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ASSOCIATION BETWEEN VEGF +936C>T GENE POLYMORPHISM WITH GASTRIC PREMALIGNANT LESIONS AND VEGF LEVELS IN *HELICOBACTER PYLORI* PATIENTS

Gontar Alamsyah Siregar* — Division Of Gastroentero-hepatology, Department Of Internal Medicine, Faculty Of Medicine, Universitas Sumatera Utara, Medan, Indonesia *Corresponding Author

Zaimah Z Tala — Department of Nutrition, Faculty of Medicine, Universitas Sumatera Utara, Medan, Indonesia

Ginanda Putra Siregar — Division of Urology, Department of Surgery, Faculty of Medicine, Universitas Sumatera Utara, Medan, Indonesia

ABSTRACT

The objective of this study was to evaluate the association between VEGF +936 C>T gene polymorphism with gastric premalignant lesions as well as the serum VEGF levels. This cross-sectional study included 80 *H. pylori* patients at Haji Adam Malik General Hospital and Permata Bunda General Hospital, Medan, Indonesia. Diagnosis of *H. pylori* infection was made if positive results of ^{14}C-UBT and/orCLO test were found. If any findings such as chronic atrophic gastritis, intestinal metaplasia, or dysplasia were present in microscopic evaluation, a diagnosis of gastric premalignant lesion was made. Real time polymerase chain reaction (RT-PCR) was used to examine VEGF +936C>T gene polymorphism. Level of circulating VEGF was determined using serum samples. Data were collected and analyzed using SPSS version 22. There was no significant association between VEGF +936 C>T polymorphism and gastric premalignant lesions (p>0.05). Difference in serum VEGF levels between genotypes and alleles of VEGF +936 C>T polymorphism were not significant (p=0.179 and p=0.076 respectively). VEGF +936 C>T polymorphism was not associated with an increased risk of gastric premalignant transformation and serum VEGF levels in H.pylori patients.

KEYWORDS : Gastric Premalignant Lesion, *Helicobacter Pylori*, Vegf, Polymorphism

INTRODUCTION

Gastritis can be caused by *Helicobacter pylori* (*H. pylori)* infection, bile reflux, nonsteroidal anti-inflammatory drugs, autoimmune, and allergic response[1]. *H. pylori* has been identified as a type 1 carcinogen according to International Agency for Research on Cancer (IARC). *H. pylori* infection is estimated to occur in 50% of the world's population where most of these infections occur in developing countries of 70-90% and only 40-50% in industrialized countries[2]. In Japan as well as Eastern Asia, *H. pylori* infection is highly correlated with the incidence of gastric cancer.[3]

Epidemiological studies suggest risk factors of developing gastric cancer include *H. pylori*, old-age, male gender, consumption of smoked food, marinated fish, meat, smoking habit, and obesity. Although the exposure of these risk factors are almost the same among individuals, vulnerability of each person to gastric cancer varies. Genetic factors have been suspected to play an important role in gastric cancer development.[4,5]

Infection of *H. pylori* triggers inflammation, neutrophil and monocytes recruitment, increase proinflammatory cytokines and therefore leading to gastric mucosal damage. This infection is the main cause of chronic gastritis progressing into advanced stages, such as atrophic gastritis, intestinal metaplasia, and gastric cancer. However, some of these patients might not develop gastric premalignant lesions and malignancies. Genetic factors may have an important role here.[6] *H. pylori* contributes to the pathogenesis of gastric malignancy by activating angiogenesis of the host. Among the angiogenic factors, vascular endothelial growth factor (VEGF) is the most potent neoangiogenesis stimulus.[7,8,9]

Angiogenesis is an early stage in the growth, invasion, and metastasis of the tumor. Gastric adenocarcinoma patients often exhibit increased VEGF expression followed by increased intratumor microvascular density. Increased VEGF expression that contributes to the initial process of carcinogenesis was also found in patients with gastric premalignant lesions.[10] These patients are at high risk of developing gastric cancer within 10 years.[4]

Located on chromosome 6p21.3, the highly polymorphic VEGF gene is organized into 8 exons and 7 introns (140 variants). VEGF gene polymorphisms can be found in many different locations and may play a role in carcinogenesis.[11-14] Of these variants, some of the SNPs are shown to be involved in the etiology of malignancy, one of which is VEGF +936 C>T polymorphism. A study by Bae et al (2008) of 154 gastric cancer patients in Korea showed T alleles of VEGF +936 C>T polymorphism associated with an increased risk of gastric cancer.[11]

To the authors' knowledge, there is no published studies that evaluates the association between VEGF +936C>T polymorphism with gastric premalignant lesions in *H. pylori* patients in Indonesia. Therefore this study was conducted to determine the association between VEGF +936 C>T polymorphism with gastric premalignant lesions and serum level of VEGF in *H. pylori* patients.

Methods

Patients Selection

Eighty patients from endoscopy unit of Haji Adam Malik General Hospital and Permata Bunda General Hospital from March until June 2018 participated in this cross-sectional study. Inclusion criteria include gastritis patients diagnosed based on histopathological examination, positive results of ^{14}C-UBT and/ or rapid urease test, at least 18 years old, and willing to take part in the study. Exclusion criteria within the study group were as follows: *H. pylori* eradication treatment in the last 6 months or ongoing antibiotic therapy on *H. pylori*; historical usage of proton pump inhibitors, H2 receptor antagonists, NSAIDs, steroids or alcohol within the past month; patients with systemic disease or other malignancies. The study was then approved by the local ethics committee of Universitas Sumatera Utara.

Helicobacter pylori detection

H. pylori infection can be diagnosed if positive results of ^{14}C-UBT and/or CLO test were found. Subjects undergoing ^{14}C-UBT test swallowed 37 kBq (1μCi) of encapsulated ^{14}C urea/citric acid composition with 25 ml water after overnight fasting (at least 6 hours). 10 min after the ingestion of ^{14}C urea, subjects exhaled into the Heliprobe Breath Cards (Noster system) until its color indicator changed from orange to yellow. Using the Heliprobe analyzer (Noster system), the breath samples were measured and the activity was counted for 250s. Results were expressed both as counts per

minute (cpm) and as grade. Counts of <25 cpm were defined as Heliprobe 0 (not infected), counts between 25 cpm and 50 cpm as Heliprobe 1 (equivocal) and counts of >25 cpm were defined as Heliprobe 2 (infected).[12]

The CLO test (Pronto Dry®, France) was also used to diagnose of *H. pylori* infection. A positive reaction shows an amber color turning into pink-red at room temperature within 24 hours. Yellow is considered to be negative.[13]

Diagnosis of Gastric Premalignant Lesion

Microscopic evaluation was done to find if any lesions such as chronic atrophic gastritis, intestinal metaplasia, and dysplasia were present. Any findings of these lesions confirmed the presence of gastric premalignant lesion. Histopathological examination were done by two Pathologists at Universitas Sumatera Utara in a double-blind manner. If any differences in the histopathological findings of both experts was found, a third Pathologist was asked to perform another histopathological examination.

Serum Levels of VEGF

Serum level of VEGF was determined using venous blood samples that were collected into serum separator tubes and allowed to clot at room temperaturefor at least 30-45 minutes. After 15 minutes at approximately 1,000 g of serum centrifugation, serum was immediately stored frozen in aliquots at -20°C until VEGF assay was performed. VEGF serum levels were examined using ELISA method (Quantikine(R) ELISA,Human VEGF Immunoassay R&D Systems, Inc., Minneapolis). This was done using a quantitative sandwich enzyme immunoassay technique. First of all, specific monoclonal antibody to VEGF was coated onto a microplate. Standards, samples, controls and conjugates are inserted into the well using pipettes and VEGF will be sandwiched by immobilized antibody with specific enzyme-linked antibody to VEGF. Following a wash to remove any unbonded substances and/or antibody enzyme-reagent, a substrate solution was added to the well which forms a color that is proportional to the amount of bound VEGF. The color formation is stopped and the color intensity was measured.

VEGF +936C>T Polymorphism

Extraction of DNA (Spin Column Method) using High Pure PCR Template Preparation reagent.TaqMan SNP Genotyping Assays 2010963 was used and PCR primers used for the +936C>T polymorphisms were 5'-GTAGCAAGAGCTCCAGAGAGAAGT-3' (forward primer) dan 5'-TGGACGAAAAGTTTCAGTGCGACG-3' (reverse primer). Reaction mixture contains a volume total of 25 µL TaqMan GTXpress Master Mix (2X) reagent, which consists of 12,5µL TaqMan GTXpress Master Mix (2X), plus 1,25 µL of 20X working stock SNP Genotyping Assay and 11,25 µL DNA template (DNA extract) with a final concentration of 1.20 ng DNA per well. Amplification was carried out in a C-1000 thermal cycler CFX96 Real Time System (BioRad) according to the following standard protocol: 10 minutes of enzyme activation at 95°C, followed by 40 amplification cycles consisting of 15 seconds of denaturation at 92°C, and annealing/extension at 60°C for 1 minute.

STATISTICAL METHODS

Data analysis was performed through univariate, bivariate (Chi-Square test) analyses using SPSS 22nd version (SPSS Inc., Chicago). A value of $p < 0.05$ with a 95% confidence interval was considered statistically significant.

RESULTS

Baseline and clinical characteristics of subjects

Patients' characteristics show a majority of males (58.8%) with mean age of 53 years, with mostly Batak ethnicity (58.8%), civil servants (41.3%) and high school graduates (65.0%) (Table 1).

Table 1. Baseline and clinical characteristics of subjects

Characteristics	n = 80
Gender	
Male	47 (58.8%)
Female	33 (41.3%)
Age, mean + SD (years)	53 (20 – 68)
Ethnicity	
Batak	47 (58.8%)
Javanese	20 (25.0%)
Aceh	7 (8.8%)
Malay	6 (7.5%)
Occupation	
Entrepreneur	17 (21.3%)
Housewife	29 (36.3%)
Civil servants	33 (41.3%)
University students	1 (1.3%)
Education	
Elementary school	7 (8.88%)
Middle school	9 (11.3%)
High school	52 (65.0%)
University	12 (15.0%)

Distribution of VEGF levels and VEGF +936C>T polymorphism frequency in H. pylori gastritis patients

There were 33 patients (41.25%) having CC genotype, followed by 28 patients (35.0%) CT genotype, and 19 patients (23.75%) TT genotype. The median value of VEGF levels was 399.5 pg/mL with a minimum level of 69.3 pg/mL and a maximum level of 2,000 pg/mL (Table 2).

Table 2. Distribution of VEGF levels and VEGF +936C>T polymorphism frequency in H. pylori gastritis patients

Variable	n = 80
VEGF level, median (min-max), pg/mL	399.5 (69.3 – 2,000)
VEGF +936C>T polymorphism	
CC genotype	33 (41.25)
CT genotype	28 (35.0)
TT genotype	19 (23.75)

Association between VEGF+936 C>T polymorphism and gastric premalignant lesions

There was no significant association between VEGF +936 C>T polymorphism and gastric premalignant lesions (p>0.05) (Table 3).

Table 3. Association between VEGF+936C>T polymorphism and gastric premalignant lesions

VEGF+936C>T Polymorphism	Gastric premalignant lesions		Total	p	PR (95% CI)
	Present	Absent			
CC	11 (33.3%)	22 (66.7%)	33 (100%)	0.598	1.27 (0.52 -3.09)
CT	8 (28.6%)	20 (71.4%)	28 (100%)	0.865	1.08 (0.42-2.81)
TT	5 (26.3%)	14 (73.7%)	19 (100%)		1 (ref.)
C allele	30 (31.9%)	64 (68.1%)	94 (100%)	0.528	1.17 (0.72 – 1.91)
T allele	18 (27.3%)	48 (72.7%)	66 (100%)		1 (ref.)

Difference in serum VEGF levels between genotypes and alleles of VEGF +936 C>T polymorphism

In this study, there were no significant differences in serum VEGF levels between genotypes and alleles of VEGF +936C>T polymorphism (p>0.05) (Table 4).

Table 4. Difference in serum VEGF levels among CC, CT, and TT genotypes, between C and T alleles in VEGF +936 C>T polymorphism

VEGF+936C>T Polymorphism	VEGF levels (pg/mL) median (min – max)	P
CC CT TT	623.1 (77.8 – 1150.3) 395.1 (163.9 – 2000) 348.1 (69.3 – 2000)	0.179
C Allele T Allele	551.4 (69.3 – 2000) 362.0 (77.8 – 2000)	0.076

DISCUSSION

Research suggests that VEGF plays an important role in the carcinogenesis pathway, such as inhibition of apoptosis, tumor development, angiogenesis, invasion, and metastasis. The specific function of VEGF in the formation of prostaglandins makes it a strong candidate for increasing susceptibility to cancers such as gastric, lung, breast, prostate, and other solid cancers[14]. The role of genetic polymorphism, as one of the endogenous causes of cancer, and in relation to the risk of gastric cancer has attracted interest in the field of DNA analysis technology and human genome knowledge[15].

Mechanisms in how VEGF polymorphisms contributes to carcinogenesis remain unclear. Potential mechanisms that occur are variations in DNA sequence that may alter the production and/or activity of VEGF, causing interindividual differences in tumor development. Alteration of the VEGF gene function, both activation and repression, the possibility of synergizing with co-factor molecules can be a rational cause. The location of +936C/T SNP lies in the 3 untranslated region (3'-UTR) of the VEGF gene, which acts in the stability of the mRNA and is associated with the hypoxic induction of VEGF. HuR (Human antigen R, ELAV like protein 1) proteins are considered to play a role in mRNA stabilization and prevent mRNA from being attacked by RNAse. HuR proteins also increase the binding of VEGF mRNA to the nucleus and increase the export of VEGF mRNA in hypoxic induced angiogenesis.[16,17]

Several studies have been conducted to determine whether VEGF polymorphisms confer susceptibility to gastric cancer. Bae et al found that VEGF 936 T alleles were associated with an increased risk of gastric cancer.[15]A study by Kim (2007) shows that TT genotype of 936C>T polymorphism had a worse overall survival in patients with gastric cancer than CC genotype (HR=3.23, 95% CI, 1.13-9.25, p = 0.037).[19] However, Ke et al and Guan et al reported that there was no significant association between VEGF +936C/T polymorphism and gastric cancer.[18,19] Meta analysis conducted by Liu (2018) also found no association of VEGF-936C>T polymorphism with risk and survival of gastric cancer. However, the T allele and TT genotype of VEGF-936C> T polymorphism were correlated with tumor size of gastric cancer (OR = 0.47, 95% CI: 0.29-0.77, p=0.002).[20] No published studies evaluating the association between VEGF +936 C>T polymorphism with gastric premalignant lesions in *H. pylori* gastritis patients were available. In this study, no association was found between +936 C>T polymorphism and gastric premalignant lesions.

Several polymorphisms on the VEGF gene were thought to affect its expression. Certain allele variation may lead to overexpression of the transcription factor that will bind to the promoter site, which serves as the initial RNA polymerase binding site that will initiate transcription[21]. The C allele of VEGF +936 C/T polymorphism has a potential binding site for activator protein 4 (AP-4), which is removed on T allele. AP-4 is a helix-loop-helix transcription factor that increases the expression of some viral and cellular genes by binding to a specific enhancer site; the loss of potential binding sites may be responsible for the decrease in VEGF expression by T allele. In this study VEGF levels were higher in C allele than T allele, but not significantly different.

CONCLUSION

There were no significant associations between VEGF +936C>T polymorphism with gastric premalignant lesions and serum VEGF levels in *H.pylori* patients.

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