Relationship between *Helicobacter pylori cagA* Genotypes Infection and IL-10 and TGFβ1 Genes' Expression in Gastric Epithelial Cells

Abstract

Background: The correlation of *Helicobacter pylori* infection with gastritis, peptic ulcer, and gastric cancer has been proven. The aim of this study was to determine the effects of *cagA*⁺ and *cagA*⁻ genotypes of *H. pylori* on genes expression of interleukin (IL) -10 and tumor growth factor (TGF) β1 in gastric epithelial cells of patients with gastritis and *H. pylori* infection. **Methods:** In all, 45 gastric biopsy samples were collected from patients with gastritis and *H. pylori* infection admitted to Tohid Hospital in Sanandaj city. Status of *urease* and *cagA* genes of *H. pylori* were directly determined from the biopsy samples using polymerase chain reaction (PCR) method. Expression of IL-10 and TGF-β1 genes in gastric epithelial cells of patients with gastritis and cagA⁺ and cagA⁻ genotypes of *H. pylori* infection was serveyed using real-time PCR method. **Results:** Overall, 25 samples had infection with *H. pylori cagA*⁺ and 20 with *cagA*⁻ genotypes. This study showed that there is a positive correlation between *cagA*⁻ genotypes of *H. pylori* and increasing of IL-10 gene expression in gastric epithelial cells of patients with gastritis ($P = 0.001$). **Conclusions:** Level of gene expression of IL-10 as an anti-inflammatory cytokine in gastric epithelial cells of patients with *H. pylori* infection is connected to *cagA*- genotypes.

Keywords: *Cytotoxin-associated gene A, gastric epithelial cells, Helicobacter pylori, interleukin-10, transforming growth factor-beta1*

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Introduction

Helicobacter pylori is a spiral gram-negative, microaerophile with outstanding catalase characteristic and a oxidase and urease positive bacterium that is colonized inside the stomach of almost half of the world's population, and humans are known as the only natural host.[1] *H. pylori* enters the stomach from the fecal–oral route and situates itself under the gastric mucosa and in contact with gastric epithelial cells.[1] Conducted studies this subject have shown there is a connection of *H. pylori* infection with gastritis, gastrointestinal ulcers, and gastric cancer.[1]

H. pylori can be colonized inside the human stomach for years and be cause inflammation in the gastric mucosa by impact on the patient's immune system.[2] Different factors including bacterial virulence factors (such as *cagA*, *vacA*, *ureA*, *hopQ*) and host genetic factors amongst variant ethnic groups are involved in the pathogenesis of *H. pylori* infection.[3] CagA that is a secretory cytotoxin reacts with the proteins inside the host cell and interferes with cellular signaling pathways through dependent and independent mechanisms of tyrosine phosphorylation.[4] CagA protein induce the expression of inflammatory cytokines genes by activation of NF-KB at the end of the cell signaling pathways.[5] One of the results of CagA cell signaling hijacking is the of production of neutrophil chemotaxis factors (interleukin 8) in gastric epithelial cells.[6] Therefore CagA plays the important roles in the pathogenicity that found in 30%–70% of clinical strains.[7] So *CagA*⁺ strains of *H. pylori* increase the possibility of developing gastric infection to acute gastritis, gastric atrophy, gastric ulcer, and gastric cancer.[7]

Chronic inflammatory response, follow *H. pylori* infection, be causes the call of neutrophils, T and B lymphocytes and macrophages.[7] The inflammation and tissue damage are the result of releasing inflammatory mediators, such as cytokines, oxygen free-radicals, and NO.[6] Tumor growth factor (TGF) -β1 and IL-10 are tow inflammatory cytokines in gastric epithelial cells that follow *H. pylori*

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infection. TGF-β1 has a regulatory role in triggering of immune response against bacteria by induction of *FOXP3* gene expression in CD4+ lymphocytes.[4] IL-10 is another regulator cytokine that secreted created from gastric epithelial cells during *H. pylori* infection.[4] The gastritis process upregulates *FOXP3* gene expression in Treg+ cells and causes the production of inhibitory cytokines TGF-β1 and IL-10 that decreases immune system response against colonization of *H. pylori* in the gastric epithelial cells.[4]

In this study, the relationship of *cagA* genotypes of *H. pylori* with TGF-β1 and IL-10 genes expression in gastric epithelial cells of patients with *H. pylori* infection was investigated.

Methods

Sampling

A case–control study was performed including of patients with gastritis and *H. pylori* infection, referred to Tohid Hospitalin Sanandaj city. All gastric biopsy samples were taken by endoscopy. The gastritis with by *cagA+* genotype of *H. pylori* were considered as case group, and biopsy samples infected by *cagA−* genotype of *H. pylori* were considered as control group. Patients with gastritis who had used aspirin or nonsteroidal anti-inflammatory drugs, or had malignancies, metabolic disorders, and immunosuppression were excluded from this study.[8]

A total of 25 biopsy samples collected from epithelial tissues of antrum in patients with *cagA+* genotype *H. pylori* infection and 20 biopsy samples of epithelial tissues of antrum in patients with *cagA−* genotype *H. pylori*. *H. pylori* infection in all biopsy samples were determined by rapid urease test (RUT). The *cagA* genotypes of *H. pylori* was determined using polymerase chain reaction (PCR) method on biopsy samples, directly after digestion and DNA extraction from tissue.[8]

Three biopsy samples were taken from each patients with gastritis and cagA pasitive and negative *H. pylori* infection infected by gastritis. One of the samples was used for detection of *H. pylori* infection using RUT test (after 20 min, change in color of RUT solution from yellow to any color between pink and red. The second sample was put in RNAlater solution for preparation process of measurement the genes expression of TGF-β1 and IL-10 genes in gastric epithelial cells. The gastric biopsy samples were transferred to the microbiology research laboratory in Kurdistan University of Medical Sciences. The third sample was used for DNA extraction and determination of the genotype of *cagA* and *urease* gene of *H. pylori*.[8]

H. pylori infection

The samples were transferred to a −70°C freezer; using DNA extraction kit (Bioflux, Japan), genomic DNA was extracted and kept in −70°C until for molecular diagnostic tests.[7]

Afterward, confirmation of *H. pylori* infection in biopsy samples was performed using specific primers of *urease* by PCR. The sequences for urease gene primers that were designed using Gene Runner software program (Cinnagen, Iran) were F: 5′-TTCTTCCCGCTTCCACTAA-3′, R: 5′-CTGAAGTTGTCGTTATCGCC-3′.[7] The program of PCR machine was initial denaturation 95°C, 10 min; denaturation 95°C, 30 s; annealing 52°C, 30 s; extension 72°C, 45 s; and final extension 72°C, 5 min. The PCR product length was 319 bp.

CagA genotypes

To determine of *cagA* virulence factor gene of *H. pylori*, PCR method was performed on the DNA extracted from gastric biopsy samples. Primers of *cagA* were designed using Gene Runner software program (Cinnagen). The sequences for *cagA* primers were F: 5′-GTGATGTAGGGCAAGCAGC-3′ and R: 5′-AGCTTCTGATACCGCCTGG-3′.[7] The program of PCR machine was the same as urease gene detection exept that annealing temprature was 54 and PCR product length was 236 bp.

Real-time PCR

Extraction of total RNA from gastric biopsy tissues was done using Total RNA Extraction Kit (Pars Tous Company, Iran). The method used in this kit is based on the liquefaction power of phenol that inhibits the binding ability of RNA to borosilicate columns. The compositions of kit neutralize cellular RNase and delete DNA as well.[8]

IL-10 and TGF-β1 genes expression in gastric epithelial cells with infection of *cagA+* and *cagA−* genotypes of *H. pylori* was serveyed using relative quantitative Real Time RT PCR method. PCR efficiency (E) and cyclic threshold (CT) parameters are essential for quantitative analysis of the amounts of target genes expression compared to *beta-actin* gene (as the reference gene). Finaly, the amount of changes in IL-10 and TGF-β1 genes expression was calculated using the numerical values of CT and Δ CT equation.[9]

For the accuracy and validity of real-time PCR, a standard curve drawn use four dilutions of complementary DNA including 1, 0.1, 0.01, and 0.001 and the efficiency of PCR reaction was calculated using the standard curve line slope by PCR Corbett machine (Rotor gene 6000).[9]

Primers of IL-10, TGF-β1, and B-actin genes were designed using Gene Runner software program (v 3.05). The primers were synthesized in the lyophilized form by Bioneer company in Korea. The concentration of 100 pmol of the primers was created, and for real-time PCR, 0.1 dilution was used.

The sequences for IL-10, TGF-β1, and B-actin primers were F: 5′-AAGCCTTGTCTGAGATGATC-3′, R: 5′-AC AGGGAAGAAATCGATGAC-3′, F: 5′-AATTCCTGGC GATACCTCAG-3′, R: 5′-TAGTGAACCCGTTGATG

TCC-3′, F: 5′-AGATCATTGCTCCTCCTGAG-3′, R: 5′-C TAAGTCATAGTCCGCCTAG-3′, respectively.[7] The program of PCR machine was initial denaturation 95°C, 5 min; denaturation 95°C, 5 s; annealing 55°C, 30 s; extension 72°C, 45 s, final extension 72°C, 5 min; and melt 72–95°C, 5 min. Denaturation, annealing, and extension stages were repeated for 40 cycles.

Results

cagA genotypes

The PCR products of cagA genotypes were rounded on gel agarose 1%. The results of the study showed that from 45 biopsy samples of patients with gastritis, 25 samples have infection with *cagA*⁺ genotype of *H. pylori* and 20 samples have infection with *cagA*⁻ genotype of *H. pylori*.

IL-10 and TGFβ1 genes' expression

For relative measurement of gene expression, ΔCT method was used, relative amount of expression of IL-10 and TGF-β1 genes (sample genes) was measured in comparison to B-actin gene (reference gene) using $2^{-\Delta CT}[2^{(ct\ sample\ -\ ct\ ref)}]$ formula.[9]

The results of the Real Time RT PCR revealed that *beta-actin* gene was present in all biopsy samples. There was no significant relationship between *cagA* genotypes of *H. pylori* and TGFβ1 gene expression in gastric biopsy samples; while the expression of IL-10 cytokine was decreased in gastric epithelial cells with infection of *cagA*⁻ genotype of *H. pylori* [Figures 1 and 2]. cagA+ genotypes were detected by PCR [Figure 3].

Statistical results

The amounts of changes in IL-10 and TGF-β1 genes' expression in two groups of case (biopsy samples with infection of *cagA*⁺ genotype of *H. pylori*) and control (biopsy samples with infection of *cagA*⁻ genotype of *H. pylori*) was obtained using output charts of Corbett

Device (Rotor Gene 6000 Qiagen) [Figures 2 and 3]. SPSS software program (version 21.00) was used for statistical analysis of the results. Summarization and classification of data were performed by using descriptive statistics that consist of central and dispersion indicators. Analysis of date and testing the hypotheses were performed using nonparametric *Mann–Whitney* test. The level of statistical significance was considered 0.05 in this study.

By considering the *P* value obtained from *Kolmogorov–Smirnov* test, it can be concluded that obtained data do not follow normal distribution, and *Mann–Whitney* test was used for testing the above-said hypothesis.[9] The *P* value obtained from *Mann–Whitney* test ($P = 0.189$) showed that there is no significant statistical difference between case and control groups in terms of TGFβ1 gene expression [Table 1]. Even though the amount of TGFβ1 gene expression in gastric epithelial cells with infection of *cag*⁺ genotype of *H. pylori* was more than gastric epithelial cells with infection of *cag*⁻ genotype of *H. pylori* for 1.857, this increase in expression was not statistically significant.

Prior to performing the test related to the above-said hypothesis, first, a normality test (*Kolmogorov–Smirnov*) was performed for investigating data distribution. The results showed that there is a meaningful connection

Table 1: Survey of IL-10 and TGF-β genes' expression by nonparametric Mann–Whitney test in two groups of patients with gastritis and *H. pylori cagA*⁻ and *cagA*⁺ genotypes infection

Genes	cagA genotype	Delta ct	Standard deviation	Average	Z statics	P value
IL-10	*cagA*⁻	0.020	0.20	29.61	−3.272	0.001
	cagA⁺	0.006	0.17	16.52		
TGF-β	*cagA*⁻	0.088	0.111	19.30	−1.315	0.189
	cagA⁺	0.168	0.213	24.35		

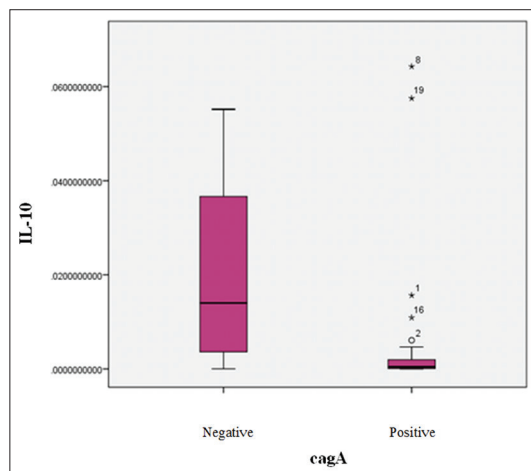IL: Interleukin; TGF: Tumor growth factor

Figure 1: Statistical box plot of IL-10 gene expression in *cagA*⁻ and *cagA*⁺ groups
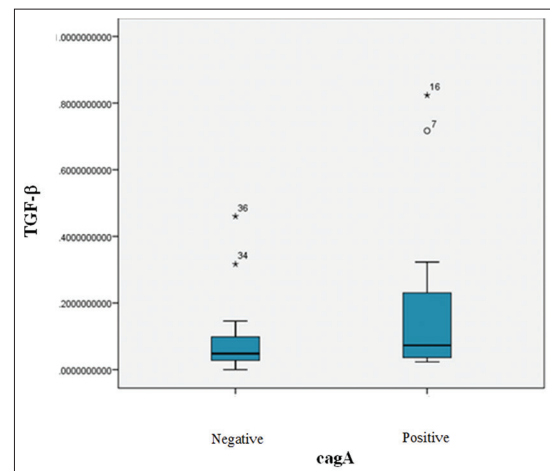

Figure 2: Statistical box plot of TGF-β gene expression in *cagA*⁻ and *cagA*⁺ groups
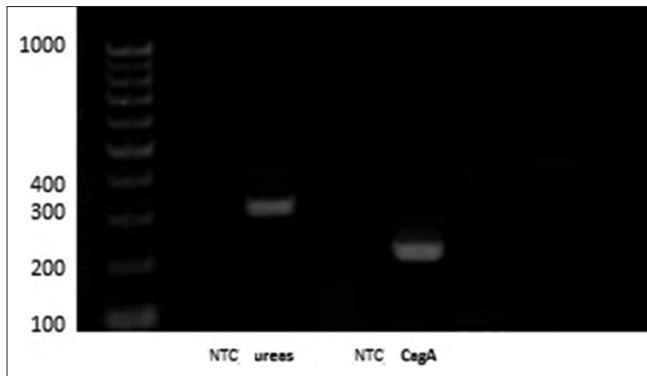
Figure 3: Detection of *Helicobacter pylori cagA* and *urease* genes in biopsy samples by multiplex PCR

between *cagA*⁺ genotype of *H. pylori* and IL-10 gene expression among gastric biopsy samples of patients with gastritis [Table 1]. With regard to the *P* value obtained from *Kolmogorov–Smirnov* test, it could be concluded that the obtained data do not follow normal distribution; therefore, *Mann–Whitney* nonparametric test was used for testing the above-mentioned hypothesis [Table 1]. The *P* value obtained from *Mann–Whitney* test ($P = 0.001$) revealed a significant difference between two groups of case and control in terms of IL-10 gene expression. The amount of IL-10 gene expression in biopsy samples of gastric epithelial cells of patients with gastritis and infection of *cagA*⁻ genotype of *H. pylori* was increased 3.073 times the gastric epithelial cells of patients with gastritis and infection of *cagA*⁺ genotype of *H. pylori*; and this increase in expression is statistically significant. Standard deviation of the results of IL-10 gene expression in the case group was high, while standard deviation of the results of IL-10 gene expression in the control group was low ($P = 0.001$) [Table 1].

Discussion

While *H. pylori* is colonized in the stomach of half the world's population, there are different information with regard to the prevalence of *H. pylori* in Iran; however, it is estimated that the prevalence rate of *H. pylori* in various regions of Iran is between 50% and 90%.[10,11] This bacterium causes colonization in the gastric mucosal tissue, damage of epithelium cells, and long-term chronic inflammation by the aid of certain virulence factors such as surface proteins, and it seems like gastric inflammation and immune response to infection are the basis of creation of disease resulting from it.[2] Colonization of *H. pylori* in stomach is due to gastritis. Despite gastritis, due to unknown reasons, gastric cancer and peptic ulcers are seen in a low number of patients with *H. pylori* infection.[1] However, a series of factors such as the host's immune system response against *H. pylori*, environmental factors, and different genotypes of *H. pylori* all play a role in creation of gastric cancer and peptic ulcers.[2]

Some genotypes of *H. pylori* are more success in colonization that CagA as an important virulence factor is noteworthy.[3] In this regard, the role of pathogenicity factors such as *cagA* and *vacA* has been studied in numerous instances and the role of these cytokines in the development of some disruptions in the way of apoptosis and cancerization of gastric epithelial cells has been proved.[7] CagA protein can affect the colonization process of bacteria indirectly and by the effect of secretion of cytokines from gastric epithelial cells.[2] Connection of *cagA* pathogenic factor with pathological factors in gastric epithelial cells shows that the level of inflammation, neutrophilic inflammation, damage of gastric epithelial cells, and also increase in the risk of gastric atrophy and intestinal metaplasia are significantly higher in patients with infection of *cagA*⁺ strains in comparison to patients with infection of *cagA*⁻ strains.[4] Nevertheless, statistical analyses of some studies such as the study by Razavi *et al.*[7] show that the amount of chronic inflammation, neutrophilic inflammation, and the level of colonization of *H. pylori* in patients with infection of *cagA*⁺ was significantly higher compared with patients with infection of *cagA*⁻.[7] In addition, the results of the study by Razavi *et al.* showed that the prevalence of Foxp3⁺ cells and IL-10 and TGF-β1 cytokines in patients with infection of *H. pylori* was significantly higher in comparison to patients without infection of *H. pylori*.[7] In that study, in which the connection of pathogenic factor of *cagA* with the amount of TGF-β1 gene expression was studied, it was determined that there is no significant relationship between the two ($P = 0.189 > 0.05$), which is compatible with the results of the present research. Previous studies show that *H. pylori* stimulates a response in Th1, Th17, and Treg cells.[6] Other studies as well have proven the role of Treg cells in immune response of the host against *H. pylori* infection and have shown that CD4⁺ C25⁺ Treg cells have a very important role in suppression of host's immune system.[12]

These studies showed that after antibiotic treatment and eradication of *H. pylori* infection, activity of Treg cells and gene expression of TGF-β1 and IL-10 shall considerably decrease in gastric mucosa, which is the result of negative feedback of *H. pylori* infection in the expression of these cytokines. In the studies conducted by Robinson *et al.*,[13] a comparison was performed between two groups of patients with infection of *H. pylori,* with and without the existence of gastric ulcer, and it was revealed that the prevalence of (IL-10⁺) Treg cells in patients suffering from gastric ulcer with infection of *H. pylori* was lower compared with infected patients who did not have gastric ulcer. Furthermore, the level of IL-10 expression has a direct relationship with the amount of bacterial colonization. IL-10 gene expression in gastric epithelial cells of patients with infection of *cagA*⁺ genotype of *H. pylori* was more than the gastric epithelial cells of patients with infection

of *cagA⁻* genotype of *H. pylori*, while no significant connection was observed between the prevalence of CD4+CD25hi cells and *cagA* genotypes.[13] A study performed by Hernandez *et al.*[14] concluded that IL-10 cytokine expression in gastric epithelial cells of children with infection of *H. pylori* was higher compared with gastric epithelial cells of children free of infection.

In addition, no significant relationship was observed in the amount of IL-10 and TGF-β cytokines in children's gastric epithelial cells with *H. pylori* infection, with or without the existence of peptic ulcer.[14] In this study, which was performed on gastric biopsy samples of patients suffering from gastritis, it was determined that there is a significant relationship between IL-10 gene expression and *cagA* pathogenic factor ($P = 0.001 < 0.05$), and IL-10 gene expression in gastric epithelial cells of patients with *cagA⁺* genotypes of *H. pylori* is less than gastric epithelial cells of patients infected by *cagA⁻* genotype of *H. pylori*. These results show the ability of *cagA⁺* strains of *H. pylori* in colonization that follows the anti-inflammatory effect of IL-10. *H. pylori* infection has a high rate or prevalence in today's societies which, if not treated, results in inflammation and increase in cytokine production. Pathogenicity factors of *H. pylori* such as *cagA* in patients are researchable, and it was determined in this study that there is no significant relationship between pathogenicity factor of *cagA* and TGFβ1 gene expression; however, there is in fact a significant relationship between pathogenicity factor of *cagA* and IL-10 gene expression, and IL-10 gene expression is found more in *cagA⁻* than *cagA⁺*.

As a conclusion, the chance of bacterial colonization and creation of mild and persistent infections such as gastritis increase by the *cagA⁻* strains of *H. pylori* cause the increase in IL-10 anti-inflammatory cytokine expression and suppression of immune system.

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Conflicts of interest

There are no conflicts of interest.

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