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 surveyed 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

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 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 low inflammatory cytokines in gastric epithelial cells that follow H. pylori
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 Hospital in 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 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.[9]

Three biopsy samples were taken from each patients with gastritis and cagA positive 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 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.[9]

**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′-TTCTTCCGCTTCCTAA-3′, R: 5′-CTGAAGTGTGCGTTACGC-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′-GTGATGTAGGGCCAC-3′ and R: 5′-AGCTTCTGATACCGCTGG-3′.[7] The program of PCR machine was the same as urease gene detection except that annealing temperature 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 surveyed using real-time 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). Finally, 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′-AAGCCTTGTCTCAGATGATC-3′, R: 5′-ACAGGGAAGAATCGATGAC-3′, F: 5′-AGCTTCTGACGCT-3′, R: 5′-TAGGACTTCCGTTATGAG
TCC-3’, F: 5’-AGATCATTGCTCCTCCTGAG-3’, R: 5’-CTAAGTCATAGTCCGCCTAG-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, \( \Delta \text{CT} \) method was used, relative amount of expression of IL-10 and TGF-β genes (sample genes) was measured in comparison to B-actin gene (reference gene) using \( 2^{-\Delta \text{CT}} = 2^{(\text{ct sample} - \text{ct ref})} \) 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 cagA+ genotype of H. pylori was more than gastric epithelial cells with infection of cagA− 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
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.[3] 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.[11] 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 canerization 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.[10] 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

Figure 3: Detection of Helicobacter pylori cagA and urease genes in biopsy samples by multiplex PCR

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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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