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TLR4 and TLR2 Expression in Biopsy Specimens from Antral and Corporal Stomach Zones in *Helicobacter pylori* Infections

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Abstract

Background: It is not yet known which types of Toll-like receptors (TLRs) are most effective in *Helicobacter pylori* (*H. pylori*) recognition. It is also not known which gastric zones have the most prominent roles in TLR-mediated bacterial recognition. The aim of this work was to analyze the expression of TLR2 and TLR4 in biopsy specimens from *H. pylori*-infected patients.

Methods: Thirty-eight patients with gastrointestinal disorders were divided into four groups in this study. The groups were: (A) *H. pylori* infection and peptic ulcer (n=15), (B) peptic ulcer only (n=5), (C) *H. pylori* infection only (n=10) and (D) control, with neither *H. pylori* infection nor peptic ulcer (n=8). Biopsy specimens from sites of redness or atrophic mucosa from gastric antrum and body in patients with gastritis were collected. RNAs from the antrum and body specimens were isolated. TLR2 and TLR4 mRNA expression was assessed by RT-PCR and quantified as densitometric ratios of TLR2 and TLR4/β-actin mRNA.

Results: In the antral zones of *H. pylori*-infected patients (Groups A and C) TLR2 and TLR4 expression was significantly greater than in uninfected patients (Groups B and D) regardless of peptic ulcers ($p < 0.05$). In the gastric body samples TLR2 expression was significantly greater in Group C (*H. pylori* infection only) than in Group B (peptic ulcer only) and TLR4 expression was significantly greater in group A (*H. pylori* infection and peptic ulcer) than in Group B (peptic ulcer only) ($p < 0.05$). No significant differences in expression of TLR4 and TLR2 were observed between samples from the antrum and body in same groups.

Conclusions: We conclude that *H. pylori* infection leads to significant increase in TLR2 and TLR4 molecules expression in antral region related to the control group. Considering the stimulatory effect of *H. pylori* on TLRs expression in the gastric tissue, we assume that colonization of *H. pylori* infection might occurs more in the gastric antral region than in the gastric body.

Keywords: *Helicobacter pylori*, Peptic ulcer, TLR2, TLR4, Toll-like receptors

Introduction

Helicobacter pylori (*H. pylori*) is a microaerophilic S-shaped or curved gram-negative bacteria that is one of the most common causes of gastric infections worldwide (1). Since 1983, when Drs. J. Robin Warren and Barry Marshall noted this causality, extensive research has further explored and explained the nature of this connection. In 1994, *H. pylori* was recognized as the main cause of gastroduodenal peptic ulcers, and more recently the International Agency for Research on Cancer named this bacteria a group I human carcinogen

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for gastric adenocarcinoma (2). In the United States, this bacterium is known to be the causative agent in 90% of duodenal and 70% of peptic ulcers (3). Several prospective case-control serological studies detected a six-fold greater risk for gastric cancer in infected than in uninfected patients, and a 50% presumable risk for all gastric cancers (2, 4, 5). The clinical consequences are different from asymptomatic gastritis to peptic ulceration and gastric malignancy, depending on host inflammatory responses (6).

Although the mechanism of *H. pylori* infection is not well recognized, the first step is bacterial binding to gastric epithelial cells (GECs) (7). It is presumed that there is a correlation between host Toll-like receptor (TLR) molecules and *H. pylori*-binding to gastric mucosa. It is not yet known which types of TLRs or which parts of the stomach are involved in the binding process (8). *H. pylori* bind to CD14 receptor cell molecules via the LPS-binding protein. After the accumulation of LPS, lipopolysaccharide-binding protein (LBP) and CD14 on the surface of the host cell, the TLR molecules close to CD14 (9) transduce intracellular signals and activate cytokine promoter genes that include IFN-γ, IL-1β, IL-6, and IL-8 (8-11). In addition, inflammatory chemokines and co-stimulatory molecules intensify inflammation and tissue necrosis (12, 13). It can be concluded that TLR stimulation leads to both innate and acquired immune responses.

The clinical aspect of *H. pylori* infection is mediated via the interaction between host TLRs and *H. pylori.* As the bacterium is minimally invasive, it seems that the clinical outcome is determined by the interplay between *H. pylori* virulence factors, host gastric mucosal factors, and the environment (14). The first step in *H. pylori* infection is bacterial binding to GECs, so it can be deduced that TLRs play a basic role in recognition of *H. pylori* by these cells (4, 5). In spite of numerous studies addressing the interaction between *H. pylori* and TLRs, a specific role for TLRs in such interactions has yet to be determined (15, 16).

Coupling TLRs with their own ligand activates NF-kB, resulting in expression of the inflammatory cytokines IL-1 and TNF-α (10). In addition, this signaling pathway results in cellular sustenance and proliferation (17). This result suggests a connection between this and tumor growth in gastro-intestinal mucosa. Furthermore, single or simultaneous body and antrum sampling is always a topic of debate. According to the Sydney classifications the biopsy locale is of utmost significance for the correct detection of gastric atrophy (18). For that reason in moderate and intense gastritis Hung-Chieg Lan suggests that biopsies should include body samples to avoid false-negative results (19).

The goal of this study was to investigate semiquantitative expression of TLR2 and TLR4 in gastric biopsies. Patients with upper abdominal pain and maldigestion were selected for this research. It is not yet known which gastric regions have the most prominent roles in bacterial recognition by TLR molecules. We compared the expression levels of TLR2 and TLR4 in antrum and body biopsy specimens of H. pylori-infected patients.

Materials and Methods

Sample collection

The sample pool included 38 patients (age: 44.1± 17.7) with digestive disorders. The antrum and the body specimens were collected from gastric tissue through endoscopic operations for pathology and gene expression studies. The specimens were chosen from regions with inflammation, redness, and tissue necrosis. Patients were divided into four groups. Patients in group A (n=15) had peptic ulcers and *H. pylori*-positive carry the cag A and vac A genes. Patients in group B (n=5) had peptic ulcers and were *H. pylori*-negative carry the cag A and vac A genes. Patients in group C (n=10) had no peptic ulcers and *H. pylori*-positive carry the cag A and vac A genes. Patients in group D (n=8) were peptic ulcer and *H. pylori* carry the cag A and vac A genes negative.

Patients with gastrointestinal hemorrhages, metabolic disorders, immunosuppression, malignancies, aspirin and non-steroidal anti-inflammatory drug users, and patients with special food habits such as alcoholic or fizzy drink users were excluded from this study (20, 21). *H. pylori* with cag A (cytotoxin associated gene) and vac A (vacuolating cytotoxin gene) are more virulent than strains that do not have these genes (17); therefore, patients who were infected with H.pylori that contained both these genes were selected for this study.

Tissue processing

After the endoscopic antral and body biopsies, tissues were divided in half; one half was used for histopathological evaluations and the other for RNA extractions. The tissues were fixed in 10%

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formaldehyde, embedded in paraffin, and cut into 4-5 µm sections. The sections were stained with hematoxylin and eosin (H&E) and May Grünwald Giemsa. For each tissue, five sections were analyzed and their average scores calculated. The tissues were evaluated by two pathologists in a double-blind study according to the Updated Sydney Classification System (USCS), for the presence or absence of *H. pylori*, density of *H. pylori* colonization, degree of atrophy, intestinal metaplasia, and inflammation.

RNA & DNA Extraction and cDNA preparation

Immediately after endoscopy, one piece of the excised tissue was immersed in RNA later® solution at –20 °C until extraction. Total RNA was isolated from biopsy samples using an RNeasy total RNA isolation kit (Qia Gene Company). DNA was also was extracted from the samples. The quality and quantity of the DNA and RNA were photometrically determined and cDNA was generated with oligo-dt16 (ParsTous, Iran). DNA samples were stored at -20 °C and cDNAs were stored at -70 °C for PCR.

Polymerase chain reaction

The presence of cytotoxin associated gene (cag A) and vacuolating cytotoxin gene (vac A) in *Helicobacter pylori* from patients were analyzed using PCR with specific primers listed in table 1.

Table 1. PCR details of genes including primer sequences, amplicon size and annealing TM

Gene	Primer	Sequence	Size bp	Annealing TM
TLR2	Forward Reverse	5'-GCTTAGTTCATTAGGAAACAGCACA-3' 5'-GCCCCAAATACTTTGCCTTG-3'	172	57
TLR4	Forward Reverse	5'-AGGATGAGGACTGGGTAAGGAATG-3' 5'-TGGATGATGTTGGCAGCAATGG-3'	124	63
Cag	Forward Reverse	5'-GTTGATAACGCTGTCGCTTC-3' 5'-GGGTTGTATGTAATTTTCCTAAA-3'	351	50&55
Vag s1/s2	Forward Reverse	5'-ATGGAAATACAACAAACACAC-3' 5'-CTGCTTGAATGCGCCAAAC-3'	259/286	55
Vag m1/m2	Forward Reverse	5'-CAATCTGTCCAATCAAGCGAG-3' 5'-GCGTCAAAATAATTCCAAGG-3'	645	50&55
β-actin	Forward Reverse	5'-GGCCAAGATCATCCATGACAACT-3' 5'-ACCAGGACATGAGCTTGACAAAGT-3'	226	57

Reverse Transcription–Polymerase Chain Reaction

cDNA samples were amplified with TLR2, TLR4, and β-actin -specific primers, listed in Table 1. RT-PCR was performed for TLR2 and TLR4 in a final volume of 20 µL containing 2 µL of 10x PCR buffer, 1.5 mmol/L magnesium chloride, 0.5 mmol/L dNTP mixture (0.5 mmol/L for TLR4), 0.25 U Hot-Start Taq DNA polymerase, 0.5 mmol/L of each primer, and 4 µL of cDNA. The TLR2 and TLR4 genes were amplified using the following conditions: an initial incubation for 10 min at 94 °C, followed by 35 cycles of 30 sec at 95 °C (denaturation), 40 sec at 57 °C (annealing), and 40 sec at 72 °C (extension),with final elongation for 10 min at 72 °C. For cag A and vac A m1/m2 initial DNA denaturation for 10 min at 94°C for 10 min; followed by 5 cycles of 30 sec at 95 °C, 30 sec at 50 °C, 30 sec at 68 °C; then 33 cycles of 30 sec at 95 °C, 40 sec at 55 °C (30 sec for vac A m1/m2), and 30 sec at 68 °C, with final extension for 5 min at 72 °C.

To normalize the expression of TLR2 and TLR4 mRNAs, a 266 bp fragment of β-actin (Table 1) was amplified as follows: initial incubation for 10 min at 94 °C, then 30 sec at 95 °C (denaturation), 40 sec at 57 °C (annealing), 40 sec at 72 °C (extension) for 35 cycles with final extension for 5 min at 72°C for 5 min.

PCR products were electrophoresed on 2.0% agarose gels and visualized under ultraviolet illumination. The mRNA expression of each gene was determined using Kodak 1D Image Analysis Software (Kodak, Stuttgart, Germany). The band intensity was expressed as an absolute integrated optical density (OD). The integrated optical density of each PCR

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product was normalized to that of β-actin for the same biopsy sample.

Histopathological examination

Peptic ulcers and infiltration of inflammatory cells were evaluated by microscopic examinations. The classification and grading of gastritis were based on the updated Sydney system (3).

Statistical analysis

Data were analyzed using SPSS version 18 (SPSS Inc., Chicago, IL) and displayed as the mean ± the standard deviation (SD). For all variables, the Kolmogorov-Smirnov (KS) test was applied to ascertain the normality of the data. The independent t-test was performed for variables with normal distribution. To compare non-normal variables, the Mann-Whitney Test was used. Distributions of optical densities for all patients with and without *H. pylori* infections were analyzed by One-way ANOVA. P values < 0.05 were considered significant.

Results

Patient selection

The presence of vac A and cag A in *H. pylori*-infected patients was assessed by PCR (data not shown).

TLR2 and TLR4 mRNA Expression in antrum and body of gastric biopsies

To determine expression changes of TLR2 and TLR4 in antrum and body of gastric tissue, we examined TLR2 and TLR4 mRNA expression from those tissues relative to β-actin by RT-PCR (Fig. 1).

TLR2 mRNA expression

Analysis of the antral region identified significantly greater TLR2 expression in the *H. pylori*-positive with peptic ulcer patients (group A) than in the *H. pylori*-negative with or without peptic ulcer patients (groups B and D) (p = 0.001 and 0.01, respectively, Fig. 2). No significant difference in TLR2 gene expression was observed between groups A and C. Also, TLR2 gene expression was significantly less in *H. pylori*-negative with peptic ulcer (group B) and *H. pylori*-negative without peptic ulcer (group D) than in *H. pylori*-positive without peptic ulcer (group C) (p = 0.002 and 0.02, respectively). No significant difference was observed between groups B and D (Fig. 2a). Furthermore, the results showed that TLR2 gene expression was significantly greater in gastric body in the *H. pylori*-positive without peptic ulcer patients (group C) than the *H. pylori*-negative with peptic ulcer patients (group B)(p = 0.03). Even though TLR2 gene expression was greater in group C than in groups A and D the differences were not statistically significant (Fig. 2). In addition there were no significant differences in TLR2 expression ratios between the same groups in antrum and body regions.

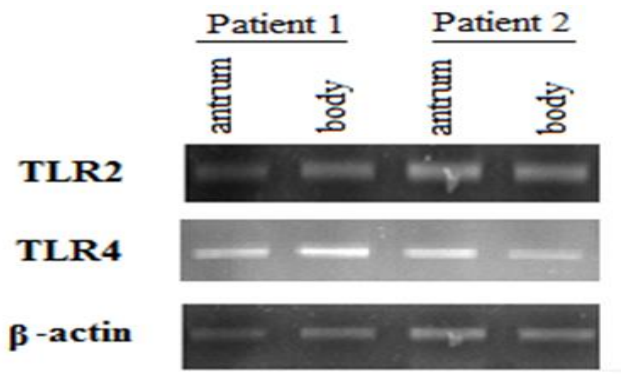

Fig. 1. RT-PCR analysis of TLR2 and TLR4 mRNA expression in the antrum and body biopsy specimens of patients and normal groups. Total RNA was extracted from Gastric biopsies and analyzed by RT-PCR using TLR-2- and TLR4-specific primers. Levels of mRNA were semiquantitated by densitometry as the ratio of TLR4 to β-actin. The results shown here are representative of two patients.

TLR4 mRNA expression

The TLR4 expression ratios in the antrum of *H. pylori*-positive with peptic ulcer patients (group A) and *H. pylori*-positive without peptic ulcer patients (group C) were similar. However, TLR4 gene expression was significantly greater in groups A and C than in the *H. pylori*-negative with peptic ulcer (group B; p = 0.01) or the *H. Pylori*-negative without peptic ulcer patients (group D; p = 0.01). No significant differences between groups B and D were observed in antral regions (Fig. 2c). In the body region, TLR4 expression was significantly greater in *H. pylori*-positive with peptic ulcer patient (group A) and *H. pylori*-positive without peptic ulcer patients (group C) than in *H. pylori*-negative with peptic ulcer patients (group B, P = 0.01 and 0.03 respectively). Furthermore, TLR4 expression was similar in groups A and C. When compared to *H. pylori*-negative without peptic ulcer patients (group D), the A and C groups showed greater TLR4 expression however, the differences were not statistically significant (Fig. 2d). No significant differences in TLR4 expression were observed between the same groups in antrum or body.

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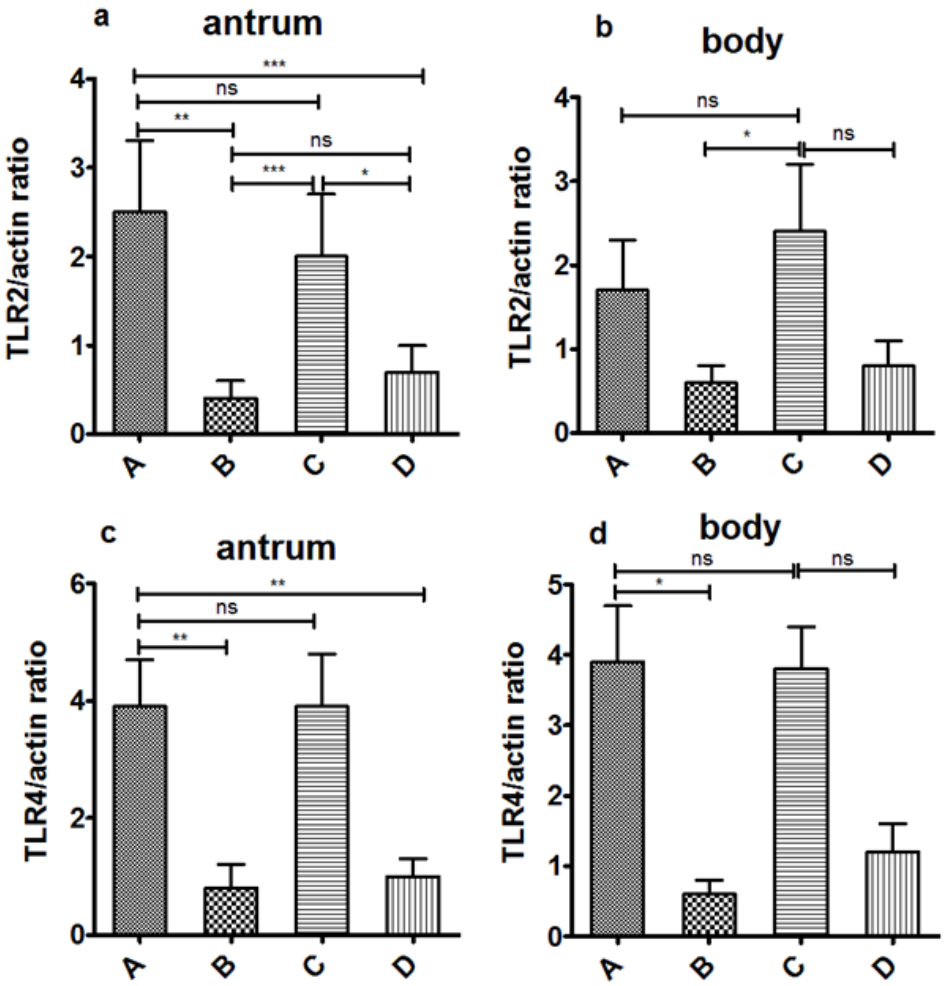

Fig. 2. The mean TLR2 and TLR4 gene expression in antrum and body biopsy specimens. Changes in mRNA expression were assessed by RT-PCR for TLR2 and TLR4 and quantified as densitometric ratios of TLR2 and TLR4/β-actin mRNA. (A) With H. pylori infection and peptic ulcer (n=15), (B) without H. pylori infection and with peptic ulcer (n=5), (C) with H. pylori infection without peptic ulcer (n=10) and (D) control group (n=8). Values are expressed as means ± SE from three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001, and ns: not significant

Discussion

In this study we investigated semiquantitative expression of TLR2 and TLR4 in gastric biopsies of patients with upper abdominal pain and maldigestion. Originally, TLRs were regarded as the molecules involved in innate immune response with physiological duties (22). They are found not only on the surface of mucosal cells but also on macrophages, dendritic cells, and mast cells (8). Immunohistochemical investigation determined that TLR2 gene expression increased significantly in the apical regions of patients with *H. pylori* infections (18, 19).

In our experiment, TLR2 and TLR4 gene expression in *H. pylori*-positive with or without peptic ulcer patients (groups A and C; respectively increased relative to the *H. pylori* negative, with (B), or without (D), peptic ulcer patients in the antral region and gastric body. However, the results were significant for the antral region only. We deduced that after *H. pylori* colonization on the surface of gastric mucosal cells, TLR2 gene expression increased because of immunological responses to the *H. pylori* infection, but it could take months or years for a peptic ulcer form. Our results support this hypothesis because we found no significant differences in TLR2 and TLR4 expression in biopsy specimens from either the *H. pylori*-negative with peptic ulcer patients (group B) or *H. pylori*-negative without peptic ulcer patients (group D) in antrum or body.

H. pylori LPS is 1000-10000-fold less toxic than other gram-negative bacterial LPSs (23). Interestingly, even with this attenuated toxicity, LPS can stimulate the innate immunological response in gastric mucosa (21). Toll-

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like receptor 4 molecules that induce innate immunological responses specifically recognize LPS-containing bacteria (24, 25). In the present study, we investigated TLR2 and TLR4 gene expression in antral and gastric body regions. As the clinical aspect of *H. pylori* infection in the interaction between TLR molecules and gastric epithelial cells, it seems logical that TLRs play a basic role in *H. pylori* recognition (13, 26, 27), but despite numerous investigations on TLRs and bacterial interactions, agreement has not yet been reached on the specific TLR or TLRs that play a basic role on *H. pylori* recognition (28, 29).

It has been indicated that *H. pylori* mediates immune tolerance through TLR2-derived signals and inhibits Th1 immunity, thus evading host defense. Hence, TLR2 may be an important target in the modulation of the host response to *H. pylori* (23). Furthermore, *H. pylori* LPS can increase TLR2 expression via TLR4 signaling (26). The incorporation of TLR2 and TLR4 results in iNOS expression (30, 31). iNOS gene expression results in nitric oxide (NO) production, which can play an important role in the pathological effects of gastric mucosa in *H. pylori* infections (31). An increase in NO is accompanied by gastric mucosa apoptosis, which may lead to pre-neoplastic changes in patients (26, 31). Therefore, it seems that incorporation of TLR2 and TLR4 in LPS recognition may result in a consistent immunological response that results in tissue degeneration in gastric mucosa (26).

H. pylori LPS stimulates O2 production in guinea pig gastric cells via TLR4 molecules (32). Although the roll of TLRs is well-understood in the ulcer's pathogenesis, this study provides evidence that TLRs as bio-markers for cancer. This explains why TLR mediators have successfully been used as treatments for *H. pylori* infections, and also are effective in the treatment of stomach cancer (33). TLR4 molecules, which are LPS receptors, can be found on the surface of gastric epithelial cells and also in gastric cancer cells (34). Therefore, it is possible that *H. pylori* may affect gastric cancer cell growth through TLR4 molecules.

In other studies, it has been shown that LPS, especially LPS derived from E. coli, has strong anti-tumor activity through macrophages activation. LPS also stimulates lymphocytes and NK cells. Anti tumor activity of these cells is mediated via TNF-α, perforin, and granzyme B production (35). Several investigations determined that *H. pylori* LPS has less biological effect than LPS from other sources such as E. coli (36) and cell line stimulation with *H. pylori* and its LPS resulted in down regulation of perforin production in NK cells co-cultured with cancer cells (37). Therefore, it is possible that *H. pylori* LPS affects gastric cancer cells via TLR4 (38). It is a matter of consideration that LPS, particularly E. coli LPS, has strong anti-tumor effects via activation of specific pre-inflammatory macrophage cytokines such as IL-2. These cells mediate their anti-tumor activities via TNF-α, perforin, or granzyme B (39).

Because *H. pylori* acts as a weak macrophage stimulator and weakens the anti-tumor responses of host NK cells (37), it therefore, strengthens host immune responses against the *H. pylori*-induced immune suppression and be an effective way to limit the *H. pylori*-dependent gastric cancer (37). It seems that TLR4 leads to gastric cancers by two mechanisms; first by acting as an effective molecule in bacterial capture, leading to primary infection, and second by suppressing host anti-tumor responses. It seems possible that *H. Pylori*-TLR4 regulation may be an effective treatment tool for prevention or delay of *H. pylori*-induced gastric cancers. It has been demonstrated that adding anti-TLR4 antibodies to cell cultures with active *H. pylori* cells stop cancer cell proliferation (37). *H. pylori* lipid A can be a strong stimulator for innate immune responses in gastric mucosa stimulated by TLR4 (41). Other studies suggest that after the infection first step, acquired immunity response and anti-cancer activities can be suppressed by TLR4 (42).

Our microscopic examination showed mild to severe infiltration of the lamina propria by polymorphonuclear leukocytes (PMN) and mononuclear (MN) cells in both the antral and body mucosa.(data not shown).

Our finding suggests that *H. pylori* infection leads to increase in TLR4 and TLR2 molecules expression in both gastric antral and body regions. However, comparisons in antrum and body showed that, TLR2 and TLR4 expression changes related to the control group were significant just in antral region. Therefore, considering the stimulatory effect of *H. Pylori* on TLRs expression in the gastric tissue, we assume that colonization of *H. pylori* infection might occurs more in the gastric antral region than in the gastric body.

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