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Association between *VEGF-634G>C* Gene Polymorphism with Gastric Premalignant Lesions and Serum VEGF Levels in *Helicobacter pylori* Gastritis Patients

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Keywords: Gastric premalignant lesion; Helicobacter pylori; VEGF; polymorphism

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Abstract

AIM: To evaluate the association between *VEGF-634G>C* gene polymorphism with premalignant gastric lesions as well as the level of VEGF.

METHODS: This cross-sectional study included patients with *H. pylori* gastritis at Haji Adam Malik General Hospital, Permata Bunda General Hospital, and Universitas Sumatera Utara Hospital, Medan, Indonesia. Detection of *H. pylori* infection was made using positive results of ^{14}C-UBT, rapid urease test, and/or immunohistochemistry. Gastric premalignant lesion diagnosis was made when one or more of the following were present: chronic atrophic gastritis, intestinal metaplasia, or dysplasia. Real-time polymerase chain reaction (RT-PCR) was used to examine VEGF-634G>C gene polymorphism. Additionally, serum samples of patients with *H. pylori* gastritis were obtained to determine the level of circulating VEGF. Data were analysed using SPSS version 22.

RESULTS: A total number of 87 patients with *H. pylori* gastritis were included in this study. Of all participants, 26 patients (29.9%) showed gastric premalignancy. There was a significant association between *GG+GC* genotype of *VEGF-634G>C* and gastric premalignant lesions (P = 0.003; OR (CI 95%) = 6.07 (1.88-41.71)). *VEGF-634 G>C* polymorphism also showed an association with VEGF serum levels (P = 0.005). Patients with the *GG+GC* genotype would be at risk of 3.16 times to have high VEGF levels compared to *CC* genotypes.

CONCLUSION: *VEGF-634G>C* polymorphism, in particular, *GG+GC* genotype was associated with an increased risk of gastric premalignant transformation as well as having high VEGF levels in patients with *H.pylori* gastritis.

Introduction

Gastric cancer remains to be the second leading cause of all cancer mortality, causing approximately 700.000 death worldwide [1]. Gastric carcinogenesis is a continuous process commonly initiated with atrophic gastritis [2]. Chronic atrophic gastritis is considered the first step in a sequence of mucosal changes in the stomach leading to cancer. The current model for stomach carcinogenesis begins with gastritis, proceeding to chronic atrophic gastritis, then to intestinal metaplasia, dysplasia and, finally, carcinoma [3]. Risk factors of gastric cancer are *Helicobacter pylori* (*H. pylori*) infection, salt intake, smoking, alcohol, family history of gastric cancer, and presence of premalignant gastric lesions such as atrophic gastritis, intestinal metaplasia, and dysplasia. Gastric premalignant lesions are well-known risk factors for the development of gastric cancer [4] [5] [6] [7] [8] [9] [10] [11] [12].

H. pylori infection has been well studied as the main aetiology of chronic gastritis. Subsequently, atrophic gastritis, intestinal metaplasia, dysplasia, and gastric cancer may follow. Not all patients with *H. pylori* infection would develop such progression. One

other factor that may contribute to the malignant progression is genetic [13].

Previous evidence suggests the Vascular Endothelial Growth Factor (VEGF) may play a role in gastric carcinogenesis, as VEGF promotes angiogenesis that is required for tumour survival and metastasis [14]. Patients with premalignant lesions such as atrophic gastritis, intestinal metaplasia, and dysplasia also show overexpression of VEGF that may contribute in the early step of carcinogenesis [15].

The VEGF gene, located at 6p21.3 chromosome, composed of 8 exons separated by 7 introns, is a very polymorphic gene (about 140 variants) [16] [17]. Previous studies show *VEGF-634G>C* polymorphism to be associated with an increased risk of gastric cancer as well as elevated VEGF levels. This polymorphism is influenced by ethnicity to previous studies showed different results [18] [19] [20] [21]. There were no similar studies in Southeast Asia after searching on PubMed. To the authors' knowledge, no published studies evaluating the association between *VEGF-634G>C* polymorphism with gastric premalignant lesions and serum VEGF levels in *H. pylori* gastritis patients are available. This study was conducted to determine the association between *VEGF-634G>C* polymorphism with premalignant gastric lesions and serum level of VEGF in *H. pylori* gastritis patients.

Methods

Patients Selection

This study was a cross-sectional study on 87 consecutive *H. pylori* gastritis patients that were admitted to the Endoscopy Unit at Haji Adam Malik General Hospital, Permata Bunda General Hospital, and Universitas Sumatera Utara Hospital, Medan, Indonesia between October and December 2017. Inclusion criteria include gastritis patients diagnosed based on histopathological examination, at least 18 years old, and willing to take part in the study. Patients with one of the following criteria were excluded: a history of *H. pylori* eradication treatment in the last 6 months or currently on antibiotics therapy commonly used in *H. pylori* eradication; history of proton pump inhibitor, H2 receptor antagonist use within the past 1 month; patients with systemic disease, malignancy or currently pregnant. This study was approved by the Institutional Review Board of Universitas Sumatera Utara.

Endoscopy was conducted to evaluate gastric mucosae such as the presence of oedema, erythema (spotted, patchy, linear), exudate, bleeding, erosion; as well as to take a tissue sample for the rapid urease test, immunohistochemistry test for *H. pylori* and histopathology. Tissue biopsy was performed within the greater and lesser curvature of the distal antrum, the lesser curvature at incisura angular, the anterior and posterior wall of the proximal corpus. An additional biopsy was also done in suspicious regions that were not included in the areas mentioned previously.

Diagnosis of Gastric Premalignant Lesion

Microscopic evaluation was done to diagnose premalignant gastric lesions such as chronic atrophic gastritis, intestinal metaplasia, and dysplasia. The presence of one or more findings is positive for a premalignant gastric lesion. Histopathologic examination was done by two pathologists blindly at Universitas Sumatera Utara Medan. If there were differences in the results of the examination of both experts, then a third pathologist was required to perform the histopathological examination.

Helicobacter pylori detection

The diagnosis of *H. pylori* infection was made using positive results of ^{14}C-UBT, rapid urease test, and/ or immunohistochemistry. Before the ^{14}C-UBT examination, the subjects fasted for at least 6 h, usually overnight. Patients swallowed 37 kBq (1 μCi) of encapsulated ^{14}C urea/citric acid composition in 25 ml water. Breath samples of patients were collected into Heliprobe Breath Cards (Noster system) 10 min after administration of ^{14}C urea. Patients exhaled into the breath card until its colour indicator changed from orange to yellow. The breath samples were measured using the Heliprobe analyser (Noster system), and the activity was counted for 250 s. Results were expressed as counts per minute (cpm) and counts < 25 cpm were defined as Heliprobe 0 = not infected, counts between 25 cpm and 50 cpm as Heliprobe 1 = equivocal and counts > 50 cpm as Heliprobe 2 = infected [22].

The rapid urease test (Pronto Dry®, France) was also used to establish the diagnosis of *H. pylori* infection. The positive result is indicated by the colour changing of the indicator from amber to pink-red at room temperature within 24 hours. The yellow indicator colour is considered to be negative [23].

Immunohistochemical (IHC) staining for evaluation of *H. pylori* status carried-out with the procedure as follows [24]. Tissue sections were deparaffinized, rehydrated, and pretreated with Proteinase K for 8 min and incubated with ChemMate peroxidase blocking solution at room temperature for 10 min. The slides were subsequently incubated with the polyclonal rabbit anti- *H. pylori* primary antibody (B0471: Dako Corporation, Glostrup, Denmark) with a dilution of 1:50 was conducted at room temperature for 1 hour. After samples had been washed 3 times with phosphate-buffered saline, the Dako EnVision

Dual Link System–HRP (K4065: Dako Corporation) was applied for 30 minutes. Finally, sections were incubated in diaminobenzidine for 10 minutes, followed by hematoxylin counterstaining and mounting. *H. pylori*-infected gastric mucosa from chronic gastritis patients served as positive controls. Negative controls were obtained by replacing the primary antibody with phosphate-buffered saline. *H. pylori* infection in the tissue sections was confirmed when short, curved or spiral bacilli resting on the epithelial surface, in the mucus layer, or deep in the gastric pits could be observed by light microscopy.

Serum Levels of VEGF

Venous blood samples were drawn into serum separator tubes and allowed to clot for 30-45 minutes at room temperature, before being centrifuged for 15 minutes at approximately 1,000 g. Serum was immediately stored frozen in aliquots at -20^{o}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).

VEGF-634 G>C Polymorphism

Genomic DNA was extracted and purified from peripheral blood using the High Pure PCR Template Preparation Kit (Roche Applied Science) and stored until processed for genotyping. Analysis of the *VEGF* SNP *-634G>C* was performed using real-time polymerase chain reaction (RT-PCR). The PCR primers used for the *–634G>C* polymorphisms were 5'-CGACGGCTTGGGGAGATTGC-3' (forward) and 5'-GGGCGGTGTCTGTCTGTCTG-3' (reverse). The PCR cycle conditions consisted of an initial denaturation step at 94°C for 5 min, followed by 35 cycles of 30 s at 94°C, 30 s at 62°C, 30 s at 72°C and a final elongation at 72°C for 10 min.

Statistical Methods

Data analysis was performed through univariate and 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

A total number of 87 *H. pylori* gastritis patients were included in this study. The diagnosis of *H. pylori* infection was made using positive results of ^{14}C-UBT, rapid urease test, and/ or IHC. Figure 1 shows *H. pylori* in the gaster tissues by IHC.

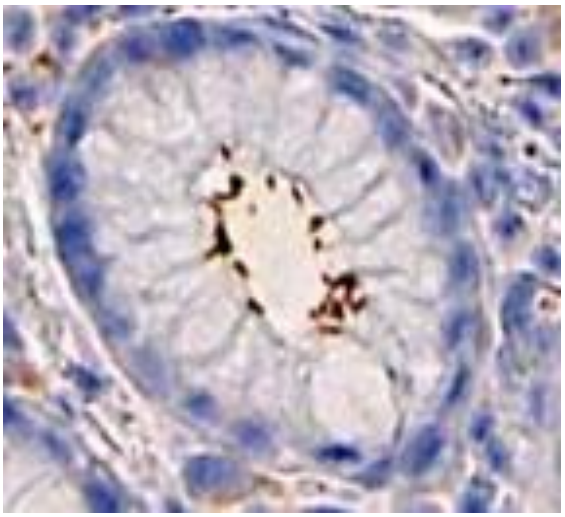

Figure 1: H. pylori in the gaster tissues by immunohistochemistry (IHC)

Patients' characteristics show a majority of males (63.2%) with a mean age of 50.8 years, with mostly Batak ethnicity (52.9%). There were 46 patients (52.9%) having *VEGF-634G>C* polymorphism *GC* genotype, followed by 24 patients (27.6%) *GG* genotype and 17 patients (19.5%) CC genotype. The median value of VEGF levels was 441.7 pg/mL with a minimum level of 80.7 pg/mL and a maximum level of 2185.2 pg/mL. We grouped VEGF levels into 2 categories based on the median value, ≥ 442 and <442. As many as 48.3% of patients had VEGF ≥442 pg/mL, 51.7% of patients had VEGF <442 pg/mL. There were 26 patients (29.9%) with premalignant gastric lesions (Table 1).

Table 1: Baseline and clinical characteristics of subjects

Characteristics	n (%)
Gender	
Male	55 (63.2%)
Female	32 (36.8%)
Age, mean ± SD (years)	50.8 ± 12.2
≥51 years	58 (66.7%)
<51 years	29 (33.3%)
Ethnicity	
Batak	46 (52.9%)
Javanese	20 (23%)
Aceh	12 (13.8%)
Malay	9 (10.3%)
Education	
Elementary school	7 (8%)
Middle school	12 (13.8%)
High school	57 (65.5%)
University	11 (12.6%)
BMI, mean ± SD (kg/m^2)	22.2 ± 3.8
Overweight	39 (44.8%)
Not overweight	48 (55.2%)
Gastric Premalignant Lesion	
Yes	26 (29.9%)
No	61 (70.1%)
VEGF-634 G>C polymorphism	
GG genotype	24 (27.6%)
GC genotype	46 (52.9%)
CC genotype	17 (19.5%)
VEGF level, median (min-max) (pg/ml)	441.7 (80.7 – 2185.2)
High (≥ 442)	42 (48.3%)
Low (< 442)	45 (51.7%)

Association between patient's characteristics and VEGF levels with premalignant gastric lesions

There was a significant association between age and gastric premalignant lesions (P = 0.020), patients ≥ 51 years were at risk of 2.75 times to get premalignant gastric lesions compared to age < 51 years. There was a significant association between ethnic group and gastric premalignant lesions (P = 0.016), where the Batak ethnic group had a 2.97 times risk of having premalignant gastric lesions compared to non-Bataks. There was a significant association between VEGF levels and premalignant gastric lesions (P <0.001), where patients with high VEGF levels had a 12.86 times risk of having gastric premalignant lesions compared to patients with low VEGF levels. There were no associations between gender, education, and overweight with premalignant gastric lesions (P > 0.05) (Table 2).

Table 2: Association between patients' characteristic and VEGF level with premalignant gastric lesions

Variables	Gastric premalignant lesions		Total	p	OR (95% CI)
	Present	Absent			
Gender					
Male	14 (25.5%)	41 (74.5%)	55 (100%)	0.237	0.68 (0.36-1.28)
Female	12 (37.5%)	20 (62.5%)	32 (100%)		
Age					
≥51 years	22 (37.9%)	36 (62.1%)	58 (100%)	0.020*	2.75 (1.05-7.24)
<51 years	4 (13.8%)	25 (86.2%)	29 (100%)		
Education					
Low level	7 (36.8%)	12 (63.2%)	19 (100%)	0.454	1.32 (0.65-2.66)
High level	19 (27.9%)	49 (72.1%)	68 (100%)		
Ethnic group					
Batak	20 (43.5%)	26 (56.5%)	46 (100%)	0.016*	2.97 (1.32-6.67)
Non-Batak	6 (14.6%)	35 (85.4%)	41 (100%)		
Overweight					
Present	10 (2.6%)	29 (74.4%)	39 (100%)	0.436	0.77 (0.4-1.5)
Absent	16 (33.3%)	32 (66.7%)	48 (100%)		
VEGF level					
High	24 (57.1%)	18 (42.9%)	42 (100%)	<0.001*	12.86 (3.24-51.1)
Low	2 (4.4%)	43 (95.6%)	45 (100%)		

*P < 0.05; Low level of education : elementary school + middle school; High level of education: high school + university.

Association between VEGF-634G>C polymorphism and premalignant gastric lesions

There was a significant association between *VEGF-634G>C* polymorphism and premalignant gastric lesions. *GG* genotype increased the risk of 7.08 times for premalignant gastric lesions compared to *CC* genotype (P = 0.014). *GC* genotype increased the risk of 5.54 times for premalignant gastric lesions compared to *CC* genotype (P = 0.048). *GG* + *GC* genotype was 6.07 times more likely to have premalignant gastric lesions than patients with *CC* genotypes (P = 0.003). Patients with the *G* allele were at risk of 1.75 times for premalignant gastric lesions compared to *C* allele (p = 0.022) (Table 3).

Table 3: Association between *VEGF-634G>C* polymorphism and premalignant gastric lesions

VEGF-634G>C Polymorphism	Gastric premalignant lesion		Total	p	OR (95% CI)
	Present	Absent			
GG	10 (41.7%)	14 (58.3%)	24 (100%)	0.014*	7.08 (1.2-50.26)
GC	15 (32.6%)	31 (67.4%)	46 (100%)	0.048*	5.54 (1.79-38.82)
CC	1 (5.9%)	16 (94.1%)	17 (100%)		1 (ref.)
GG+GC	25 (35.7%)	45 (64.3%)	70 (100%)	0.003*	6.07 (1.88-41.71)
CC	1 (5.9%)	16 (94.1%)	17 (100%)		1 (ref.)
Allele *G*	35 (37.2%)	59 (62.8%)	94 (100%)	0.022*	1.75 (1.07 – 2.88)
Allele *C*	17 (21.3%)	63 (78.8%)	80 (100%)		1 (ref.)

*P <0.05.

Association between VEGF-634G>C polymorphism and serum VEGF levels

Further analysis was done to evaluate the association between *VEGF-634G>C* polymorphism and serum levels of VEGF. *GG* genotype increased the risk of 3.54 times to have high VEGF levels compared to *CC* genotype (P = 0.004). GC genotype increased the risk of 2.96 times to have high VEGF levels compared to *CC* genotype (P = 0.014). *GG* + *GC* genotype was 3.16 times more likely to have high VEGF levels than patients with *CC* genotypes (P = 0.005). Patients with *G* allele were at risk 1.53 times to have high VEGF levels compared to *C* allele (P = 0.009) (Table 4).

Table 4: Association between VEGF -634 G>C polymorphism and the level of serum VEGF

VEGF-634G>C Polymorphism	VEGF Level		Total	P	OR (95% CI)
	High	Low			
GG	15 (62.5%)	9 (37.5%)	24 (100%)	0.004*	3.54 (1.21-10.35)
GC	24 (52.2%)	22 (47.8%)	46 (100%)	0.014*	2.96 (1.02-8.56)
CC	3 (17.6%)	14 (82.4%)	17 (100%)		1 (ref.)
GG+GC	39 (55.7%)	31 (44.3%)	70 (100%)	0.005*	3.16 (1.11-9)
CC	3 (17.6%)	14 (82.4%)	17 (100%)		1 (ref.)
Allele G	54 (57.4%)	40 (42.6%)	94 (100%)	0.009*	1.53 (1.1 – 2.13)
Allele C	30 (37.5%)	50 (62.5%)	80 (100%)		1 (ref.)

*P < 0.05.

Discussion

H. pylori is a type 1 carcinogen according to the International Agency for Research on Cancer (IARC). It promotes malignant changes through triggering an inflammatory cascade, including neutrophil and monocyte recruitment and upregulation of proinflammatory cytokines destroying the gastric mucosa. Epidemiological studies have shown an association between chronic inflammation and malignant transformation. *H. pylori* may also play a role in angiogenesis, essential for malignant cells survival. Among angiogenic factors, VEGF is known to be the most potent stimulus for neoangiogenesis [3]. This VEGF has a mitogenic effect on endothelial cells, promotes tumour cell growth, stimulates cell migration, and metastasis from the primary site [25].

The previous meta-analysis by Chen et al. reported higher VEGF expression in Asian patients with gastric cancer compared to controls (OR=

112.41, 95% CI= 64.12 – 197.06). High expression of VEGF also showed poor 5-year survival rates (RR= 2.45, P = 0.000), indicating its potential use as a marker for gastric cancer prognosis [26]. In gastric adenocarcinoma, a higher level of VEGF was also observed along with the increased density of intratumour small vessels. Moreover, higher expression of VEGF was also seen in gastric premalignant lesions such as chronic atrophic gastritis, intestinal metaplasia, and gastric dysplasia; as well as gastric carcinoma [15] [27] [28]. Raica et al also reported there was an association between increased levels of VEGF expression with gastric carcinogenesis [28]. These results are consistent with this study, that there was a significant association between high VEGF levels and premalignant gastric lesions (P <0.001).

Several SNP on the VEGF gene is 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 [29]. A previous study by Oh et al. evaluated 190 gastric cancer patients in Korea reported that the GG genotype of *VEGF-634G>C* polymorphism was associated with higher VEGF serum levels as well as poor outcome in patients with advanced stage [21]. Similar with that study, this present study also found a significant association between *VEGF-634G>C* polymorphism and premalignant gastric lesions, in particular, those with the *GG+GC* genotype had an increased risk of 3.16 fold to have high VEGF levels compared to those with the CC genotype.

A gene polymorphism was not only related to an increase or decrease in serum levels, but also with susceptibility to certain diseases [16] [30]. A study in Texas by Guan et al. included 171 gastric cancer patients (vs 353 controls) found that *VEGF-634CG+CC* showed an increased risk of gastric cancer compared to *-634GG* genotype [18]. Patients with VEGF heterozygous *-634GC* showed a poorer 1-year survival compared to patients with *VEGF-634GG* genotype [31]. Similar findings were reported by Tzanakis et al. (2006) in Greece that evaluated 100 gastric cancer patients. Tzanakis et al. found the *VEGF-634CC* genotype was associated with increased risk of gastric cancer and lower survival rates [19]. Meanwhile, a study by Chae et al. (2006) in Korea on 413 gastric cancer patients and 413 subjects as controls showed that *VEGF-634CC* was significantly associated with a lower risk of gastric cancer. *VEGF-634 C* allele was associated with a significant reduction in gastric cancer susceptibility (OR 0.686; 95% CI 0.564-0.834) [20]. Another study in Oman by Al-Moundhri (2009) found a significant association between *VEGF-634 CC* genotype with poor tumour differentiation and lymph nodes metastasis [32]. There were still limited studies that evaluate the relationship of *VEGF* polymorphism with premalignant gastric lesions. Tahara et al. reported that *1612G>A* polymorphism of the *VEGF* gene was associated with premalignant gastric lesions in older individuals. The *1612 GA* genotype showed a significantly higher incidence of intestinal metaplasia in *H. pylori*-positive individuals whose age was more than 65 years old [13]. This current study showed that *VEGF-634G>C* polymorphism was associated with premalignant gastric lesions, where *-634GG+GC* genotype increased the risk of 6.07 times for premalignant gastric lesions, presumably due to elevated serum *VEGF* levels in those genotypes.

These study indicated that older age was associated with an increased risk of premalignant gastric lesions. Previous studies reported a similar result [33] [34]. Benberin et al. showed the prevalence of premalignant gastric lesions increased with age. This condition was rarely seen in individuals under the age of 40 years [35]. The majority ethnic group in this study were Bataks (52,9%) because Bataks inhabit most of the North Sumatera region. There was a significant association between ethnicity and premalignant gastric lesions. Batak ethnic group increased the risk of 2.97 times for premalignant gastric lesions (P = 0.016). Further study is required to evaluate the high prevalence of premalignant gastric lesions in Bataks. Bataks have a habit of consuming alcohol both in a traditional ceremony and daily life [36]. Background of genetic factors, nutritional factors or lifestyle, immune responses to *H. pylori* infection may be considered. Ethnic differences may influence the risk of premalignant gastric lesions, which may be due to genetic variation [37].

In conclusion, the polymorphism of VEGF-634 G>C, in particular, GG+GC genotype, was associated with an increased risk of premalignant gastric lesions and high serum levels of VEGF in patients with *H. pylori* gastritis in Medan, Indonesia. There was a significant association between age, ethnic, and VEGF levels with premalignant gastric lesions.

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