Serum TNF-α levels and *Helicobacter pylori* cagA and vacA genes

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Abstract. *Helicobacter pylori* is associated with higher virulence. TNF-α has an important role in host defense against *H. pylori* infection. The aim of this study was to investigate the relationship between TNF-α serum levels with cagA and vacA genes in *H. pylori* infection. This was a cross-sectional study involving 80 patients that consecutively admitted to endoscopy unit. Diagnosis of *H. pylori* infection was based on rapid urease test. Serum samples were collected to determine circulating TNF-α level. Polymerase chain reaction was done to examine *H. pylori* vacA and cagA genes. Data analysis was carried out using SPSS version 22 with 95%CI and p<0.05 was considered statistically significant. About 45 (56.3%) patients infected with *Helicobacter pylori*. There were 33 (73.3%) patients with *H. pylori* cagA positive. Serum level of TNF-α was significantly higher in cagA positive than negative. Subjects with *H. pylori* cagA gene positive were more likely to have higher level of serum TNF-α than *H. pylori* cagA gene negative.

1. Introduction

*Helicobacter pylori* (*H. pylori*) is a spiral-shaped, gram-negative bacterium that establishes chronic colonization in the human stomach and is a causative pathogen of various gastroduodenal diseases, including gastritis, peptic ulcer disease (PUD) or mucosa-associated lymphoid tissue (MALT) lymphoma.[1] *H. pylori* infection is generally asymptomatic. *H. pylori* can induce increased proinflammatory cytokines, macrophages, lymphocytes, and neutrophils.[2] Individuals infected with *H. pylori* positive cagA/vacA status susceptible to severe gastritis that induce peptic ulcer and gastric malignancies.[3] *H. pylori* virulence factors induce pro-inflammatory cytokines such as TNF-α, which influence mucosal inflammation and/or gastric acid secretion.[4] TNF-α has an important role in host defense against *H. pylori* infection, but a high concentration of this cytokine may cause severe pathology.[5]

Many studies reported relationship of *H. pylori* infection with increased expression of TNF-α in gastric mucosa. However, the studies that discussed the relationship of serum levels of TNF-α with *H. pylori* virulence were limited. The study aimed to investigate the relationship between TNF-α serum levels with cagA and vacA gene in *H. pylori* infection.
2. Methods

2.1 Patient Selection
This study was a cross-sectional study of eighty gastritis patients that were consecutively admitted to 2 hospitals, *Adam Malik General Hospital* and *Permata Bunda Hospital*, Medan, Indonesia between May and December 2016. Inclusion criteria are male or female aged ≥18 years old, diagnosed with gastritis by endoscopy and histopathologic examination, willing to be recruited in the study and signed the patient consent forms. None of the patients had received antibiotics, a bismuth compound, H2 antagonists, proton pump inhibitors or immune modulating drugs within the last 1 month before endoscopy. Exclusion criteria were patients with malignancy, immunosuppression, metabolic disorders, or gastrointestinal hemorrhage, and patients who had a history of gastric surgery. This study has been approved by the local ethics committee. During endoscopy examination, gastric biopsy specimens were taken for rapid urease test, histopathology and polymerase chain reaction (PCR) test.

2.2 Histological Assessment of Gastritis
Gastritis was diagnosed by histopathologic examination. The following procedure was done by taking a biopsy from gastric antrum and corpus, staining them using a Hematoxylin-Eosin, and analyzing the pathology of gastric mucosa referring to the visual analog scale of the updated Sydney System.[6]

2.3 Helicobacter pylori detection
The rapid urease test (Pronto Dry®, Gastrex, France) was used to establish the diagnosis of *H. pylori* infection. Results were read within 24 hours. We reported it as positive if the color changed from amber to pink-red within 24 hours of incubation at room temperature. The yellow color is considered a negative result.[7]

2.4 Polymerase Chain Reaction
During endoscopy, antral gastric biopsy specimens were taken. Biopsies are done to extract DNA, by the QIAmp DNA Mini Kit (Qiagen, Valencia, CA, USA). Extracted DNA was used for subsequent PCR experiments.

2.5 Serum Levels of TNF-α
Venous blood was drawn using a serum separator tube. It allowed clotting for 30-45 minutes at room temperature. Then, centrifugation for 15 minutes at approximately 1,000 g. Serum then was stored frozen in aliquots at -20°C until assay for TNF-α was performed. Circulating TNF-α levels were measured by means. It used a high sensitivity ELISA that uses an additional amplification step (HS Quantikine, R&D Systems, Inc., Minneapolis).[8]

2.6 Statistical Methods
Data analysis was performed through univariate, bivariate (Mann Whitney U-test), and logistic regression analyses using the SPSS 22nd version (SPSS Inc., Chicago) with a 95% confidence interval. P<0.05 was considered statistically significant.

3. Results
The major occupations of subjects were employees (43.7%), housewife (33.7%) and entrepreneurs (11.3%). The mean age of the subjects was 46.73±13.19 years. About 45 (56.25%) subjects were male patients and 35 (43.75%) of female patients. Around 45 (56.3%) *H. pylori*-infected patients. The median of TNF-α serum was 1.72 pg/mL (0.70–37.76 pg/mL). (Table 1)

| Table 1. Basic characteristics of the subjects. |
From 45 patients infected with *H. pylori*, 33 (73.3%) patients had *H. pylori* with *cagA* gene positive. All of them had *H. pylori* with *vacA* gene due to the whole *H. pylori* strains carrying *vacA* gene. (Table 2)

Table 2. Distribution of *H. pylori* *cagA* and *vacA* gene status.

| *H. pylori* Status | *cagA* Gene | *vacA* Gene |
|--------------------|-------------|-------------|
| Positive           | 33 (73.3%)  | 45 (100%)   |
| Negative           | 12 (26.7%)  | 0 (0%)      |

There was a significant difference in the mean serum TNF-α levels between patients with *H. pylori* positive and negative (p=0.001), while patients with *H. pylori* positive had serum levels of TNF-α significantly higher than *H. pylori* negative (5.01±6.89 vs 2.09±2.73 pg/mL). (Table 3)

Table 3. Comparison of serum TNF-α levels between patients with *H. pylori* positive and negative (n = 80).

| *H. pylori* Status | TNF-α Serum (Mean ± SD) | p     |
|--------------------|-------------------------|-------|
| Positive           | 5.01 ± 6.89 pg/mL       | 0.001*|
| Negative           | 2.09 ± 2.73 pg/mL       |       |

*p<0.05

There was a significant difference in the mean serum TNF-α levels between patients with *H. pylori* *cagA* gene positive and negative (p=0.0001). Patients with *H. pylori* *cagA* gene positive had serum levels of TNF-α significantly higher than *H. pylori* *cagA* gene negative (6.30±7.67 vs. 1.47±0.47 pg/mL). (Table 4)

Table 4. Comparison of serum TNF-α levels between patients with *H. pylori* positive and negative (n = 80).

| *H. pylori* Status | TNF-α Serum (Mean ± SD) | p  |
|--------------------|-------------------------|----|
| Positive           | 6.30 ± 7.67 pg/mL       | 0.0001*|
| Negative           | 1.47 ± 0.47 pg/mL       |    |

*p<0.05
H. pylori cagA gene positive and negative (n = 45).

| cagA Gene | Serum TNF-α (Mean ± SD) | p     |
|-----------|-------------------------|-------|
| Positive  | 6.30 ± 7.67 pg/mL       | 0.0001a |
| Negative  | 1.47 ± 0.47 pg/mL       |       |

*p<0.05

Logistic regression was performed to ascertain the effect of cagA gene status on the likelihood that subjects have a high level of serum TNF-α. The logistic regression model was statistically significant (p=0.002). Patients with H. pylori cagA gene positive were 11.13 times more likely to have a higher level of serum TNF-α than H. pylori cagA gene negative. (Table 5)

Table 5. Logistic regression for the association between H. pylori cagA gene and serum TNF-α levels (n=45).

| Variable | OR (95% CI)     | P     |
|----------|-----------------|-------|
| cagA gene | 11.13 (2.34 – 52.89) | 0.002b |

*a*adjusted for age and sex;

*b*p<0.05

4. Discussion

The average age of H. pylori positive patients was 50.44±12.44 years old and 41.94±12.72 years old for H. pylori negative. Our results were comparable to a study conducted by Chai FY et al., which reported that the average age of H. pylori-positive patients was 47.74±14.93 years old and 50.50±15.02 years old for H. pylori negative.[9]

The prevalence of H. pylori in this study (56.5%) was comparable to a study conducted by Pan KF et al., which reported that the prevalence of H. pylori among Chinese was 57.6%.[10] Myint T et al. revealed that the prevalence of H. pylori in Myanmar was 48.0%.[11] In Indonesia, Syam AF et al. reported the prevalence of H. pylori was 22.1%.[12]

Various virulence factors are involved in H. pylori-mediated pathogenicity in gastric epithelial cells. One of them was cagA that encoded at one end of the Cytotoxin-associated genes pathogenicity island (cagPAI). CagA gene was more frequently associated with severe gastric inflammation, ulceration, and an increased risk of gastric cancer.[13,14] In the present study, cagA gene was found in 33 (73.3%) H. pylori positive patients. Our result was comparable to a study conducted by Salimzadeh et al., which reported that 71.2% H. pylori positive patients had cagA gene.[15] Trang et al. conducted a study in Bhutan, Vietnam and Myanmar, reported that all H. pylori (100%) had cagA gene in Bhutan, but in Vietnam and Myanmar were 95.1% and 88.4% respectively.[16]

The vacuolating cytotoxin (vacA) is also one of the major virulence factors released by H. pylori. VacA causes the formation of large vacuoles and the induction of apoptosis in gastric epithelial cells. [13] Almost all H. pylori contain the vacA gene that encodes a vacuolating cytotoxin.[14,17] In this study, vacA gene was found in all H. pylori, positive patients.

The immune response is induced by the contact of H. pylori with gastric cells and is followed by the stimulation of pro-inflammatory cytokine production such as TNF-α.[5] TNF-α has many biological activities, including the stimulation of the expression of adhesion molecules, such as intercellular adhesion molecule 1, on endothelial cells (which facilitates the extravasation of neutrophils into the lamina propria of the mucosa), activation of leukocytes and T-lymphocytes, stimulation of the production of cytokines by macrophages and monocytes and the induction of apoptosis. H. pylori infection elevates TNF-α in tissues and induces cytotoxicity and apoptosis of gastric epithelial cells.[18] This study also found that serum TNF-α level in the infected group significantly higher compared to H. pylori negative (p<0.05). The previous study also suggested that H. pylori can upregulate the VEGF serum levels.[19]

Cytotoxin-associated genes pathogenicity island (cagPAI) expresses a needle-like structure, type IV secretion system (T4SS) that is required for the injection of the protein of cytotoxin-associated gene A
(cagA) or peptidoglycan into thecytosol of host cells. *H. pylori* peptidoglycan is recognized by a cytosolic receptor, nucleotide-binding oligomerization domain (NOD) 1, which leads to NF-κB activation.[13] A study by Lin Q et al. showed that *Helicobacter pylori* CagA induced the expression of P300/CBP-associated factor (PCAF) in gastric epithelial cells. PCAF was able to physically associate with the NF-κB p65 sub-unit and enhance its acetylation. More importantly, PCAF-induced p65 acetylation was shown to contribute to p65 phosphorylation and further upregulation of TNF-α. [20] In this study, there was a significant difference in TNF-α serum levels between cagA positive and cagA negative [6.30±7.67 vs. 1.47±0.47 pg/ml; p=0.0001]. CagA-expressing *H. pylori* are associated with an enhanced host inflammatory response.[14] Subjects with *H. pylori* cagA gene positive were 11.13 (p=0.002) times more likely to have a higher level of serum TNF-α than *H. pylori* cagA gene negative. The limitation of this study was that the diagnosis of *H. pylori* only used one method (rapid urease test) while other methods may give different results. In addition, the sample size was small.

5. Conclusion

Serum TNF-α levels were significantly higher in patients with *H. pylori* cagA gene positive compared to cagA gene negative. This result was reasonable because cagA gene of *H. pylori* associated with higher virulence of *H. pylori* and severe inflammatory responses.

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