Serum VEGF Levels in *Helicobacter pylori* Infection and Correlation with *Helicobacter pylori* cagA and vacA Genes

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Abstract

**BACKGROUND:** Helicobacter pylori vacA and cagA genes are associated with higher virulence. Vascular Endothelial Growth Factor (VEGF) is one important marker for neoangiogenesis.

**AIM:** The purpose of this study was to investigate the relationship between VEGF serum levels with cagA and vacA genes in *H. pylori* infection.

**METHODS:** A cross-sectional study was done on eighty patients that consecutive admitted to endoscopy unit. The diagnosis of *H. pylori* infection was based on rapid urease test. Serum samples were obtained to determine circulating VEGF level. Polymerase chain reaction was done to examine *H. pylori* cagA and vacA genes. Data analysis were carried-out using SPSS version 22.

**RESULTS:** A total of 80 patients were examined. There were 45 (56.3%) patients infected with *Helicobacter pylori*. There were 33 (73.3%) patients with *H. pylori* cagA positive. Serum VEGF levels in patients with the *H. pylori* positive were significantly higher compared to the patients that have no *H. pylori*. Serum levels of VEGF were significantly higher in cagA positive than negative.

**CONCLUSION:** Serum VEGF level is correlated with *H. pylori* infection and its virulence status. The more virulence of *H. pylori*, cagA gene, the higher serum VEGF levels were found.

Introduction

*Helicobacter pylori* (*H. pylori*) infection is estimated occurred in 50% of the population in the world where the majority of these infections occur in developing countries with a percentage between 70-90% while only 40-50% occur in industrialised countries [1, 2]. The prevalence of *H. pylori* in Western countries continues to decline due to the improvement of living standards, good hygiene, low population density, and the use of antibiotics, while in Asia including in Indonesia, *H. pylori* infection rate is very high [3, 4].

*H. pylori* infection is the most common cause of chronic gastritis in worldwide. *H. pylori* which colonize in the human stomach can cause chronic gastritis, peptic ulcer disease, gastric cancer, lymphoma mucosa related tissue (MALT). Status of vacA and cagA *H. pylori* most associated with higher virulence of *H. pylori*. Individuals infected with *H. pylori* positive cagA / vacA status susceptible to severe gastritis that induce peptic ulcer and gastric malignancies [5].

Gastritis inflammatory response can occur either in acute or chronic condition. General mechanisms involved in the pathogenesis of inflammatory and ulcerative epithelial lesion is neoangiogenesis which is the development of new blood vessels from existing endothelial precursors. Vascular Endothelial Growth Factor (VEGF) is one important marker for neoangiogenesis. Tucillo et al. reported an increased expression of VEGF mucosa in *H. pylori* gastritis [6]. Caputo et al. report the *H. pylori* vacA gene can induce the expression of VEGF mucosa in patients with gastric malignancy [7]. Many types of research on the relationship of *H. pylori* virulence with increased expression of VEGF in the gastric mucosa have been done, which the expression of VEGF-related to angiogenesis and contributed to
the occurrence of gastric malignancy. However, the
studies discussed the relationship serum levels of
VEGF with *H. pylori* virulence were limited. The
purpose of this study was to investigate the
relationship between VEGF serum levels with *cagA*
and *vacA* gene in *H. pylori* infection.

**Material and Methods**

**Patient Selection**

This study was a cross-sectional study on
eighty consecutive gastritis patients that were
admitted to Endoscopy Unit at Adam Malik General
Hospital and Permata Bunda Hospital, Medan,
Indonesia between May and December 2016. Inclusion
criteria are stated as followings: male or female aged ≥ 18 years old, patients were diagnosed
with gastritis on 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 four weeks before
endoscopy. Patients with evidence of malignancy,
immunosuppression, metabolic disorders, or
gastrointestinal haemorrhage, and patients who had a
history of gastric surgery excluded. This study was
approved by the local ethics committee. During
endoscopy examination, gaster biopsy specimens
were taken for rapid urease, histopathology and
polymerase chain reaction tests.

**Histological Assessment of Gastritis**

A diagnosis of gastritis was made by a
histopathologic examination. The following procedure
was done by taking a biopsy from the gastric antrum
and corpus, staining them using a Hematoxylin-Eosin
stain, and analysing the pathology of the gastric
mucosa referring to the visual analogue scale of the
updated Sydney System [8]. All specimens were
examined by the same professionals at the laboratory
of anatomical pathology in the University of Sumatera
Utara.

**Helicobacter pylori detection**

The rapid urease test (Pronto Dry®; Gastric,
France) was used to establish the diagnosis of *H.
pylori* infection. The results were read within 24 hours.
The yellow colour is considered a negative result. A
positive result was reported if the colour changed from
amber to pink-red within 24 hours of incubation at
room temperature [9].

**Polymerase Chain Reaction**

Antral gastric biopsy specimens were
collected during endoscopy. DNA was extracted from
the biopsies by the QIAmp DNA Mini Kit (Qiagen,
Valencia, CA, USA) following the manufacturer’s
instructions. Extracted DNA was used for subsequent
PCR experiments. Amplification was conducted in a
total volume of 25 µL. The reaction mixture contained
12.5 µL, 2X ready PCR mix (Thermo Scientific) and
consisted of 1.25 U Taq-Pol, 75 mM Tris-HCl (pH 8.8),
1.5 mM MgCl₂, and 0.2 mM of each dNTP. The
reaction mixture contained 12.5 µL master mix, 1.0
µM of each forward and reversed primers, 1 µg DNA
template, and 8.5 µL RNase-free water to a total
volume of 25 µL. The amplification was carried out in
a C-1000 thermal cycler (Bio-Rad, USA) according to
the following program: an initial denaturation step at
95°C for 10 min, followed by 35 cycles of denaturation
at 95°C for 30 s, annealing, primer specific for 1 min,
and a final extension step at 72°C for 5 min. Amplified
PCR products were resolved by agarose gel
electrophoresis (5V/60 min) using 1.5% agarose in
Tris-Acetate-EDTA (TAE) buffer containing 0.5 µg/mL
of ethidium bromide. Molecular size ladder of 1 kb
(Fermentans, Germany) was used to determine the
size of the bands. The gel was observed and
photographed on a Gel-Doc System (Bio-Rad, USA).

**Serum Levels of VEGF**

Venous blood was drawn using a serum
separator tube and allowed to clot for 30-45 minutes
at room temperature before centrifugation for 15
minutes at approximately 1,000 g. Serum was
immediately stored frozen in aliquots at -20°C until
assay for VEGF was performed. Circulating VEGF
levels were examined in serum using the Quantikine
Human VEGF-ELISA (Quantikine, R&D Systems, Inc.,
Minneapolis).

**Statistical Methods**

Data analysis was performed through
univariate and bivariate analyses using the SPSS 22nd
version (SPSS Inc., Chicago) with a 95% confidence
interval. Bivariate analysis was performed using a
Mann-Whitney test and logistic regression with
significance p<0.05.
Results

The mean age of the 80 subjects was 46.73 ± 13.19 years, with a range between 19-68 years. There were 45 (56.25%) male patients and 35 (43.75%) female patients. Three major occupations of the patients were employees (43.7%), housewife (33.7%) and entrepreneurs (11.3%). There were 45 (56.3%) H. pylori-infected patients. The median of VEGF serum was 390.2 pg/mL (65.3 – 2526.9 pg/mL) (Table 1).

| Table 1: Basic characteristics of the subjects |
|---------------------------------------------|
| Characteristics                          | H. pylori Positive | H. pylori Negative | Total n=80 |
|-------------------------------------------|--------------------|-------------------|------------|
| Sex                                       |                    |                   |            |
| Male                                      | 29 (64.4%)         | 45 (100%)         |            |
| Female                                    | 16 (45.7%)         | 35 (100%)         |            |
| Age (years)*                             | 50.44 ± 12.44      | 41.94 ± 12.72     | 46.73 ± 13.19 |
| Occupation                                |                    |                   |            |
| Employee                                  | 20 (57.1%)         | 35 (100%)         |            |
| Housewife                                 | 16 (59.3%)         | 27 (100%)         |            |
| Entrepreneur                              | 5 (55.6%)          | 9 (100%)          |            |
| Others                                    | 4 (44.4%)          | 5 (55.6%)         |            |
| Educational status                        |                    |                   |            |
| Primary school                            | 4 (66.7%)          | 6 (100%)          |            |
| Junior high school                        | 5 (71.4%)          | 7 (100%)          |            |
| Senior high school                        | 30 (57.7%)         | 52 (100%)         |            |
| College                                   | 6 (40%)            | 15 (100%)         |            |
| VEGF serum (pg/mL)*                      | 338.23 ± 2182.2    | 293.4 ± 2526.9    | 390.2 ± 2526.9 |

n: Total number of subjects; * mean ± SD; # median (min – max).

Logistic regression was performed to ascertain the effect of cagA gene status on the likelihood that subjects have a high level of serum VEGF. The logistic regression mode was statistically significant (p = 0.037). Patients with H. pylori cagA gene positive were 10.82 times more likely to have a higher level of serum VEGF than H. pylori cagA gene negative (Table 4).

| Table 4: Comparison of serum VEGF levels between patients with H. pylori cagA gene (n=45) |
|---------------------------------------------|
| cagA Gene     | Serum VEGF (Mean ± SD) | p     |
|----------------|-------------------------|-------|
| Positive       | 399.08 ± 350.42 pg/mL   | 0.017*|
| Negative       | 338.23 ± 2182.2 pg/mL   |       |

n: Total number of subjects; *p<0.05

| Table 5: Logistic regression for the association between H. pylori cagA gene and serum VEGF levels (n=45) |
|---------------------------------------------|
| Variable     | OR (95% CI) | p     |
|-----------------|------------|-------|
| cagA gene       | 10.82 (1.14 – 101.93) | 0.037*|

n: Total number of subjects; * adjusted for age and sex; p<0.05

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 patients. Our results were comparable to a study conducted by Salimzadeh L et al., which reported that the average age of H. pylori positive patients were 46.74 ± 16.79 years old and 48.56 ± 19.82 years old for H. pylori negative [10]. Meanwhile, in Laos, the mean age of H. pylori positive patients was 46 years old [11].

The prevalence of H. pylori in this study (56.5%) was higher than other studies. Salimzadeh L et al. reported that the prevalence of H. pylori among Iran was 44.4% [10]. While Myint T et al. revealed that the prevalence of H. pylori in Myanmar was 48.0% [12]. In Indonesia, Syam AF et al. reported the prevalence of H. pylori was 22.1% [13]. The difference occurred as this study was not population-based study.

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 [14, 15]. In the present study, cagA gene was found in 33 (73.3%) H. pylori positive patients. A study by Yakut M et al. in Turkey reported that 38 of 98 (38.7%) H. pylori positive patients had cagA gene [16]. Trang et al. conducted a study in Bhutan, Vietnam and Myanmar, reported that negative (656.89 ± 497.95 vs. 399.08 ± 385.00 pg/mL) (Table 4).
all *H. pylori* (100%) had cagA gene in Bhutan, but in Vietnam and Myanmar were 95.1% and 88.4% respectively [17].

The vacuolating cytotoxin A (*vacA*) is also one of major virulence factors released by *H. pylori*. *VacA* causes the formation of large vacuoles and the induction of apoptosis in gastric epithelial cells [14]. Almost all *H. pylori* contain the *vacA* gene that encodes a vacuolating cytotoxin [15, 18]. In this study, *vacA* gene was found in all *H. pylori*, positive patients.

Vascular endothelial growth factor (VEGF) is a central regulator of angiogenesis and vasculogenesis. There are evidence showing that VEGF expression is closely associated with poor prognosis and adverse clinical characteristics of gastric cancer such as tumour invasion and lymph node metastasis and *H. pylori* upregulates VEGF expression in gastric epithelial cells. Several mechanisms such as NF-κB, cyclooxygenase-2 (COX-2), and epidermal growth factor receptor (EGFR) signalling are considered to mediate *H. pylori*-induced VEGF production in gastric epithelial cells [14]. This study also found that serum VEGF 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 the cytosol of host cells. *H. pylori* peptidoglycan is recognised by a cytosolic receptor, nucleotide-binding oligomerization domain (NOD) 1, which leads to NF-κB activation and IL-8 production. A study conducted by Kang et al. reported that *H. pylori* could induce VEGF production in gastric epithelial cells via both T4SS-dependent and T4SS-independent pathways [14]. In this study, there was a significant difference in VEGF serum levels between cagA positive and cagA negative [719.27 ± 525.60 vs. 402.80 ± 442.67 pg/ml; p = 0.002]. CagA-expressing *H. pylori* are associated with an enhanced host inflammatory response [15]. Subjects with *H. pylori* cagA gene positive were 10.82 times more likely to have a higher level of serum VEGF 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) whiles other methods may give different results. Also, the sample size was small.

In conclusion, serum VEGF level is correlated with *H. pylori* infection and its virulence status. The more virulence of *H. pylori*, cagA gene, the higher serum VEGF levels were found.

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