***Open Access Maced J Med Sci* electronic publication ahead of print,**
published on March 19, 2017 as https://doi.org/10.3889/oamjms.2017.031

ID Design 2012/DOOEL Skopje, Republic of Macedonia
Open Access Macedonian Journal of Medical Sciences.
https://doi.org/10.3889/oamjms.2017.031
eISSN: 1857-9655
Basic Science

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

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Citation: Siregar G, Sari D, Sungkar T. Serum VEGF Levels in Helicobacter pylori Infection and Correlation with Helicobacter pylori cagA and vacA Genes. Open Access Maced J Med Sci. https://doi.org/10.3889/oamjms.2017.031

Keywords: *Helicobacter pylori*; Vascular endothelial growth factor; *cagA gene; vacA gene*; Gastritis.

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Received: 25-Jan-2017; **Revised:** 09-Feb-2017; **Accepted:** 10-Feb-2017; **Online first:** 19-Mar-2017

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Funding: This research did not receive any financial support.

Competing Interests: The authors have declared that no competing interests exist.

Abstract

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

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 vacA* and *cagA* 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 c*agA 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 $MgCl_2$, 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).

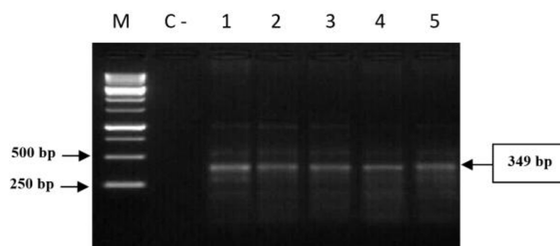

Figure 1: PCR Amplification product of cagA gene 349 bp of H. pylori. (Lane M: Ladder marker, Lane 1 to 5 biopsy samples, C- negative control)

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 (n = 45)	*H. pylori* Negative (n = 35)	Total (n = 80)
Sex			
Male	29 (64.4%)	16 (35.6%)	45 (100%)
Female	16 (45.7%)	19 (54.3%)	35 (100%)
Age (years)[a]	50.44 ± 12.44	41.94 ± 12.72	46.73 ± 13.19
Occupation			
Employee	20 (57.1%)	15 (42.9%)	35 (100%)
House wife	16 (59.3%)	11 (40.7%)	27 (100%)
Entrepreneur	5 (55.6%)	4 (44.4%)	9 (100%)
Others	4 (44.4%)	5 (55.6%)	9 (100%)
Educational status			
Primary school	4 (66.7%)	2 (33.3%)	6 (100%)
Junior high school	5 (71.4%)	2 (28.6%)	7 (100%)
Senior high school	30 (57.7%)	22 (42.3%)	52 (100%)
College	6 (40%)	9 (60%)	15 (100%)
VEGF serum (pg/mL)[b]	441.7 (80.7–2182.2)	293.4 (65.3–2526.9)	390.2 (65.3–2526.9)

n: Total number of subjects; [a] mean ± SD; [b] median (min – max).

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 cagA & *vacA* Gene Status	n = 45
cagA Gene	
Positive	33 (73.3%)
Negative	12 (26.7%)
vacA Gene	
Positive	45 (100%)

n: Total number of subjects.

There was a significant difference in the mean serum VEGF levels between patients with *H. pylori* positive and negative (p = 0.026), while patients with *H. pylori* positive had serum levels of VEGF significantly higher than *H. pylori* negative (586.14 ± 462.8 vs. 416.39 ± 400.42 pg/mL) (Table 3).

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

H. pylori Status	VEGF Serum (Mean ± SD)	p
Positive	586.14 ± 462.80 pg/mL	0.026*
Negative	416.39 ± 400.42 pg/mL	

n: Total number of subjects; *p<0.05

There was a significant difference in the mean serum VEGF levels between patients with *H. pylori cagA* gene positive and negative (p = 0.017). Patients with positive *H. pylori cagA* gene had serum levels of VEGF significantly higher than *H. pylori cagA* gene negative (656.89 ± 497.95 vs. 399.08 ± 385.00 pg/mL) (Table 4).

Table 4: Comparison of serum VEGF levels between patients with *H. pylori cagA* gene (n = 45)

cagA	Serum VEGF (Mean ± SD)	p
Positive	676.28 ± 503.15 pg/mL	0.017*
Negative	338.23 ± 169.75 pg/mL	

n: Total number of subjects; *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 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 5).

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; [an] 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

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 (c*agA*) 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 c*agA* positive and c*agA* 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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