Int J Clin Exp Med 2018;11(6):6099-6105
www.ijcem.com /ISSN:1940-5901/IJCEM0019901

Original Article

Influence of VEGF polymorphisms on ischemic stroke in Chinese Han population: a case-control study

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Received November 16, 2015; Accepted August 21, 2016; Epub June 15, 2018; Published June 30, 2018

Abstract: Aim: Our aim was to investigate the association of interactions between vascular endothelial growth factor (*VEGF*) gene rs3025039 and rs3025021 polymorphisms and environmental factors with ischemic stroke (IS) and its subtypes in Chinese Han population. Methods: We adopted a case-control study in 120 IS cases and 140 healthy controls frequency-matched in age and gender. Taqman probe allelic discrimination technique was used to detect the genotypes of *VEGF* polymorphisms (rs3025039 and rs3025021) and conduct the genotyping. The χ^2 test conducted the differences comparison in genotype and allele frequencies of the two polymorphisms between the case and control subjects. The association strength of gene polymorphism and disease was evaluated by odds ratio (OR) with 95% confidence interval (CI). In addition, crossover analysis was conducted to detect the potential gene-environment interaction. Results: The differences in distributions of CC and CT+TT genotypes of *VEGF* rs3025039 polymorphism between the case and control groups were found statistically significant (OR=2.25, 95% CI=1.29-3.94), after adjusted by clinical basic information. Subtype analysis indicated that CT+TT genotype of *VEGF* rs3025039 was also associated with large artery atherosclerosis (LAA) risk (*P*=0.036). Interactions existed between *VEGF* rs3025039 polymorphism and smoking and drinking. Conclusions: In Chinese Han population, *VEGF* rs3025039 may be correlated with the increased risk of IS and LAA, especially in smokers and drinkers. More gene-environment association studies are hoped to further explore this issue.

Keywords: *VEGF*, polymorphism, IS, environmental factors

Introduction

Ischemic stroke (IS) is a disease characterized by high incidence, wide distribution and severe social influence, and has become a serious threat to the health of Chinese people [1]. It can lead to a long-term and even permanent disability and affect the life quality of patients and their families [2]. Approximately 80% of IS patients are caused by the blocking of the cerebral blood supply [3]. It is a multifactorial disease caused by genetic and environmental factors [4]. Life styles, such as the smoking, drinking and dietary habit, form the person's environment, and might involve in the disease occurrence. But some traditional risk factors such as age, high blood pressure (HBP), diabetes, hyperlipidemia, and smoking don't completely explain the occurrence and development of IS. At present, it has been identified that atherosclerosis (AS) is a main cause of IS. Blood vessels in atheromatous plaque have active proliferation, and most of the new vessels are immature and microthrombus tends to occur. As a result, the atheromatous plaque may become instable and even broken [5-8].

Vascular endothelial growth factor (VEGF) encoded by *VEGF* gene which is located in chromosome 6p12 is a factor most closely related to neovascularization in the AS region [9]. Studies have demonstrated that VEGF mainly interacts with tyrosine kinase receptor-vascular endothelial growth factor receptor-2 (VEGFR-2), which can induce the proliferation and metastasis of endothelial cells, increase the vascular permeability, and promote the progression of atherosclerotic lesions [7, 8, 10, 11]. Recent studies have indicated that *VEGF* can promote the vascular proliferation in ischemic regions of the brain, improve the blood supply for microcirculation in local regions, and reduce the infart

area, so it can be as a marker for the prediction of IS in elders [12-16].

Some single nucleotide polymorphisms (SNPs) of *VEGF* gene have been revealed to closely associate with IS [17], however, most of the SNPs studied were located in the promoter region of *VEGF* gene. Therefore, we carried out a case-control study in Chinese Han population to explore the association between two SNPs (rs3025021 in intron 7 and rs3025039 in exon 8) and IS as well as its subtypes, and further studied the interactions of the two SNPs and environmental factors based on IS.

Materials and methods

Clinical materials

In our case-control study, 120 IS patients (86 men and 34 women) were collected from Neurology Department in Yidu Central Hospital from June, 2011 to August, 2013, and 140 healthy controls (98 males and 42 females) whose age and gender matched with the case subjects were randomly selected from the Physical Examination Center during the same period of time. Mean age of the cases and the controls was respectively 60.8 ± 7.6 and 62.6 ± 9.3 years. The patients were diagnosed by pathobiology and in accordance with NINDS (National Institute of Neurological Disorders and Stroke) standard. Participants with the following diseases would be excluded: subarachnoid hemorrhage, thromboembolic cerebral infarction, severe infections, malignant tumors, and severe chronic diseases. All study subjects were from Affiliated to Weifang Medical College, and unrelated with each other by blood. The present study was authorized by the Ethics Committee of each clinical center and obtained the informed consent of all subjects.

Data collection

Questionnaires and inquiries concerning the following items were conducted based on the cases and controls in the same form: history of present illness and past medical history, physical examination result, and personal physical condition, including body mass index (BMI), histories of high blood pressure (HBP), coronary heart disease (CHD), cigarette smoking, alcohol drinking, and family history of IS. We divided the IS patients into small artery occlusion (SAO), cardioembolism (CE) and large artery atherosclerosis (LAA) groups after excluding the IS cases caused by other etiologies or other unknown reasons according to the Trial of Org 10172 in Acute Stroke Treatment (TOAST).

Definitions

HBP patients referred to those with a ≥140 mmHg systolic pressure or a ≥90 mmHg diastolic pressure, or those who took antihypertensive medications once. People with CHD referred to those who has identified histories of angina pectoris and myocardial infarction. Those who smoked an average of at least 1 cigarette a day within a three-year period were as the smokers. Persons who drank an average of more than 50 g liquor each day for more than three continuous years were thought to have a drinking history. People at least one immediate family members suffering from cerebral infarction were defined to have a family history of IS.

Extraction of DNA

2 ml of morning fasting elbow vein blood was collected from each subject and put in tubes with Ethylene Diamine Tetraacetic Acid (EDTA) anticoagulant on the first day in hospital. Genomic DNA was extracted from the blood with a standard DNA extraction kit (Shanghai Sangon Biotech Service Co., Ltd.).

Selection of SNPs

Based on the data in NCBI SNP Database, we chose the SNPs with a minor allele frequency (MAF) larger than 18% or those had been reported as our objective SNPs, and finally determined rs3025021 and rs3025039 of *VEGF* gene.

Genotyping of VEGF rs3025039 and rs3025021

The design and synthesis of primers and probes of *VEGF* rs3025039 and rs30250321 were performed by Applied Biosystems Company. Genotyping of the two SNPs was conducted with the Taqman probe method and a Roche LightCycler480 real-time fluorescent

Table 1. Comparison of clinical characteristics between the case and control groups

Materials	Case group (n=120)	Control group (n=140)	*P*
Age ($\bar{x}$ ± s, year)	60.8 ± 7.6	62.6 ± 9.3	0.480
Male/Female	86/34	98/42	0.923
Smoking history	63 (52.5)	45 (32.1)	0.039
Drinking history	58 (48.3)	39 (27.9)	0.024
CHD history	23 (19.2)	16 (11.4)	0.169
HBP history	73 (60.8)	57 (40.7)	0.068
IS family history	29 (24.2)	19 (13.6)	0.084
BMI (≥25 kg/m^2)	34 (28.3)	26 (18.6)	0.154

quantitative PCR instrument. The volume of PCR was 12.5 μl, and the reaction conditions included predenaturation at 95°C for 10 minutes; 40 cycles of denaturation at 92°C for 15 seconds, annealing at 60°C for 1 minute, extension at 72°C for 1 minute; and finally long extension at 72°C for 5 minutes. Some samples were sequenced to verify whether each reaction plate with 96 holes included standard samples of the three known genotypes (wild homozygote, mutation homozygote, and heterozygote).

DNA sequencing

To test the accuracy of Taqman probe method in detecting the genotypes of *VEGF* rs3025039 and rs3025021, we conducted DNA sequencing of PCR products representing different genotypes. About 50 μl of PCR amplification products were obtained from each genotype sample, and then cloned into vectors and sequenced (Shanghai Sangon Biotech Service Co., Ltd.).

Statistical methods

SPSS 18.0 software was used to conduct the statistical analysis, and Hardy-Weinberg Equilibrium (HWE) test was applied to detect the representativeness of the samples. The data were represented by $\bar{x}$ ± s or %. We adopted χ^2 test to analyze the differences in genotype and allele frequencies of *VEGF* rs3025039 and rs3025021 polymorphisms between the cases and the controls, the results were adjusted by the logistic regression to comprehensively analyze the role of each SNP in IS. In the meanwhile, the crossover analysis was used to assess the gene-environment interactions. The difference was statistically significant when $P<0.05$.

Results

Comparison of clinical materials

After examined by t-test and χ^2 test, differences in age, gender, histories of CHD and HBP, family history of IS, and BMI between the case and control subjects had no statistical significance ($P>0.05$). However, cigarette smoking and alcohol drinking in the case group were more common compared to those in the control group, with statistical significance ($P<0.05$) (**Table 1**).

Comparison of genotype and allele distributions

Genotype distributions of *VEGF* rs3025039 and rs3025021 polymorphisms in the control group were in accordance with HWE (**Figure 1**, $P>0.05$). No obvious correlation was found to exist between *VEGF* rs3025021 polymorphism and IS susceptibility respectively before and after adjusted by confounding factors (**Table 2**, $P>0.05$). CT+TT genotype of *VEGF* rs3025039 polymorphism had a higher frequency in the cases than the controls and might positively associated with the risk of IS (OR=2.04, 95% CI=1.23-3.41). After through the adjustment of related confounding factors (age, gender, histories of cigarette smoking, alcohol drinking, HBP, CHD, BMI, and family history of IS) with logistic regression analysis, the effect of *VEGF* rs3025039 polymorphism CT+TT genotype still caused an increased risk of IS (OR=2.25, 95% CI=1.29-3.94, $P=0.007$).

Relationship between two SNPs and subtypes of IS

We found a statistical correlation between the mutant genotype CT+TT of *VEGF* rs3025039 and the susceptibility to LAA ($P=0.036$), but the significant association was not found in genotypes of *VEGF* rs3025021 (**Tables 3**, **4**).

Interactions of VEGF rs3025039 and environmental factors

Significant interactions existed between VEGF rs3025039 polymorphism and smoking and drinking status, the interaction positively correlated with the IS risk. Compared with non-

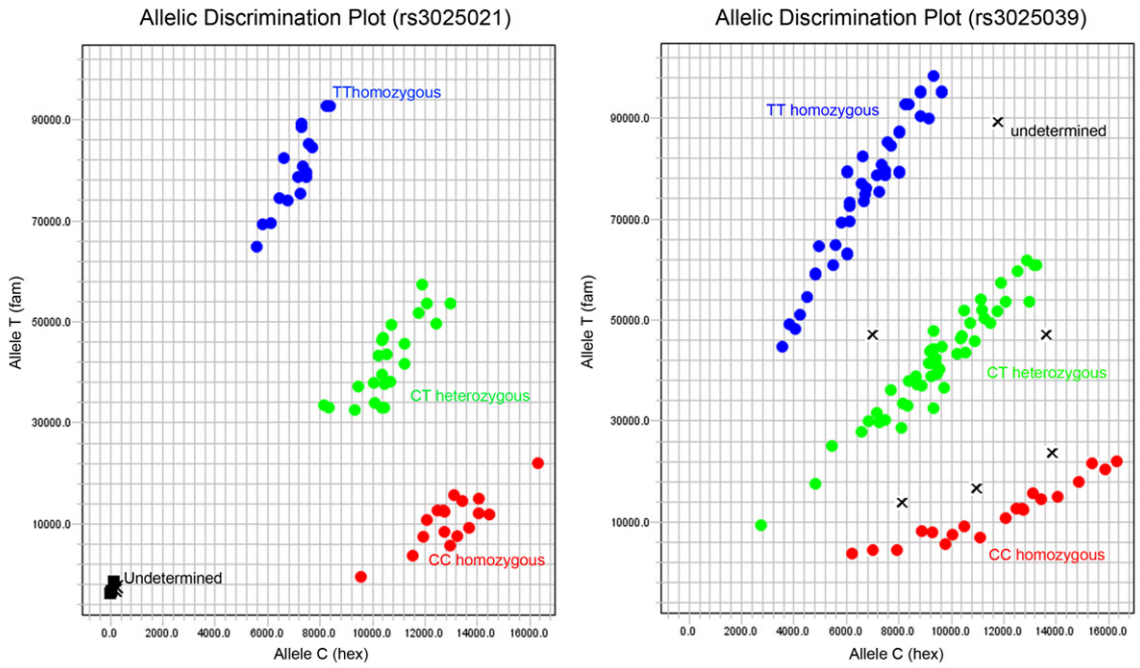

Figure 1. The genotyping results for *VEGF* polymorphisms.

Table 2. Distribution of genotypes and alleles of *VEGF* rs3025039 and rs3025021

Genotype	Case (n=120)	Control (n=140)	*P*	OR (95% CI)	OR (95% CI)#	*P*#
rs3025021						
CC	90 (75.0)	98 (70.0)	-	1.00	1.00	-
CT+TT	30 (25.0)	42 (30.0)	0.406	0.78 (0.45-1.35)	0.61 (0.33-1.14)	0.123
rs3025039						
CC	65 (54.2)	99 (70.7)	-	1.00	1.00	-
CT+TT	55 (45.8)	41 (29.3)	0.007	2.04 (1.23-3.41)	2.25 (1.29-3.94)	0.004

Note: #: the value after adjustment of clinical information (age, sex, BIM, the histories of smoking, drinking, CHD, HBP and IS family history).

Table 3. Comparison of genotypes of *VEGF* rs3025021 based on the subtypes of IS

Group	Number	rs3025021		
		CC	CT+TT	*P*
LAA	62	43	19	1.000
SAO	36	26	10	0.841
CE	22	16	6	1.000
Control group	140	98	42	-

Note: LAA refers to large artery atherosclerosis, SAO refers to small artery occlusion, and CE refers to cardio-embolism.

Table 4. Comparison of genotypes of *VEGF* rs3025039 based on the subtypes of IS

Group	Number	rs3025039		
		CC	CT+TT	*P*
LAA	62	34	28	0.036
SAO	36	23	13	0.425
CE	22	13	9	0.322
Control group	140	99	41	-

Note: LAA refers to large artery atherosclerosis, SAO refers to small artery occlusion, and CE refers to cardio-embolism.

smokers carrying *VEGF* rs3025039 CC genotype, the smokers with CC and CT+TT genotypes (OR=2.23, 95% CI=1.17-4.23; OR=4.00, 95% CI=1.79-8.92), as well as the nonsmokers with CC genotypes (OR=2.32, 95% CI=1.19-4.51) all had higher risk for IS development (**Table 5**). Similar results were discovered between the interactions of rs3025039 genotypes and

Table 5. Analysis of interactions of *VEGF* rs3025039 and environmental factors in IS

SNP	Risk factor	Case group	Control group	Original OR (95% CI)	Corrected OR (95% CI)#	*P*#
rs3025039	Smoking					
CC	-	30	65	1.00	1.00	0.002
CC	+	35	34	2.23 (1.17-4.23)	2.68 (1.32-5.46)	0.006
CT+TT	-	31	28	2.32 (1.19-4.51)	2.63 (1.27-5.47)	0.009
CT+TT	+	24	13	4.00 (1.79-8.92)	4.74 (1.95-11.53)	0.001
rs3025039	Drinking					
CC	-	34	67	1.00	1.00	0.006
CC	+	31	32	1.91 (1.00-3.63)	2.30 (1.14-4.66)	0.020
CT+TT	-	28	23	2.40 (1.21-4.78)	2.85 (1.33-6.14)	0.007
CT+TT	+	27	18	2.96 (1.43-6.11)	3.39 (1.50-7.63)	0.003

Note: #: the value after adjustment of clinical information (age, sex, BIM, the histories of smoking, drinking, CHD, HBP and IS family history).

drinking (OR>1.91). After the adjustment of related risk factors, the OR values were respectively increased (**Table 5**, OR>2.3, $P<0.05$).

Discussion

IS is the most important cause of disability in adults across the world [18], and the incidence thereof in Asians or only in Chinese populations is higher than that in Europeans or Americans. There are over 1,500,000 new cases of IS in China every year, but the pathology and ethology of IS still remain mysterious and the outcomes of treatment in current medical methods are also unsatisfactory. It has been suggested that there exists a close relationship between IS and AS, and one important mechanism of the thrombogenesis and rupture of atherosclerotic plaque is the neovascularization in atheromatous plaque. *VEGF* and its receptor signaling pathways play a crucial role in vasculogenesis as well as in AS [19-21].

Numerous studies have demonstrated that *VEGF* modulates the development and maturity of blood vessels through *VEGF*R-2 [15]. The autophosphorylation of tyrosine residue *VEGF*R-2 in the cytoplasm can activate the motigen-activated protein kinase (MAPK) and endothelial nitric oxide synthase (eNOS) in downstream signal transduction pathways, which promotes the proliferation, and metastasis of endothelial cells and induces neovascularization [16]. Chen et al. have indicated that *VEGF* and *VEGF*R-2 are highly expressed in the atheromatous plaque of human coronary artery and carotid artery [20]. In contrast, angiogenesis inhibitors can greatly inhibit the development of atheromatous plaque lesions so that the plaque volume is reduced [21, 22]. IS is not a monogenic disease, it is affected by interactions of genetic and environmental factors such as HBP, diabetes, and abnormal blood lipid [23-25].

Our study demonstrated that the genotype distribution of *VEGF* rs3025039 was different between the case and the control groups and the individuals with genotype CT+TT of *VEGF* rs3025039 had a higher IS risk than those with genotype CC; and the CT+TT genotype was correlated with the susceptibility to LAA but not SAO and CE. The pathogenesis of LAA is that the excessive lipid accumulated on endarterium and neurovascular dysfunction makes the arterial vascular wall become thickened and hardened, lose elasticity and lead to a shrinking lumen. Many current studies have also shown a relationship between the *VEGF* gene and AS. The interactions of *VEGF* rs3025039 and rs3025021 polymorphisms and environmental factors was also analyzed based on IS in this article. The results showed that there existed interactions between smoking and drinking and CT +TT genotype of *VEGF* rs3025039. Individuals with genotype CT+TT of *VEGF* rs3025039 as well as smoking and drinking histories had a higher IS risk than those with genotype CC but with no smoking and drinking histories, which revealed genetic factors didn't play an indenpendent role in the onset of IS.

The present study selected IS which had a high morbidity and severe social influence as the research object, used reasonable experi-

mental methods, and finally obtained credible results. Overall, genotype CT+TT of *VEGF* rs3025039 may be associated with the increased risk of IS and and its subtype LAA, especially in smokers and drinkers. But due to some limitation conditions, our conclusion may exist little deviation, for example, the sample size is too small to represent the whole race and the gene polymorphism is also possibly different in different populations and races. Moreover, the power for present study is low because the small sample size. The association of between the interaction of *VEGF* rs3025039 polymorphism and environmental factors with the pathogenesis of IS needs to be further verified in the further with enough large sample size and various populations.

Disclosure of conflict of interest

None.

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