp3^+$, $P < 0.001$; $CD25^+/CD4^+$, $P = 0.040$; $Foxp3^+/CD4^+$, $P = 0.003$). Thus, IM was associated a reduced number of Tregs in patients with gastroduodenal diseases.

DISCUSSION

Most studies investigating the role of Tregs in gastroduodenal diseases have used fluorescence-activated cell sorting (FACS) to directly identify $CD4^+CD25^+$ Tregs in lymphocytes from peripheral blood or lamina propria[15,24-27]. Recently, Perrone *et al*[28] investigated tumor-infiltrating $Foxp3^+$ Tregs in radically resected (R0) gastric cancer by immunohistochemistry, and suggested that $Foxp3^+$ Tregs may be a novel in situ marker for identifying high-risk gastric cancer patients. In the present study, we investigated the expression of two markers for Tregs, namely Foxp3 and CD25, in relation to CD4 expression by immunohistocytochemistry in three consecutive sections of a panel of normal and diseased specimens. We confirmed a significantly high correlation between the numbers of $CD25^+$ and $Foxp3^+$ T lymphocytes, and between the $CD25^+/CD4^+$ and $Foxp3^+/CD4^+$ ratios.

The retrospective study of 135 non-cancer subjects in a single clinical institute demonstrates a positive correlation between the number of $Foxp3^+$ Tregs and histological grade, including AIS, CIS, and LFs. Furthermore, the presence of *H. pylori* was positively associated with the number of Tregs. These results suggest that gastritis can be induced by *H. pylori* infection, which contributes to the occurrence of lymphoid tissue hyperplasia and recruits Tregs. This result agrees with previous evidences for a positive link between *H. pylori*-induced gastritis and recruitment of $Foxp3^+$ Tregs[21,29-31]. It is interesting that Tregs isolated from gastric adenocarcinoma patients are able to suppress *H. pylori*-induced T cell responses *in vitro*, supporting the role of Tregs in facilitating persistent *H. pylori* colonization and hence gastric carcinogenesis[15].

The number of Tregs in our gastric cancer group was significantly higher than that of the peptic ulcer group or the gastritis group. Additionally, the number of $Foxp3^+$ Tregs and the $Foxp3^+/CD4^+$ ratio in antral mucosa were increased progressively from healthy controls to gastritis patients to gastric cancer patients. Interestingly, this sequential change corresponds to the sequential clinical sequelae of gastric carcinogenesis, or Correa's cascade[32,33]. Increasing evidence has indeed demonstrated the presence of significantly elevated numbers of $CD4^+$ Tregs in various types of cancers[15,24,34-39]. It has been hypothesized that Tregs suppress anti-tumor immunity, which leads to immune tolerance of tumor cells[40]. Recently, advanced tumor/node/metastasis (TNM) stage in gastric cancer patients was found associated with elevated expression of Foxp3 in tumor-infiltrating Treg cells[25]. Of note, cyclooxygenase-2/prostaglandin E_2 production might be involved in Treg-based immune suppression, which may have new implications for gastric cancer therapy.

We further examined the distribution of Tregs in patients with IM, a precancerous lesion of the intestinal-type gastric adenocarcinoma[32,33,41-46]. Notably, gastritis and peptic ulcer patients with IM had significantly fewer

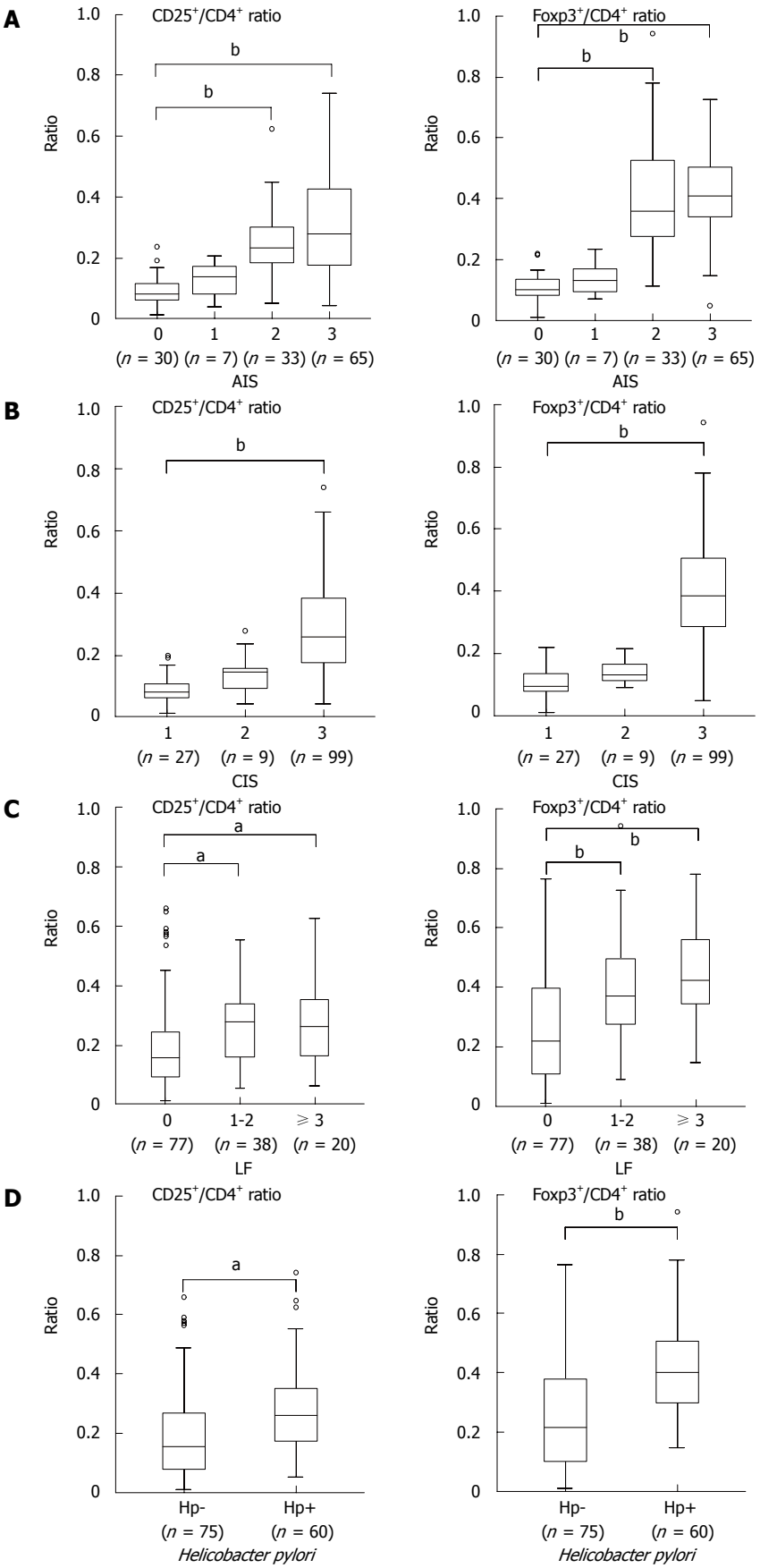

Figure 4 Box plots for the ratio of $CD25^+/CD4^+$ and of $Foxp3^+/CD4^+$ according to acute inflammatory score, chronic inflammatory score, lymphoid follicle number, and *Helicobacter pylori* infection. Box plots for the ratio of $CD25^+/CD4^+$ and of $Foxp3^+/CD4^+$ according to A: Acute inflammatory score (AIS); B: Chronic inflammatory score (CIS); C: Lymphoid follicle number (LF); D: *Helicobacter pylori* infection. Data were analyzed using the Mann-Whitney *U* test. $^aP < 0.05$ and $^bP < 0.001$. HP: *Helicobacter pylori*.

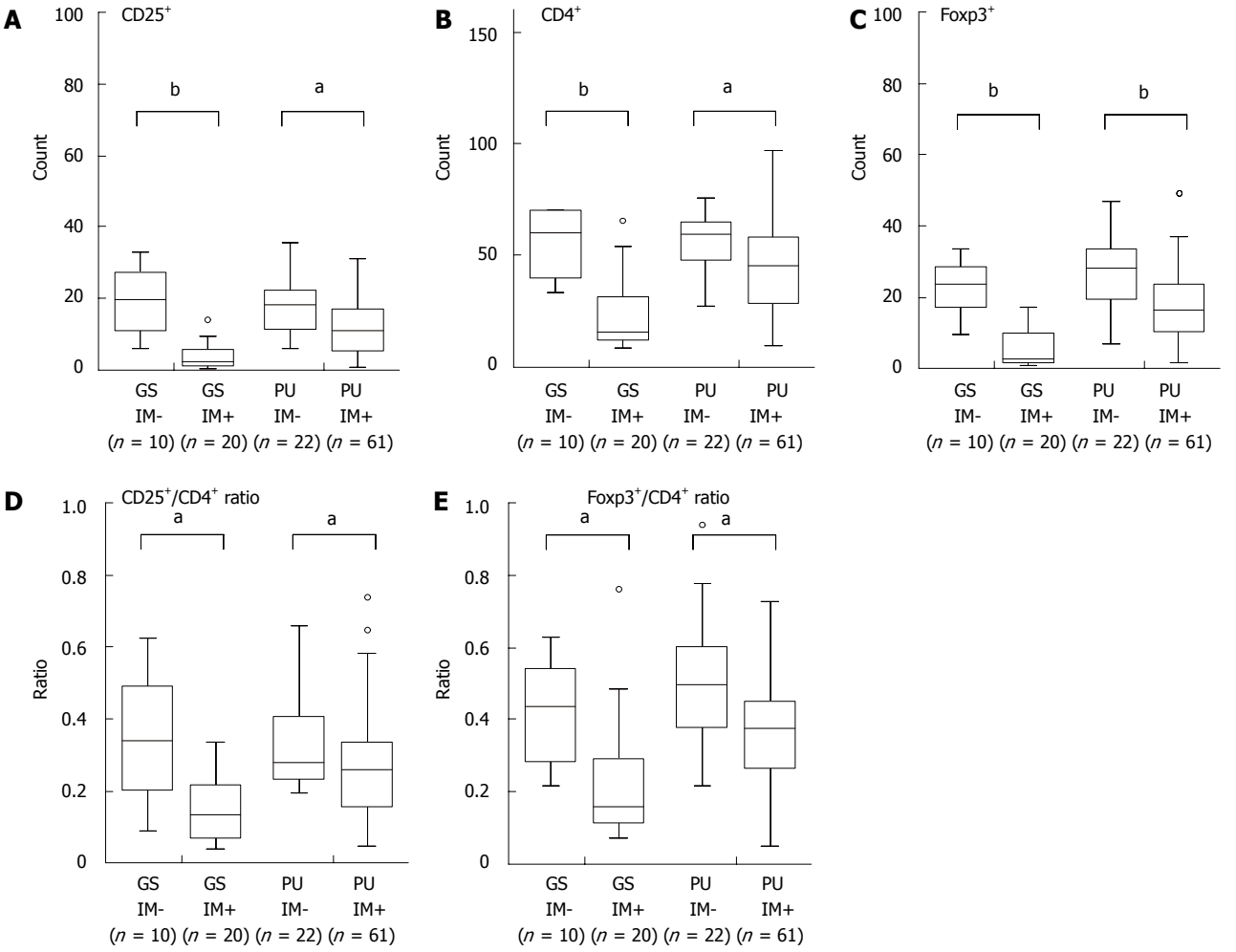

Figure 5 Box plots for the number of CD25$^+$, CD4$^+$, and Foxp3$^+$ cells, and the ratio of CD25$^+$/CD4$^+$ and of Foxp3$^+$/CD4$^+$ in gastritis and peptic ulcer patients with and without intestinal metaplasia. Box plots for A: The number of CD25$^+$ cells; B: The number of CD4$^+$ cells; C: The number of Foxp3$^+$ cells; D: The ratio of CD25$^+$/CD4$^+$; E: The ratio of Foxp3$^+$/CD4$^+$ in non-intestinal metaplasia (IM) areas of antral gastric mucosa from gastritis (GS) and peptic ulcer (PU) patients with and without IM. Data were analyzed using the Mann-Whitney *U* test. $^aP < 0.05$ and $^bP < 0.001$.

Tregs than patients without IM. Previous studies showed an increased level of TGF-β as well as Foxp3$^+$ Tregs in patients with *H. pylori*-induced gastritis[30], which supports the notion that TGF-β is a critical differentiation factor for Treg cells within a local microenvironment[47,48]. It is interesting that Mutoh *et al*[49] recently reported a lower level of TGF-β expression in the IM subjects, which was comparable to that in the normal tissues as opposed to a 6.5-fold increase in gastric carcinoma. Such a lower level of TGF-β expression might contribute to the lesser numbers of Foxp3$^+$ Tregs in GS/PU patients with IM. However, further investigations will be needed to explore this possibility.

There are limitations in our study, all stemming from its retrospective design. First, there is a possible selection bias, even though at least 30 cases were included in each disease group. Second, it is unlikely to rule out the remote NSAIDs used in our enrolled subjects based on reviewing their medical records. The potential effect of NSAIDs on Tregs in our patients thus might be undetermined. Third, it was not possible to use a functional assay for Treg expression to address detailed mechanisms.

In conclusion, the number of Tregs is elevated and positively correlated with histological grade of chronic gastritis, atrophic gastritis and adenocarcinoma, but is decreased and negatively correlated with histological grade of IM.

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COMMENTS

Background

Helicobacter pylori (*H. pylori*) are associated with chronic gastroduodenal inflammation, atrophy, intestinal metaplasia, and gastric cancer. The role of CD4$^+$CD25$^+$ Tregs in suppressing the immune response to *H. pylori*, and increased populations of CD4$^+$CD25$^+$ T regulatory cells (Tregs) link to the *H. pylori*-infected pathologies have been reported.

Research frontiers

CD4$^+$CD25$^+$Foxp3$^+$ Tregs are expressed in various types of cancers including gastric cancer. Tregs is also elevated in *H. pylori*-associated gastritis. However, the relationship between Tregs with precancerous lesions of the stomach remains unclear. In this study, the authors analyzed the number of CD4$^+$, CD25$^+$,

or Foxp3$^+$ T cells by immunohistochemistry in the antrum mucosa and examined the relationship between marker expression and precancerous lesions.

Innovations and breakthroughs

Recent reports have highlighted the association between Tregs and precancerous lesions of the stomach. In this study, the authors report that the number of Tregs is positively correlated with histological grade of chronic gastritis, atrophic gastritis and adenocarcinoma, and negatively correlated with histological grade of IM, suggesting that Tregs may play a role in the progression of gastric cancer.

Applications

Foxp3$^+$ Tregs may be a novel *in situ* marker for identifying gastric cancer patients.

Terminology

Tregs are a small population of T lymphocytes that may induce and maintain immunologic self-tolerance to prevent the development of autoimmune diseases. Forkhead box p3 (Foxp3) has been identified as a crucial transcription factor that regulates the development of CD4$^+$CD25$^+$ Tregs function and thus may serve as a reliable marker for CD4$^+$CD25$^+$ Tregs.

Peer review

This manuscript is a well written contribution and the data merit publication. Significant patient material has been collected and relevant data have been retrieved. The immuno-based analysis is well carried out and justifies the conclusions reached by the authors.

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