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Evaluating the MicroRNA Expression of IL-35 and IL-37 in *Helicobacter Pylori*-infected Patients with Gastritis and Gastric Ulcer

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ABSTRACT

Interleukin (IL)-35 and IL-37 are two anti-inflammatory cytokines. IL-35 inhibits the development of T-effector cells such as Th1, and Th17; while increasing regulatory T cells (Tregs). IL-37 causes the suppression of inflammatory cytokines. Regarding the positive impact of *Helicobacter pylori (H. pylori)* infection on inflammation and considering the anti-inflammatory effects of IL-35 and IL-37, this study aimed to evaluate the expression of these two cytokines in H. pylori-infected patients with gastrointestinal problems.

The case group consisted of *H. pylori*-infected individuals with gastric ulcer and/or gastritis (n=50) and the control group consisted of cases with gastric ulcer and/or gastritis non-*H. pylori*-infected (n=50). Sampling and classification of patients were based on pathology findings. A real-time polymerase chain reaction was performed for evaluating the IL-35 and IL-37 expression levels.

H. pylori-infected gastritis patients showed lower expression of IL-35 and IL-37 than the non-infected group. There was a significant difference between the expression levels of IL-35 and IL-37 in patients with gastric ulcers and/or gastritis who were infected and non-infected by *H. pylori*. There were no significant differences in the expression level of IL-35 and IL-37 in H. pylori-infected patients with gastric ulcer or gastritis.

Interleukins 37 and 35 were less expressed in patients with *H. pylori*-infection. In differentiation between patients with gastrointestinal symptoms who have *H. pylori* infection or with similar symptoms who do not have *H. pylori*-infection, mentioned interleukins can be used as diagnostic markers.

Keywords: Gastritis; Helicobacter pylori; Human interleukin-35; Human IL-37 protein; Stomach ulcer

INTRODUCTION

Helicobacter pylori (*H. pylori*) is a gram-negative, microaerophilic, curved helical-shaped bacterium with 3 to 5 flagella. It is the specific pathogenic bacterium of the human stomach that is often accompanied by acute and chronic inflammation. At first, Warren and Marshall identified it in 1983 in the microscopic

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examination of the gastric epithelium of the patients with active chronic gastritis.[1] Then, in 1994, *H. pylori* was recognized as a first-line carcinogen and today it is the most common cause of gastrointestinal complications and cancers.[2,3] Most of the infected patients are asymptomatic and have no specific gastrointestinal symptoms. The infection mostly occurs in childhood and is caused by fecal-oral transmission.[4,5] The *H. pylori* membrane consists of "lipopolysaccharides (LPS)" which restrict the host inflammatory response.[6] Decreased host immune system allows the bacteria to survive with minimal pathological risk.[7] Gastritis is commonly found in all patients infected with *H. pylori*. In people with acute gastritis, the entire stomach is involved and is often associated with a decrease in stomach acidity (Hypochlorhydria).[8,9] These diseases are divided into acute and chronic forms. The statistics indicate that about 50% of the world's population is infected with this bacterium and about 2 to 5% leads to gastric cancer.[10] Acute gastritis becomes chronic when lymphocytes are replaced by neutrophils. Chronic gastritis is usually a sign of underlying diseases such as gastric ulcers and gastric cancer. *H. pylori* cause 70-85% of gastric ulcers and 90-95% of duodenal ulcers. Pathological changes that lead to gastric cancer begin with gastritis and are followed by atrophic gastritis, metaplasia, dysplasia, and ultimately turn into carcinoma.[11,12] This process happens over several years which is why most patients are middle-aged or old. In addition to *H. pylori*, environmental factors such as smoking and diet contribute to the creation and development of adenocarcinoma.[13] Interleukin (IL)-35 is a member of the IL-12 family that has been recently recognized as an anti-inflammatory cytokine that can modulate immune and inflammatory responses during infections.[14] IL-35 is a heterodimer molecule including Epstein-Barr virus-induced gene 3 protein (EBI3) and P35 protein subunits. There is little information about the mechanism of this molecule. IL-35 is not expressed in all tissues and is produced only by regulatory T cells (Tregs).[15] The biological function of IL-35 includes inhibiting both T effector proliferation and T-helper cell 17 (Th17) development.[16] IL-37 is a new member of the IL-1 family and is expressed as IL-1F7. It has anti-inflammatory effects and has five different isoforms (from IL-1F7a to 1F7e). IL-1F7 is found in bone marrow, testis, lymph node, thymus, lung, colon, uterus, and skin. IL-37b is the largest isoform of IL-1F7 which is expressed by five out of six exons in the *IL-37* gene area. Also, it has a great common sequence with IL-18. Similar to IL-1 and IL-18, IL-37 is initially synthesized as a zymogen and is activated by caspase1 after being secreted. Studies that were performed on transgenic rats have shown that this cytokine causes a negative regulation of inflammation.[17,18] While *H. pylori* cause inflammation in the stomach, IL-35 and IL-37 have anti-inflammatory activities. So, it is hypothesized that these two cytokines may be effective in reducing gastric inflammation in *H. pylori*-infected patients. We decided to study evaluate the mRNA expression of IL-35 and IL-37 cytokines in *H. pylori*-infected patients with gastritis and gastric ulcer compared with non-infected patients.

MATERIALS AND METHODS

Study Design

This case-control study was an *Ex-vivo* study conducted in 2015. The ethical committee of Shahrekord University of Medical Sciences approved the study (ethical code number: SKUMS.RCE.1394.156). The studied patients consisted of two groups: *H. pylori*-infected patients with gastrointestinal problems including ulcer and gastritis (case group) and the non-infected subjects (control group).

Patients who took aspirin or non-steroidal anti-inflammatory drugs (NSAIDs) or those who had malignancy, metabolic and immunosuppressive disorders were excluded from the study. The sample size was 50 patients with gastritis and gastric ulcer infected with *H. pylori* with a 95% level of confidence (α=0.05), test power 80% (ß=0.2), and frequency of P1=0.3 and P2=0.01. A total of 50 non-infected individuals who were admitted to Hajar clinic of Chaharmahal and Bakhtiari province for endoscopy were included as the control group.

Sampling and Classification of Patients Based on Pathology Finding

Biopsies were taken from several sites of 100 patients without *H. pylori*-infection (n=50) and individuals with gastritis and/or gastric ulcer (n=50) infected with *H. pylori*. These cases did not receive any medication in the past two weeks and the procedure was performed by a gastroenterologist. Moreover, a consent letter was taken from patients. Signs of gastritis

included black and tarry stool, bloating, nausea and vomiting, feeling extra full during or after a meal, loss of appetite, stomach ulcers, losing weight, upper abdominal pain, and hematemesis. After biopsies, a rapid urease test (RUT) (Cat number; 014, IPK, Tehran, Iran) was performed on one of the biopsies and another sample was sent for histopathological evaluation and confirmation of *H. pylori* existence by a pathologist. Inflammatory cells with damage and loss of structure in the epithelium**,** lymphocytes, and plasmocytes infiltration indicate chronic inflammation of *H. pylori* and polymorphonuclear (PMN) cell infiltration were characteristics of *H. pylori* activity. Electron microscopy showed that *H. pylori* have caused these damages by attaching to the superficial cell membrane. *H. pylori* infection causes chronic active gastritis which is characterized by a striking infiltration of the gastric epithelium and the underlying lamina propria by neutrophils, T and B lymphocytes, macrophages, and mast cells. Examination of gene expression was performed on samples that were RUT-positive and were evaluated by a pathologist for diagnostic confirmation.[19-21]

RNA Extraction

After sampling, biopsies were put into a lysis solution containing an RNase inhibitor (Cat number; N2615, NORDIC BIOLABS AB, Stockholm, Sweden) to stabilize the mRNA. Extraction was performed by Trizol (a phenol-guanidine isothiocyanate solution) (Cat number; BSC29S1, BioFlux, Tokyo, Japan) reagent according to the manufacturer's instruction. Extracted RNA was transferred to the liquid nitrogen tank until the next step. Revert Aid First cDNA synthesis kit (Cat number; 11117831001, Fisher Scientific Ltd., Vantaa, Finland) was used to prepare cDNA according to the manufacturer's instructions. The program of the cycles used to prepare cDNA was as follows: cycle 1: 25°C for 5 minutes; cycle 2: 42°C for 60 minutes, cycle 3: 70°C for 5 minutes.

Real-time Polymerase Chain Reaction (RT-PCR)

The *IL-35* and *IL-37* as analytical, and β-actin as reference genes were proliferated; using SYBR Green PCR MasterMix (Cat number; 330620, Qiagen, Hilden, Germany). Then, the mRNA expression of *IL-35* and *IL-37* genes were analyzed in comparison with β-actin as the reference gene (Supplementary Table 1). All RT-PCR reactions were performed in Rotor-Gene TM 3000 (Corbett device, Australia). The heat-time schedule was set up in multi-steps. The first stage which leads to the denaturation of complementary DNA (cDNA) molecules, was performed at 95°C for 10 minutes. The next stage was 45 cycles and each cycle comprised of two steps including denaturation followed by annealing and extension. These reactions were performed in duplicate in a final volume of 25 µL in 0.1-micron microtubs. The final volume of each reaction was 12 µL, containing 0.4 µL of forwarding and 0.4 µL of reverse primers the concentration of each one was 10 Picomoles, 0.2 µL of 10 Picomoles probes, 7 µL of RNase-free distilled water, and 2 µL of pattern cDNA. Relative quantification of the cytokine to β-actin (cytokine mRNA/β-actin mRNA) was determined; using the $2^{-\Delta Ct} = 2^{-(Ct,\ cytokine - Ct,\ \beta\text{-actin})}$ method.[22]

Data Analysis

Data were analyzed by SPSS version 16. Mann-Whitney U tests (for gene expression in two different groups) were used to analyze the data. After statistical analysis, the required graphs were plotted; using GraphPad Prism 5 Demo software. A p-value≤0.05 was considered significant.

RESULTS

The results showed that the expression of *IL-35* and *IL-37* can be used as a biomarker in the histopathological diagnosis of chronic active gastritis and gastric ulcers of infected and non-infected *H. pylori* groups. Decreased expression of *IL-35* and *IL-37* was observed in patients with *H. pylori* infection compared with the non-infected group. A significant reduction in the expression of *IL-35* (p =0.02) and *IL-37* (p =0.01) was observed in patients with gastric ulcers who were infected by *H. pylori* compared to the non-infected group (Figure 1). Besides, a significant reduction in the expression of *IL-35* (p=0.0012) and IL-37 (p=0.0021) was observed in patients with gastritis who were infected by *H. pylori* compared to the non-infected group (Figure 2). Statistical analysis also showed no significant differences in expression of *IL-35* (p=0.81) and *IL-37* (p=0.33) in *H. pylori*-infected patients suffering from gastric ulcer or gastritis (Figure 3).

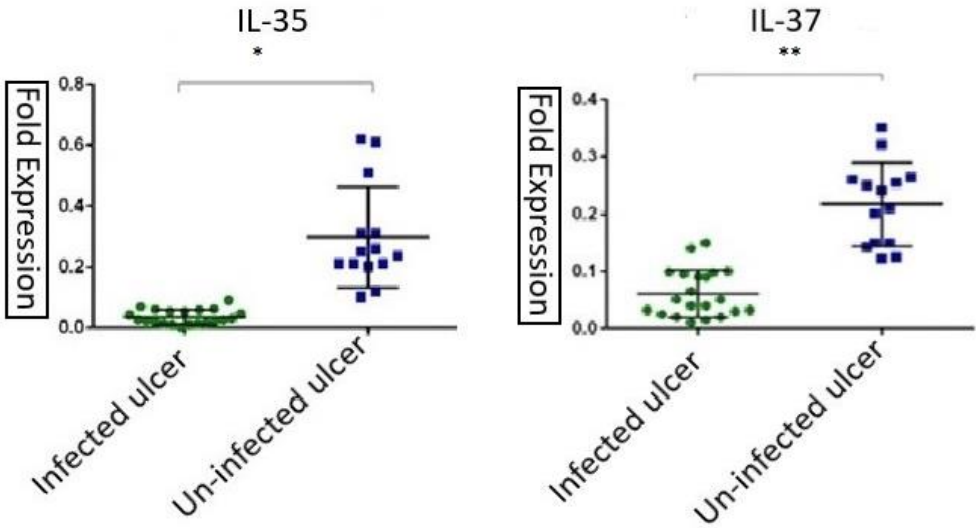

Figure 1. Expression of interleukin (IL)-35 and IL-37 in *H. pylori*-infected and uninfected patients with gastric ulcers. Reduction in the expression level of IL-35 and IL-37 in patients with gastric ulcers who were infected by *H. pylori* was compared with the non-infected group (p*≤0.05, ***p*≤0.01).**

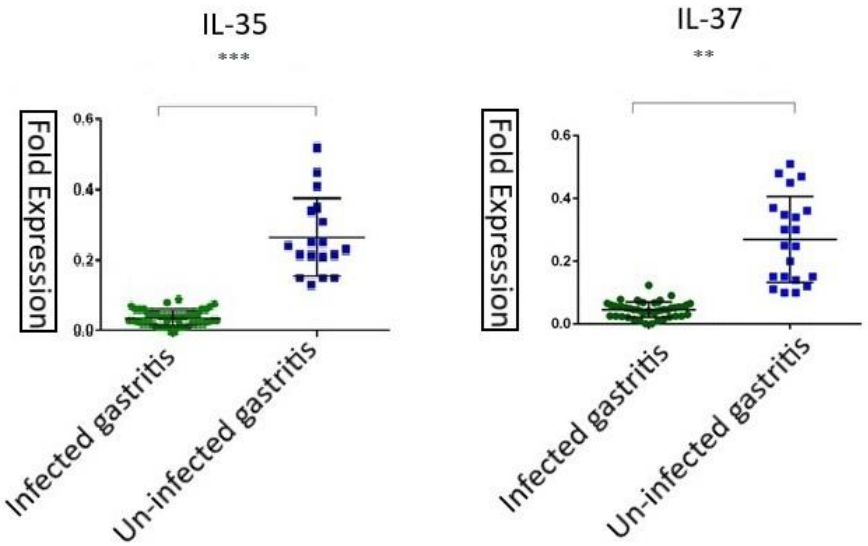

Figure 2. Expression of interleukin (IL)-35 and IL-37 in *H. pylori*-infected and non-infected patients with gastritis. Reduction in the expression of IL-35 and IL-37in patients with gastritis who were infected by *H. pylori* was compared to the non-infected group (**p*≤0.001, ***p*≤0.01).**

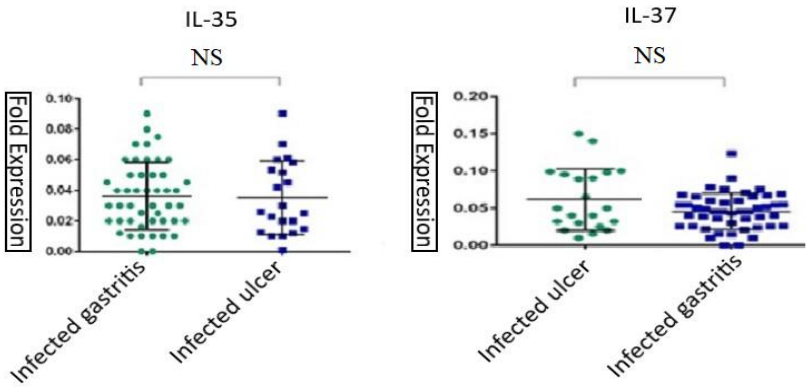

Figure 3. Expression of interleukin (IL)-35 and IL-37 in *H. pylori*-infected patients with gastritis and gastric ulcer. No significant differences were observed in the expression level of IL-35 and IL-37 in *H. pylori*-infected patients with gastric ulcer or gastritis (p>0.05), NS: non-significant.

DISCUSSION

The study showed that *H. pylori* infection, with an unknown mechanism, probably reduces the expression of *IL-35* and *IL-37*. Moreover, our studies showed that *IL-35* expression in *H. pylori*-infected patients with gastric ulcers was lower than in non-infected patients. Some recent studies indicated the relationship between IL-35 and other infectious diseases which emphasizes on anti-inflammatory effect of IL-35. A study by Zandian et al, was performed on rats with herpes simplex virus (HSV)-IL2 induced demyelination. Accordingly, they proved that IL-35 plays an anti-inflammatory role in this disease, whereas interferon-gamma deteriorates the condition.[19]

Another study by Liu et al, showed that the P35 subunit of IL-35 had been detected in the peripheral blood of patients with chronic hepatitis B (CHB). This finding suggests that P35 may prevent the occurrence of hepatic fibrosis in these patients. In various studies, the role of IL-35 in reducing and suppressing inflammatory cells, such as Th1 and Th17 had been investigated.[20]

Sawant et al proved that IL-35 is more powerful than IL-27 in inhibiting the proliferation of Th1 and Th17; leading to reducing experimental colitis and protecting the intestine against the immune response in rats.[21] The subunit of P35 in the rats leads to the development of herpes simplex keratitis (HSK). Both subunits of IL-35 can equally regulate the immune system and the inflammatory process.[16] IL-35 messengering route has not been specified well yet, but studies have shown that IL-35 applies its effect via a heterodimer containing IL-12Rβ2 and gp130. IL-35 messaging pathway activates STAT1 and STAT4 proteins and provides a unique heterodimer that attaches to specific sites on encoding promoters of IL-12, P35, and EBI proteins. This complex directly suppresses the proliferation of T cells and causes the conversion of the immature T cells into activated T cells by IL-35 (iTreg 35). Moreover, it suppresses the activity of Th17 and stimulates the production of IL-10 which is a modulator of the immune system. IL-37 has anti-inflammatory effects. *IL-37* expression in macrophages and epithelial cells causes the suppression of inflammatory cytokines such as IL-1α, IL-1β, IL-6, and TNFα. IL-37 attaches to IL-18Rα, IL-18BP, and the IL-37-IL-18Rα complex and activates an anti-inflammatory response. IL-37 is widely expressed in the synovial fluid of patients who are suffering from rheumatoid arthritis. IL-37 has a high expression in the skin cells of patients with psoriasis and Crohn's disease. IL-37 is synthesized as a zymogen which is activated by caspase1 after stimulation. IL-37 has various roles such as antibacterial, antiviral, neutralizing endotoxin, and anti-carcinogenic effects, which are mostly completed by changing the permeability of the cell membranes.[21-23] Our study showed that *H. pylori* infection reduced *IL-37* expression in patients with gastritis and gastric ulcer.

Roberta Caruso et al, showed that the expression of IL-17 and IL-23 cytokine was significantly higher in *H. pylori*-infected individuals compared to the healthy group. Moreover, they showed that IL-23 protein levels

were significantly higher in infected individuals than in healthy cases. So far, the precise mechanisms of IL-35 and IL-37 have not been identified but studies have shown that EBI (a component of IL-35) leads to the downregulation of IL-17 and IL-22 cytokines as well as Th17 cells which can suppress the immune system.[23,24] IL-17 and IL-23 are involved in defense against some gastrointestinal mucosal infections and this role was performed by attracting neutrophils to the site of the infection by IL-17.[23-25]

In conclusion, we compared IL-35 and IL-37 expression in patients with gastric ulcer and gastritis in both *H. pylori*-infected and non-infected cases. Our study showed that the mean expression of *IL-35* and *IL-37* in patients with *H. pylori* infection was significantly lower than in non-infected subjects. This study showed that *H. pylori* reduced the expression level of IL-35 and IL-37 as strong anti-inflammatory cytokines that are probably practical for the progress of the infection, disease, and inflammation by unknown mechanisms.

CONFLICT OF INTEREST

There is no conflict of interest in this article.

ACKNOWLEDGEMENTS

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