Association Between Expression of Interleukin-32 Gene and Various *Helicobacter pylori* Virulence Factors in Human Infected Gastric Biopsy

Heshmat Shahi, Fatemeh Kiaee

Department of Immunology, School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran

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Abstract- *Helicobacter pylori* (*H. pylori*) is a spiral bacterium that infects the human gastric mucosa. Various clinical aspects of the infection may mirror distinctive forms of cytokine expression. It correlates with immune cell penetration to the gastric mucosa with numerous cytokines production and gastric inflammation. IL-1 and IL-8 directly contribute to *H. pylori* effected gastritis. IL-32 is a pro-inflammatory cytokine categorized by the training of Immune cell activation, which has a vital role in human immunity. *H. pylori* virulence and danger factors are critical in gastritis, such as the outer inflammatory protein (OipA) and the cytotoxin associated gene a (cagA). We aimed to study the IL-32 mRNA expression in *H. pylori*-positive and negative patients as well as its relation with bacterial cagA, oipA, and severity of gastritis. Endoscopic biopsies were taken from the antrum of 60 *H. pylori*-infected patients and 62 uninfected individuals. Mucosal IL-32 mRNA expression was assessed by real-time PCR. With PCR, the *H. pylori* virulence factors were evaluated. Showed that the mRNA expression of IL-32 levels was significantly lower in biopsies of *H. pylori*-uninfected patients compared to positive individuals (P=0.01). A straight communication between virulence factor up, cagA, and heightening in IL-32 mRNA expression (P<0.001) was observed. Furthermore. IL-32 mRNA expression levels were approximately equal in both chronic and active gastritis (P=0.1). IL-32 may have a critical role in different situations like inflammation and the severity of inflammatory changes in the gastric mucosa.

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Keywords: Interleukin-32; *Helicobacter pylori*; Virulence factors; Gastritis

Introduction

Helicobacter pylori (*H. pylori*) is a type of bacterium that has a spiral shape and Gram-negative that penetrates to the gastric cells and localizes in the stomach in more than 50% of the world human population and can live for a long time in the stomach and creates a gastric inflammation with host immune involvement (1-3). *H. pylori* infections are associated with immune cells infiltration into the mucosa and gastritis, which may result in chronic inflammation (gastritis) and secretion of inflammatory markers such as IL-1 and TNFα, and finally, it can change to peptic ulcer and cancer (1,4,5). IL-32, formerly named NK-4 and in current years, IL-32 is a recently defined pro-inflammatory cytokine that is commonly produced by epithelial cells, T cells, and natural killer (NK) cells. IL-32 was first started as a transcript in IL-2 activated NK and T cells. Newly a synergism between IL-32 and other well-characterized players in innate immunity has been recognized. IL-32 has been concerned in inflammatory conditions, such as bacterial infections, and gastrointestinal disease (6). Virulence factors of the bacterium are principle agent for starting to gastric inflammation or gastritis, atrophy, and metaplasia that result in malignancy in the stomach. In several studies has been observed that levels of inflammatory cytokines in the penetration site with *H. pylori* have a relationship with the *H. pylori* virulence factors (7). This study, mucosal mRNA expression of IL-32 were assessed in both groups (in and un-patients) and measured its correlation with danger elements like virulence factors OipA, CagA, and type of gastritis.

Materials and Methods

One hundred and twenty-two specimens were collected from patients with dyspepsia referred to endoscopy center of Shahrekord Hajar Hospital, Iran, from June 2013 to March 2014. All patients were given consent about the procedure before their inclusion, in

Corresponding Author: H. Shahi
Department of Immunology, School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran
Tel: +98 9117579584, Fax: +98 2122439970, E-mail address: heshmat.shahi@gmail.com

accordance with the Helsinki Declaration (8). Exclusion criteria were: age lowers than 18-year-old or more than 70, pregnancy, diabetes type-1, systemic infection, use of drugs effective against *H. pylori* in 1 month ago, alcohol abuse and chronic corticosteroid or nonsteroidal anti-inflammatory drug use. Gastric biopsy specimens were taken from the antrum. Gastritis was investigated by endoscopy. *H. pylori* infection was detected by the polymerase chain reaction (PCR), pathological examination (PE), and rapid urease test (RUT), of three biopsies taken from the antrum. The procedure was according to the Helsinki Declaration ethics and approved by the Ethics Committee of Shahrekord University of Medical Sciences (1,9).

Histological examination

The paraffin compound gastric biopsy specimens were slice into 5-µm-thick sections after that for *H. pylori* detection stained with silver and to the grade of gastritis prepare by hematoxylin and eosin (H&E). Gastritis was scored according to the infiltration of immune cells like PMNs and MNCs in 0(None) 1(Mild) 2(Moderate) 3(Severe) degrees and dysmorphic according to the updated Sydney system (10).

The bacterial virulence factors were detected by specific primers using the PCR test (Table1). DNA extraction kit (BioFlux, Japan) was used For Genomic DNA extraction from all samples.

Table 1. Specific primers for *H. pylori* and its virulence factors

Genes	Primer sequence (5'-3')	Size (bp) of PCR product	Reference
16SrRNA	HP-1: CTGGAGAGACTAAGCCCTCC HP-2: ATTACTGACGCTGATTGTGC	109	(1)
glmM (ureA)	F: AAGCTTTTAGGGGTGTTAGGGGTTT R: GCA TTC ACA AAC TTA TCC CCA ATC	161	(1)
oipA	F: GTTTTTGATGCATGGGATTT R: GTGCATCTCTTATGGCTTT	401	(11)
cagA	F: ATGACTAACGAAACTATTGATC R: CAGGATTTTTGATCGCTTTATT	244	(1)

The PCR protocol for CagA, OipA gene evaluation was done according to the previous study (8,12,13). To measure IL-32 mRNA expression, real-time PCR was performed, the designed primers and probes were shown in table 2.

Table 2. Primer and probe sequences employed in this study

Genes	Primer and probe sequence (5'-3')
IL-32	F: GAATCAGGACGTGGACAGGTG
	R: CTCCTCATAATAAGCCGCCACTG
	P: FAM-CCCTCTTTGAAGTCGTCCTCCAGCTCTG-TAMRA
β-actin	F: AGCCTCGCCTTTGCCGA
	R: CTGGTGCCTGGGGCG
	P: FAM-CCGCCGCCCGTCCACACCCGCC-TAMRA

RNA extraction bioZol® kit was used for total RNA extraction. The Real-Time PCR protocol was used for the evaluation of the IL-32 mRNA expression levels in the biopsies according to the previous study (8). The Oligo.7 software was used for designing the sequences of β-actin and IL-32 primers and probe that are shown in Table 2 (1). IL-32 mRNA expression was compared to β-actin mRNA expression by using the $2^{-\Delta}$Ct method for Relative quantification (1,14). The whole protocol was done in duplicate.

Statistical examines

For determination data normal distribution, the normality Shapiro-Wilk test was used. For the determination of differences between mRNA expression in the infected and uninfected groups, student's t-test was used by presented as mean. $P<0.05$ were considered significant.

Results

Recognition of *H. pylori* in gastric mucosa by PCR

Patients were positive RUT, PCR, PE tests were considered as positive for *H. pylori* infection. The oipA gene was found in 71% of the *H. pylori* positive biopsies, and cagA gene was detected in 67% of *H. pylori* positive

specimens. Sixty-seven *H. pylori*-infected gastritis patients, including 33 men and 34 women and Sixty two uninfected gastritis patients, 32 men and 30 women, involved in this study.

Mucosal IL-32 mRNA expression levels in gastric mucosa biopsy

Figure 1 shows the expression ratio of IL-32 in the gastric mucosa. Mucosal IL-32 levels were significantly elevated in *H. pylori*-positive samples compared to negative samples (P=0.01) by 2.23 fold enhancement.

Prevalence of inflammation severity in active and chronic gastritis

The levels of inflammation were measured and sorted as follows: 30% (1), 40% (2), 30% (3), and approximately 33% of gastritis samples were in a chronic phase. Also the levels of inflammatory activity were: 30% (1), 37.5% (2) and 32.5% (3). Higher than 66% of gastritis samples were in the active stage (Table 4).

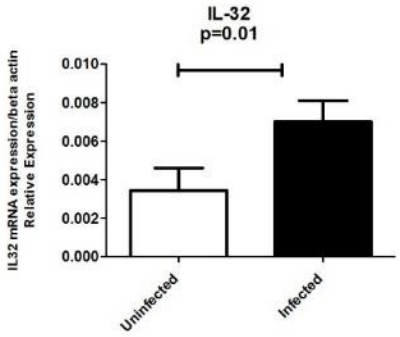


Figure 1. Comparison of Mucosal IL-32 mRNA expression level in infected and non-infected patients. 67 *H. pylori*-infected and 62 *H. pylori* non-infected patients with gastritis were analyzed for IL-32 expression by real-time PCR. Levels are normalized to β-actin

Table 3. Frequency of the cagA and the OipA in studied groups

Genotype	CagA		OipA	
	Positive	Negative	Positive	Negative
Frequency (%)	40(67)	27(33)	44(71)	23(29)

Table. 4. *H. pylori* gastritis status according to updated Sydney classification

	H. Pylori	Man/woman	Active	Chronic
Severe N (%)	+	8/11	13 (32.5)	6 (30)
Moderate N (%)	+	13/10	15 (37.5)	8 (40)
Mild N (%)	+	8/10	12 (30)	6 (30)
Total N (%)	+	29/31	40 (66.6)	20 (33.4)

The relation between mucosal expression ratio of IL-32 mRNA levels and gastric inflammation grouping

The mucosal IL-32 mRNA expression levels and mononuclear cell infiltration as chronic inflammation have a relation that was shown in Figure 2. Between the active inflammation and mucosal IL-32 mRNA expression levels not observed any significant correlation with P=0.1 and score 1.06 fold.

Effect of virulence factors oipA and cagA on the expression levels of mucosal IL-32 mRNA in *H. pylori*-positive samples

The presence of virulence factors can affect mucosal IL-32 mRNA levels. After the determination of the mean level of IL-32 in *H. pylori*-cagA positive and negative group, observed that IL-32 mRNA expression levels in CagA-positive samples are significantly higher than the negative group by 2.57 fold. Also, in the OipA-positive samples, the IL-32 mRNA expression levels significantly higher than Oip-A negative samples by 2 fold. Both P-value is shown in (Figure 3).

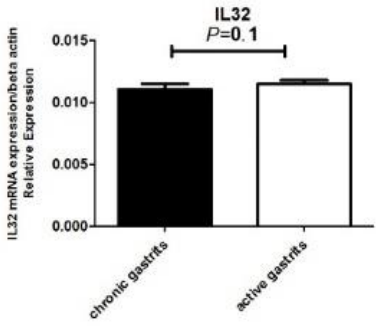
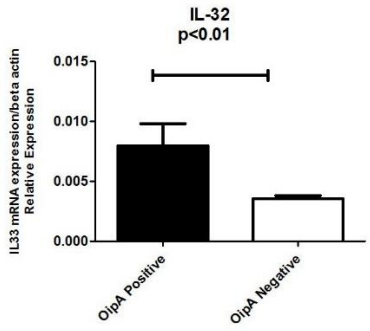


Figure 2. Comparison of Mucosal IL-32 mRNA expression level in active gastritis and chronic gastritis patients. Levels are normalized to β-actin



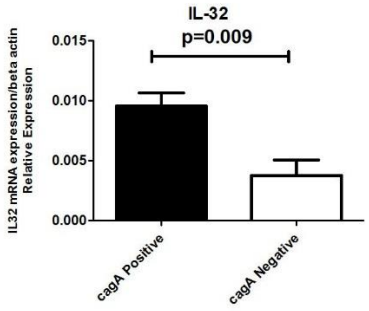


Figure 3. Mucosal IL-32 mRNA expression in *H. pylori*-infected patients according to virulence factors. IL-32 mRNA expression level was significantly lower in cagA and oipA negative samples than CagA and OipA positive samples

Discussion

H. pylori infection is a prevalent infection in the world. The instant changes in the epidemiology of clinical outcomes caused by this bacteria suggest communication between host, microbes, and environmental factors that leads to a revolution in strains differing in virulence (11). *H. pylori* pathogenesis is linked to its own virulence factors, including OipA and cagA. The OipA is a member of the large outer membrane. The prevalence of OipA virulence factor in samples that gather from Iranian people has been shown in the wide range from 33% to 71% in different studies (11,15). In this study, according to other Iranian studies, approximately 71% of the samples were positive for OipA. By using primers for detection of the CagA gene, we showed 67% of isolated strains contain this gene, which is according to the previous studies that showed the CagA prevalence varies between 44 to 91% in the Iranian population based on different ethnic background. Also according to other Iranian studies, 67% of the samples were positive for cagA (2,16,17). IL-32 has an important roles in the several inflammatory illnesses, such as T.B infection, arthritis and GI inflammatory disease (18-20). In our study, IL-32 mRNA expression levels were significantly lower in *H. pylori*-uninfected samples than infected samples. Similar to our results, sakitani *et al.*, indicated that IL-32 mRNA expression increased with a bacterial infection in the stomach (21). Opposite by our results, shown in another study that IL-32 was not detectable by ELISA in supernatants of AGS cells cultured with *H. pylori* (21). In the present study, IL-32 mRNA expression was significantly low in OipA-negative *H. pylori*-positive patients as well as cagA-negative *H. pylori*-positive patients. According to presented report, in the another study observed that IL-32 mRNA expression was raised in cagA-positive infected patients (6). To the good point of our knowledge, this report is one of the first studies about associations between IL-32 mRNA expression and OipA virulence factors and forms of gastritis. In several studies, authors showed increase IL-32 mRNA expression in chronic gastric lesions compared to primary lesions (6,21,22). Carmi *et al*., showed that IL-32 promotes angiogenesis and controls the balance between inflammation and antitumor immunity in specific tumor microenvironments (23). Also in the Another study observed thaFt levels of IL-32 in the serum were significantly lower in healthy people than patients with gastric cancer, suggesting that serum IL-32 levels may have a closer correlation with gastric cancer (24). IL-32 expression in tumor cells mainly occurs in the advanced stages of cancer (25). Cytokines and interleukins have different roles for defense against microbes by activation or suppression the immune system. Microbes induce the system by their pathogen-associated molecular patterns (PAMP) by attach to certain receptors such as Toll-like receptors (TLRs) and induce signaling pathways in both immune and non-immune cells. These signals induce the production of inflammatory cytokines like IL-32 that is a new member in the family and have relation with several pro-inflammatory diseases as well as microbial infections (26-28). Inspiration through both Toll-Like receptors (TLRs) and Nod-Like receptors (NLRs) are all essential

for processing and release of IL-32 from normal monocytes and LPS from Escherichia coli rouse host immune cells via TLR4 and TLR2 and increase IL-32 gene expression level in monocytes. Several data suggest that engagement of both TLR2 and TLR4 pathways stimulates pro-inflammatory cytokines such as IL-32 mRNA expression to respond to diverse pathogens and pathogen-associated microbial patterns (PAMPs) (29,30).

Helicobacter pylori is a human gastric pathogenic micro-organism that localized around half of the world's population. *H. pylori infection* induces chronic inflammation, like, increases the risk of duodenal ulcer, gastritis and, gastric cancer. In this study, we suggested that the increased IL-32 mRNA expression levels may be the main marker for forecasting prognosis of gastritis and has a relative role by the presence of virulence factors, especially cagA and OipA. According to the current knowledge, evaluation of the human and *H. pylori* genome sequences and animal models might be useful to know the biological basis of *H. pylori*-associated disorders, especially in early age.

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