Suez Canal University Medical Journal Vol. 27 (3), 2024
Pages 13-22

Association between Vascular Endothelial Growth Factor Gene +405 C/G Polymorphism and Acute Coronary Syndrome

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Abstract

Background: **Acute coronary syndrome (ACS) is an acute condition that results from decreased blood flow to the coronary arteries. According to the WHO 2020 census, 32.40% of total deaths in Egypt are due to coronary heart diseases, ranking Egypt #15 in the world. ACS is a wide spectrum including ST-segment elevation myocardial infarction, non-ST segment elevation myocardial infarction, and unstable angina. Many risk factors contribute to the etiology of ACS including lifestyle, environmental, and genetic factors. Vascular endothelial growth factor gene polymorphism is one of the genetic elements that may share in the progression of ACS.** ***Aims:*** **to identify the association between *VEGF* gene +405 C/G polymorphism and acute coronary syndrome.** ***Methods:*** **We designed a descriptive, case-control study. Patients were randomly recruited from March 2021 to October 2021. Fifty patients diagnosed with ACS (35 STEMI_ 10 NSTEMI_ 5 unstable angina) by laboratory tests and imaging and 50 healthy individuals, both groups were age and sex-matched. Initially, we isolated the total DNA from peripheral blood, then we investigated single nucleotide polymorphisms of *VEGF* (rs 2010963) and the genotyping was performed by PCR-RFLP technique.** ***Results:*** **Our results revealed a significant association between the +405 CC genotype (p=0.025) and acute coronary syndrome. Patients with ACS had significantly more history of diabetes mellitus in comparison with the non-ACS group (p=0.037), while regarding smoking and lipid profile, there was no statistically significant difference between the two groups.** ***Conclusion:*** **This study proves the existence of an association between *VEGF* gene polymorphisms (rs2010963) and susceptibility to ACS, so SNPs in *VEGF* need further investigation as prognostic markers and indicators of angiogenic potential stimulating the formation of collaterals.**

Keywords: STEMI- NSTEMI- Atherosclerosis- risk factors- RFLP

Introduction

Acute coronary syndrome (ACS) is characterized by impaired perfusion of the muscles of the heart, and it includes myocardial infarction and unstable angina. The main pathological event of ACS is the erosion of an unstable plaque. This leads to complete or partial occlusive thrombosis in the coronary arteries[1,2]. ACS is classified into three conditions, ST-segment elevation myocardial infarction (STEMI), non-ST-segment elevation myocardial infarction (NSTEMI), and unstable angina pectoris (UAP)[3]. ACS

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is a serious fatal disease with acute course, characterized by acute onset with rapid progression and poor prognosis[4]. Many studies investigated the risk factors of ACS[5] which include smoking, obesity, hyperglycemia, hyperlipidemia and hypertension[6,7]. However, in recent years, important independent factors have been identified for ACS[8], such as fibrinogen levels, blood uric acid levels, serum cystatin C level, and mean platelet volume[9–13]. An important process that subtatialy decreases the adverse effects after acute coronary occlusion is the development of functional collateral circulation around occluded arteries. The development of collateral arteries are associated with improved survival rate in patients diagnosed with coronary artery disease[14]. VEGF is a key factor in the angiogenesis process and collateral circulation (arteriogenesis) formation. It is mediated by VEGF binding to VEGFR-1 (Flt-1) and VEGFR-2 (KDR) receptors[15]. Thus, polymorphisms in *VEGF* genes affect the expression and alternative splice decisions of *VEGF-A*[16–18] and therefore the ability to form collateral circulation decreases. Numerous single nucleotide polymorphisms (SNPs) of *VEGF-A* are identified by single-stranded conformational polymorphism analysis and sequencing is associated with susceptibility to numerous atherosclerotic events such as coronary artery disease, peripheral artery disorder, and lung cancer[19,20]. Other than CAD, *VEGF* polymorphism has a major role in the development of various vascular-dependent events including diabetic nephropathy and retinopathy, tumor growth, and tissue expansion through involvement in normal and pathogenic vascular development and formation of new blood vessels[21]. Accordingly, the current study aimed to assess the association between *VEGF* gene polymorphism and the development of acute coronary syndrome. This could help in more understanding of the underlying pathogenesis of ACS to improve the prediction of ACS, which can contribute to early management decisions and better outcomes.

Methods

Study population

A case-control study consists of two groups. The first group consisted of 50 patients, who were randomly recruited from the CCU department and diagnosed with acute coronary syndrome according to the diagnostic criteria of ACS[22] from March 2021 to October 2021, patients were selected according to the following inclusion criteria: Adult male and female patients (age older than 18 years). Patients diagnosed with Ischemic heart disease were confirmed by lab results (elevated CK-CKmb- LDH- Troponin I) and ECG changes. The second group included 50 healthy control individuals randomly recruited from the outpatient lab, who were not recently diagnosed or previously had ACS. Both groups were age and sex-matched. Patient history and clinical information were collected by interviewing or reviewing their medical history. Patients with shocks like cardiogenic, septic, or hypovolemic shock and malignancies were excluded from the study, also patients below 18 years were not included in our study. The research protocol was approved by the medical ethics committee of the Faculty of Medicine Suez Canal University research 4376# and all patients provided informed consent for participation.

Laboratory investigations

The concentration of serum total cholesterol, triglycerides, high-density lipoprotein (HDL), low density lipoprotein (LDL), and triglycerides of all the blood samples were collected from the subjects following a 12 hour fasting, were measured. Cardiac markers (CK, CKmb, LDH and troponin I)

were measured to ACS patients within the first six hours from admission. All biochemical tests were done using Cobas 6000 automated auto-analyzer kits supplied by Roche Diagnostics (Mannheim, Germany). Measurement of glycosylated hemoglobin (HBA1C) and Complete blood count (CBC) were also done to all participants.

VEGF +405 C/G polymorphism genotyping

Total DNA was isolated from peripheral blood leukocytes using Qiagen genomic DNA extraction kit (Cat: 69504)[23]. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was used to determine the +405 *VEGF* C/G (rs2010963) polymorphism status. PCR was performed in a volume of 50 ul; 10 ul of sample including 100 ng DNA, 25 ul Taq PCR master mix, 2.5 ul primers, and 12.5 ul deionized water. Genomic DNA was amplified using the following conditions: denaturation at 95°C for 5 min followed by 35 cycles at 94°C for 1 min, 55°C for 1.5 min for annealing, and 72°C for 2 min for extention. And a final extension was at 72°C for 10 min. The following primers were used: Forward 5'-TTGCTTGCCATTCCCCACTTGA-3 and reverse 5'-CCGAAGCGAGAACAGCCCAGAA-3'. The PCR product was digested with *Bsm*FI restriction nuclease enzyme. The amplification products were separated by electrophoresis according to the size by 1% agarose gel stained with ethidium bromide. According to the *VEGF* +405 polymorphism, the uncut fragment was 469 bp (C allele) and digested products were 274 bp and 195 bp (G allele).

Statistical Analysis

SPSS software version 22.0.0.0 was used as a statistical tool in the analysis and comparison of the variables between ACS and non-ACS groups. Data was presented as tables and graphs as appropriate. Mean and standard deviation represent the quantitative data, while qualitative data is expressed as percentages and numbers. The categorial variables were analyzed using the chi-squared test or Fisher's exact test if required while the continuous variables were analyzed using the student's t-test. One-way analysis of variance (ANOVA) was used for parametric variables and Kruskal-Wallis test was used for non-parametric variables. The logistic regression analysis, odds ratio (OR), and 95% confidence interval (CI) for OR determine the genotype association between the two groups. A *P* value of ‹0.05 was considered significant. The balance of the allele frequencies of the polymorphism was investigated according to the Hardy-Weinberg equilibrium law.

Results

In this case-control study, a total of 100 subjects (65 males, 35 females). Fifty patients were diagnosed with ACS and 50 non-ACS individuals. ACS patients were diagnosed according to history, Lab. investigations, and imaging techniques. The mean age was 59.1 ± 11.2 [Range: 36 – 78] for non-ACS individuals and 61.0 ± 12.2 [Range: 28 -85] for ACS patients.

Prevalence of +405 C/G gene polymorphism

The genotypic distribution in our study population agreed with Hardy- Weinberg equilibrium. Among the 100 study subjects, 20% had *VEGF* +405 CC, 47% had GC and 33% had GG genotype. The analysis showed an association between mutated alleles of *VEGF* +405 C/G polymorphism (CC) and the presence of ACS (OR= 4.07, 95% CI= 1.20 - 13.86, P=0.025; Table 2). No significant association was found among the heterozygote model (GC) of *VEGF* +405 C/G polymorphism and ACS P=0.841. In addition, CC/GC genotype in C allele subjects greatly increased the susceptibility of ACS P=0.033.

Table 1: Prevalence of +405 C/G gene polymorphism among study groups				
Genotype of +405 C/G polymorphism	Study Groups		OR (95% CI)	p-value
	Non-ACS (n = 50)	**ACS** (n = 50)		
Wild (GG)	19 (38.0%)	14 (28.0%)	REF	
Heterozygous (GC)	26 (52.0%)	21 (42.0%)	1.10 (0.45, 2.69)	0.841 [b]
Homozygous mutant (CC)	5 (10.0%)	15 (30.0%)	4.07 (1.20, 13.86)	**0.025*** [b]
MAF	36 (36.0%)	51 (51.0%)	1.85 (1.05, 3.26)	**0.033*** [b]

*Wild "GG"; Heterozygous "GC"; Homozygous mutant "CC"; Minor allele frequency "MAF"; *. Statistically significant p-value (<0.05); [b]. Chi-square test.*

Table 2: Distribution of +405 C/G gene polymorphism by the study variables in ACS group				
Variables	Genotype of +405 C/G gene polymorphism			p-value
	Wild (n =14)	**Heterozygous** (n =21)	**Homozygous mutant** (n =15)	
Cigarette Smoking				
Nonsmoker	7 (50.0%)	11 (52.4%)	3 (20.0%)	0.118 [b]
Smoker	7 (50.0%)	10 (47.6%)	12 (80.0%)	
History of Chronic Illnesses				
None	6 (42.9%)	7 (33.3%)	5 (33.3%)	-
Hypertension	2 (14.3%)	1 (4.8%)	2 (13.3%)	0.837 [f]
Diabetes Mellitus (DM)	1 (7.1%)	7 (33.3%)	2 (13.3%)	0.357 [f]
Hypertension and DM	5 (35.7%)	6 (28.6%)	6 (40.0%)	1.00 [f]
WBC ($x10^3/\mu l$)	9.58 ± 2.86	11.84 ± 4.51	10.09 ± 2.57	0.153 [d]
HbA1c (%)	7.70 ± 2.46	7.39 ± 2.66	8.52 ± 3.06	0.468 [d]
Triglyceride (mg/dL)	129.43 ± 66.2	122.67 ± 60.47	137.33 ± 87.51	0.830 [d]
Cholesterol (mg/dL)	185.3 ± 44.9	178.71 ± 51.56	170.53 ± 55.55	0.737 [d]
LDL- cholesterol (mg/dL)	117.93 ± 32.2	112.38 ± 43.43	101.93 ± 43.84	0.798 [d]
HDL- cholesterol (mg/dL)	41.21 ± 11.99	40.71 ± 10.67	38.80 ± 8.17	0.561 [d]
Cardiac Enzymes				
High CK	10 (71.4%)	19 (90.5%)	14 (93.3%)	0.260 [d]
High CK-MB	10 (71.4%)	17 (81.0%)	13 (86.7%)	0.609 [d]
High LDH	9 (64.3%)	13 (61.9%)	11 (73.3%)	0.765 [d]
High Troponin-I	11 (78.6%)	20 (95.2%)	13 (86.7%)	0.346 [d]

*Wild "GG"; Heterozygous "GC"; Homozygous mutant "CC"; *. Statistically significant p-value (<0.05); [f]. Fisher's Exact test; [b]. Chi-square test; [d]. One-way ANOVA test.*

No statistically significant associations between the +405 C/G gene polymorphism and all study variables in the ACS group. Were found (Table 2). Further analysis on the association between the +405 C/G gene polymorphism and ACS revealed that the recessive model was statistically significant with an OR of 3.86 (95% CI: 1.28-11.64, p=0.017). Although the odds ratio for the dominant model was 1.58, it was not statistically significant (95% CI: 0.68-3.65, 0.289) (Table 3).

Table 3:. Dominant and Recessive models of +405 C/G gene polymorphism among study groups				
Genotypes of +405 C/G gene polymorphism	Study Groups		OR (95% CI)	p-value
	Non-ACS (n = 50)	**ACS** (n = 50)		
Dominant model				
GG	19 (38.0%)	14 (28.0%)	REF	
GC+CC	31 (62.0%)	36 (72.0%)	1.58 (0.68, 3.65)	0.289 [b]
Recessive model				
GG + GC	45 (90.0%)	35 (70.0%)	REF	
CC	5 (10.0%)	15 (30.0%)	3.86 (1.28, 11.64)	**0.017*** [b]

GG "Wild"; GC "Heterozygous"; "CC" Homozygous mutant "CC"; Reference "REF"
**. Statistically significant p-value (<0.05); [b]. Chi-square test.*

Genotyping results of +405 C/G VEGF gene polymorphism rs2010963

The PCR product was treated by restriction enzyme (BSMF1). So, the presence of a single band of 469bp represents the presence of C allele (mutant allele), while the presence of two bands of 195bp and 274bp represents the presence of allele G (Wild type), So, there were 3 genotypes: CC, CG and GG. Allele carrier: genotype CC is a carrier for allele C only, genotype GG is a carrier for allele G only, while GC is a carrier for both C and G alleles (Fig. 1). In Table 4, the crude odds ratios of bivariate associations show that there were no statistically significant associations between mutant allele carrier and all study variables. However, statistically significant associations existed with chronic illnesses. patients with chronic illnesses showed significantly higher odds of MAF compared to those without chronic illness

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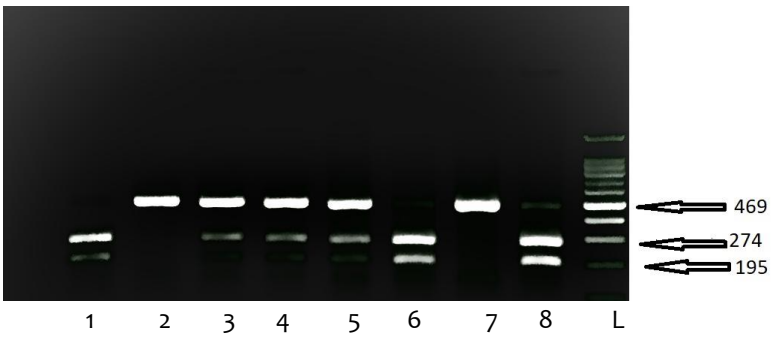

Figure 1: Electrophoretic pattern of different rs2010963 (C/G) genotypes

Photograph of a 2% agarose gel showing the digested PCR products for rs2010963 (C/G) genotyping. Lane L: DNA ladder (100bp). Lane 1,6,8: Wild GG genotype showing 2 bands size 274 and 195bp. Lane 2,7: Homozygous CC genotype showing one band size 469bp. Lane 3,4,5: heterozygous GC genotype showing bands size 469, 274, and 195bp.

Discussion

ACS is one of the chronic progressive and polygenic diseases, and atherosclerosis is the major pathological process underlying CAD[23]. This disease is considered to be the major cause of mortality in developed and developing countries. The risk factors

namely; lifestyle, environmental factors, and genetic factors are considered for the susceptibility of cardiovascular disease. The prevalence of risk factors among healthy individuals increase the probable occurrence of CAD in near future[24]. There are many studies recently demonstrated the effect of VEGF on the incidence of coronary artery disease. The polymorphism +405 C/G has been the main focus of the research into cardiovascular diseases, and the findings have shown that of this polymorphism, *VEGF* +405 C/G could be considered a more potential genetic variant for atherosclerotic CAD in different populations[25-29].

Table 4: Crude and adjusted association of the +405 C/G gene polymorphism (Carriers of the mutant allele C) and the study variables

Variables	N	Minor Allele Carriers of +405 C/G gene polymorphism				
		n (%)	**Crude OR** (95% CI)	p-value	**Adjusted OR** (95% CI)	p-value
ACS *vs.* Non-ACS	50	36 (72.0%)	1.58 (0.68, 3.65)	0.289	2.03 (0.57, 7.24)	0.274
Smoker vs. non-smoker	49	33 (67.3%)	1.03 (0.45, 2.37)	0.942	1.84 (0.39, 8.68)	0.442
Chronic Illnesses *vs.* None	56	42 (75.0%)	2.28 (0.98, 5.33)	0.057	4.15 (1.18, 14.58)	**0.026***
WBC ($x10^3/\mu l$)	100	NA	1.002 (0.88, 1.14)	0.975	1.02 (0.87, 1.21)	0.776
HbA1c%	100	NA	1.07 (0.871, 1.31)	0.533	0.83 (0.59, 1.18)	0.298
Triglyceride (mg/dL)	100	NA	0.9995 (0.99, 1.01)	0.681	1.01 (0.99, 1.03)	0.312
Cholesterol (mg/dL)	100	NA	0.995 (0.99, 1.00)	0.282	0.96 (0.90, 1.04)	0.329
HDL (mg/dL)	100	NA	1.003 (0.97, 1.04)	0.863	1.05 (0.97, 1.14)	0.235
LDL (mg/dL)	100	NA	0.995 (0.99, 1.01)	0.317	1.02 (0.94, 1.09)	0.660

*OR: Odds Ratio; CI: Confidence Interval; *. Statistically significant p-value (<0.05), binary logistic regression*

In the current study, we aimed to assess the association between *VEGF* rs2010963 (+405 C/G) polymorphism and acute coronary syndrome. Therefore, we enrolled fifty patients diagnosed of ACS (72% males, 28% females) with a mean age 61.0 ± 12.2 and fifty normal subjects (58% males, 42% females) with a mean age 59.1 ± 11.2. Both groups were sex and age-matched. In our study, most of the risk factors; hypertension, smoking, and dyslipidemia were high in both groups with no statistically significant difference in agreement with the results of different studies that addressed the same topic[30,31]. Other studies showed that the majority of cardiovascular risk factors were more frequent in ACS group than those with normal coronary artery[31]. In this regard, the prevalence of hyperlipidemia and smoking were higher in the ACS group compared to the non-ACS group. While in our study, DM was significantly higher among the ACS group (20%) than the control group with P= 0.037, these results were in agreement with the results obtained by Ferrannini G. et al. who

concluded that CAD was more prevalent among participants with Type 2 diabetes mellitus (HbA_{1c} 6.7 ± 1.1%) than those without diabetes and with findings of kalayi Nia, S. et al, who demonstrated that diabetes mellitus was higher in the CAD group compared to non- CAD group[31,32]. According to our study, WBCs count was significantly higher in ACS group than in the control group. These results are similar to the results obtained by Takeda Y. et al who showed that WBC baseline counts in patients with ACS were higher than those of the healthy participants. Also, hypertension, hyperlipidemia, and HBA1c fraction ≥ 6.1% had a significant relationship with coronary artery disease[33]. Single nucleotide polymorphisms at certain locations can predict an individual suscibtibility to environmental factors. Some SNPs can affect gene function by affecting its expression and dectating their alternative splicing decisions and post translational modifications[34]. In the current study, the percentage distribution of VEGF gene rs2010963 showed that the heterozygous variant (GC) was the most prevalent (47%) , followed by the wild type (normal variant (GG) with percentage (33%) and the mutant homozygous variant (CC) with percentage (20%) . Also, the percentage of mutant homozygous variant was higher among ACS patients than in control individuals with percentages (10%) and (30%) respectively with statistically significant differences (OR=4.07, 95%CI=1.20-13.86; P= 0.025). The wild type showed higher frequency in the control group than in ACS patients with percentages (38%) and (28%) respectively as well as the heterozygous variant was higher in the control group than in ACS patients with percentages (52%) and (42%) respectively. Also, according to our results, Minor allele frequency (C allele) was significantly higher among ACS patients compared to the control group (51% vs 36%), P=0.033. These results were similar to those showed by kalayi Nia, S. et al which concluded that there was an association between the CC genotype of *VEGF* +405 C/G polymorphism and ACS (OR=3.65, 95%CI=1.53–8.72; *P*=0.003) and no significant association between the heterozygote model (GC) of *VEGF* +405 C/G polymorphism and ACS risk (*P*=0.175)[32]. In contrast to our study, Moradzadegan A. et al concluded a strong and significant relationship between *VGEF* +405 C/G polymorphism G allele and increased risk of ACS in diabetic patients after adjustment for the presence of normal lipids and absence of history of increased blood pressure with OR=2.25 (95% CI= 1.03–4.9, p=0.043). The presence of G allele increased the risk of CAD 1.75-fold (p=0.024)[35]. While the study conducted by Wang Y. et al showed that *VEGF* +405 C/G polymorphism wasn't associated with risk of overall CAD. However, in a subgroup analysis by the type of ACS, the polymorphism was associated with MI risk (CC *vs*. GG: OR = 1.62; 95% CI, 1.05 - 2.50; *P* = 0.029; CC *vs*. CG+GG: OR = 1.51; 95% CI, 1.01 - 2.27; *P* = 0.047)[36]. Regarding our study, the recessive model was statistically significant with an OR of 3.86 (95% CI: 1.28-11.64, P=0.017) while the odds ratio for the dominant model was 1.58, it was not statistically significant (95% CI: 0.68-3.65, p= 0.289). Similar to these results, Ma, WQ. et al showed that the association between *VEGF* +405 C/G polymorphism and ACS risk was found in the recessive and homozygous genetic models (CC vs. GG + GC: OR = 1.45, 95% CI = 1.03–2.05; CC vs. GG: OR = 1.57, 95% CI = 1.02–2.42). Stratification by sample size indicated that this SNP was significantly associated with ACS risk for small sample sizes compared to large sample sizes in several genetic models (CC vs. GG + GC: OR = 1.52, 95% CI = 1.01–2.33; CC vs. GG: OR = 2.03, 95% CI = 1.26–3.28; C vs. G: OR = 1.27, 95% CI = 1.03–1.78. No significant

associations were observed following a subgroup analysis by ethnicity and control source[37]. Several limitations in our study should be addressed. First, the relatively small sample size and the study design that is confined to a single ethnic group, could limit this analysis. Also, we only analyzed the +405 C/G polymorphism regardless of other polymorphisms related to the susceptibility of CAD and its clinical outcomes.

Conclusion

The current study supports the existence of an association between *VEGF* gene polymorphisms (rs2010963) and susceptibility to ACS so it could be considered as a genetic marker for predicting ACS in the Egyptian population.

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