

Original Article

Association between VEGF polymorphisms (936c/t, -460t/c and -634g/c) with haplotypes and coronary heart disease susceptibility

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Received November 15, 2014; Accepted December 24, 2014; Epub January 1, 2015; Published January 15, 2015

Abstract: Aim: Our aim was to investigate the association between single nucleotide polymorphisms (SNPs) of vascular endothelial growth factor (VEGF) and coronary heart disease (CHD) susceptibility in Chinese Han population. Methods: 144 CHD patients and 150 healthy individuals were enrolled in the study. Three SNPs (936C/T, -460T/C and -634G/C) of VEGF were chose and then were genotyped with Sequenom time-of-flight mass spectrometry (TOFMS). Odds ratio (OR) with 95% confidence interval (CI) were used to evaluate the association of genotypes and haplotypes and CHD susceptibility. Results: The frequencies of -460T/C CC genotype (13.6%) was found higher in the case group than that of control group (6.7%), which indicated that CC genotype was a risk factor for CHD (OR=2.50, 95% CI=1.10-5.68). Correspondently, the C allele appeared to increase the risk of CHD (OR=1.54, 95% CI=1.07-2.22). For -634G/C polymorphism, the risk of the CC genotype carrier for CHD increased 2.24 fold compared to the wild genotype. Moreover, -634G/CC allele was significantly associated with CHD susceptibility (OR=1.65, 95% CI=1.15-2.36). In addition, +936C/T CT genotype and C allele appeared to be a genetic-susceptibility factors for CHD (OR=2.43, 95% CI=1.44-4.10; OR=1.95, 95% CI=1.26-3.02). The haplotype analysis showed that T-C-T, C-C-C and C-G-C haplotypes all could increase the risk for CHD (OR: 2.43, 2.77 and 2.33). Conclusion: we concluded VEGF polymorphisms were associated with CHD susceptibility. Moreover, the haplotypes of T-C-T, C-C-C and C-G-C all could increase the risk for CHD.

Keywords: Coronary heart disease, vascular endothelial growth factor, polymorphism, haplotype

Introduction

Coronary heart disease (CHD) is also named coronary atherosclerotic heart disease and occurrence of the disease is closely associated with ischemia, anoxia and necrosis of myocardial caused by vascular dysfunction and obstruction result from atherosclerotic lesions in coronary artery. CHD is a complex disease involving genetic and environment factors [1]. The risk environment factors include smoking, drinking, stress, lack of exercise and hyperlipidemia [2]. For genetic factor, genome-wide association studies (GWAS) identified many single-nucleotide polymorphisms (SNPs) associated with CHD [3-10].

Vascular endothelial growth factor (VEGF) is a kind of glycoprotein with about 64,000 molecu-

lar weight. VEGF family includes VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, VEGF-F and placental growth factor (PlGF), of which VEGF-A often refers to VEGF. VEGF plays a key role in neovascularization, regulation of vasopermeability and direct protection for nerves [11]. Moreover, VEGF has been demonstrated to promote the formation of angiocarp and associate with the risk of CHD [12]. Molecular biology studies indicated that VEGF gene includes numerous SNPs and the expression level of VEGF could be regulated by certain SNP polymorphisms [13, 14]. However, there are few studies concerning the relationship of VEGF SNPs polymorphisms and CHD.

In our study, we chose 3 SNPs of VEGF gene and investigated the association between VEGF polymorphisms and CHD susceptibility in

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Table 1. Primer sequence

SNP	Region	Primer	Length
-460T/C	Promoter	F: 5'-CCTCTTTAGCCAGAGCCGGGG-3' R: 5'-TGGCCTTCTCCCGCTCCAAC-3'	360 bp
-634G/C	Promoter	F: 5'-TTGCTTGCCATTCCCCACTTG-3' R: 5'-CCGAAGCGAGAACAGCCCAGAA-3'	326 bp
936C/T	3'UTR	F: 5'-ACCACACCATCACCATCG-3' R: 5'-CCAACCTCAAGTCCACAGC-3'	295 bp

Chinese Han population, which could provide experimental basis for the search of drug targets related to the pathogenesis of CHD.

Materials and methods

Subjects

A total of 144 CHD patients were enrolled from cardiology department in Peking University Third Hospital during Nov. 2008-Jun. 2014. The examinations included physical examination, electrocardiogram, myocardial enzyme, cardiac color ultrasound and coronary angiography. And the arteriography result was judged by two experienced physicians. Coronary angiography was identified that the stenosis in at least one of the three main blood arteries (right coronary artery, anterior descending branch and circumflex branch) was more than 50% and the stenosis was also tested in two left main coronary arteries. 150 healthy checker in the hospital were selected in the study with normal examination results except atypical chest pain and normal angiography. The coronary angiography results indicated that the stenosis of coronary artery was less than 50%. The subjects with abnormal liver functions, infectious diseases and rheumatic diseases were excluded. Clinical data were shown in **Table 1**.

DNA extraction

Five mL peripheral blood was obtained from every subject and then put in EDTA anticoagulant tube. DNA was extracted with the phenol-chloroform extraction method and was purified by ethanol. DNA samples were stored under -20°C.

PCR reaction

The primers were designed by Premier 5 software (Sequenom Company, USA). Primer sequence was shown in **Table 1**. PCR reaction

mixture included DNA 1 µL, 0.95 µL ddH₂O, 0.625 µL PCR buffer (with 15 mmol/L MgCl₂), 2.5 mmol/L dNTP 1 µL, 25 mmol/L MgCl₂ 0.325 µL, each primer 1 µL and HotStarTaq enzyme 0.1 µL. PCR was conducted under the following conditions: pre-degeneration at 94°C for 15 min, followed by 45 cycles of 94°C for 20 s, 56°C for 30 s, 72°C for 1 min and at 72°C for 3 min. After PCR amplifi-

cation, the residual dNTP was digested by dephosphorylation. Reaction mixture included ddH₂O 1.53 µL, SAP buffer 0.17 µL and alkaline phosphatase 0.3 µL. This reaction was performed at 37°C for 40 min and enzyme was inactivated by 85°C for 5 min. After the processing of alkaline phosphatase, the reaction system based on the extension primers of SNP single-base was proceeded with ddH₂O 0.755 µL, 10× iPLEX buffer 0.2 µL, termination mixture 0.2 µL, iPLEX enzyme 0.041 µL, 10 µmol/L extension primers 0.804 µL under the following condition: 40 cycles of 94°C for 30 s followed by 5 cycles of 94°C for 5 s, 52°C for 5 s and 80°C for 5 s and finally 72°C for 3 min.

Genotyping

The genotyping of SNPs was completed by Beijing Sunbiotech Co., Ltd with MassARRAY system (Sequenom Company, USA). The terminated reactants were mixed with 6 mg cation exchange resin for desalination and then 25 µL water was added into the mixture for suspension.

The genotyping products were added on a piece of spectroCHIP with 384 holes by MassARRAY Nanodispense system and analyzed by matrix assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS). The results were read timely with MassARRAY RT software (Version 3.0.0.4) and genotyping analysis was completed by MassARRAY Type software.

Statistical analysis

Chi-square (χ^2 -Test) was used to analyze distribution differences of gender, cigarette smoking and alcohol drinking between two groups and further verify whether genotype frequencies complied with HWE. MannWhitney-U was used to test the age differences between two groups. Odds ratio (OR) with 95% CI calculated by

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Table 2. Comparison of general information between two groups

	Case	Control	P value
Average age	65.54±8.63	62.36±8.14	0.097
Gender			
Male	87 (60.2)	93 (62.0)	0.811
Female	57 (39.8)	57 (38.0)	
Cigarette smoking			
Yes	94 (65.5)	103 (68.6)	0.620
No	50 (34.5)	47 (31.4)	
Alcohol drinking			0.780
Yes	113 (78.4)	115 (76.8)	
No	31 (21.6)	35 (23.2)	

χ^2 -Test was used to show the correlation between polymorphisms, haplotypes and the risk of CHD. All the statistical tests were performed by SPSS18.0 statistical software. $P \leq 0.05$ indicates statistical significance.

Results

Subject characteristics

The average age of case group was 65.54, a little higher than that of control group (62.36). And we also found that there were no significant differences of gender, cigarette smoking and alcohol drinking between two groups (P : 0.811, 0.620 and 0.780) (Table 2).

Association of VEGF polymorphisms and CHD susceptibility

In the study, genotype distribution in the control group were all consistent with HWE (-460T/C: $P=0.40$; -634G/C: $P=0.70$; 936C/T: $P=0.29$). As shown in Table 3, the results showed that VEGF polymorphisms were associated the pathogenesis of CHD. The frequencies of -460T/C CC genotype (13.6%) was found higher in the case group than that of control group (6.7%), which indicated that CC genotype was a risk factor for CHD (OR=2.50, 95% CI=1.10-5.68). Correspondently, the C allele appeared to increase the risk of CHD (OR=1.54, 95% CI=1.07-2.22). For -634G/C polymorphism, the risk of the CC genotype carrier for CHD increased 2.24 fold compared to the wild genotype. Moreover, -634G/CC allele was significantly associated with CHD susceptibility (OR=1.65, 95% CI=1.15-2.36). In addition, +936C/T CT genotype and C allele appeared to

be a genetic-susceptibility factors for CHD (OR=2.43, 95% CI=1.44-4.10; OR=1.95, 95% CI=1.26-3.02).

Haplotype analysis

Haploview software was used to test the linkage disequilibrium (LD) and the haplotype analysis of three SNPs (-460T/C, -634G/C and 936C/T). The results indicated that there was statistical significance between each locus on LD. The haplotypes analysis showed that the haplotypes were associated with CHD susceptibility, among which T-C-T, C-C-C and C-G-C haplotypes all were genetic-susceptibility factors of CHD (OR=2.43, 95% CI=1.54-3.83; OR=2.77, 95% CI=1.25-6.15; OR=2.33, 95% CI=1.45-3.76) (Table 4).

Discussion

CHD, one of the most common heart diseases, is also called ischemic heart disease (IHD), which mainly occurs when confined coronary artery and insufficient blood supply cause cardiac dysfunction and/or organic lesion. The symptoms are squeezing pain in the center of the chest and the pain can transfer to neck, jaw, arm, back and stomach. At present, it is unclear whether CHD belongs to hereditary diseases. Nevertheless, numerous epidemiologic studies at home and abroad demonstrated that the pathogenesis of CHD involves obvious familial nature. However, the etiology of CHD is still unclear and genetic factors may be the internal causes.

VEGF was separated and purified from solution of bovine pituitary follicle stellate cells by Ferrara in 1989, which can accelerate the proliferation, differentiation, and migration of microvascular endothelial cells through receptors and contribute to the formation and greater permeability of microvessels [15]. VEGF could promote the recanalization of blood vessels in completely blocked thrombus and establishment of collateral circulation and improve the dependent vasodilatation of endothelial cells, which are tightly related to CHD.

At present, studies about VEGF polymorphisms mainly focused on new organic diseases associated with blood vessels, such as the occurrence, development, invasion of malignant tumors and inflammatory diseases, which has

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Table 3. Distribution of genotypes and alleles of 936C/T, 460T/C and -634G/C in case and control group

Geno-type	CHD (n=144) n (%)	Control (n=150) n (%)	χ^2	P value	OR (95% CI)
-460T/C					
TT	72 (50.0)	90 (60.0)	-	-	Reference
TC	52 (36.2)	50 (33.3)	1.073	0.300	1.30 (0.79-2.14)
CC	20 (13.6)	10 (6.67)	5.009	0.025	2.50 (1.10-5.68)
T	196 (68.1)	230 (76.7)	-	-	Reference
C	92 (31.9)	70 (23.3)	5.459	0.019	1.54 (1.07-2.22)
-634G/C					
GG	69 (46.9)	86 (57.3)	-	-	Reference
GC	49 (34.1)	54 (36.0)	0.233	0.629	1.13 (0.69-1.86)
CC	26 (19.0)	10 (6.7)	8.971	0.003	3.24 (1.46-7.18)
G	187 (64.9)	226 (75.3)	-	-	Reference
C	101 (35.1)	74 (24.7)	7.607	0.006	1.65 (1.15-2.36)
+936C/T					
CC	84 (58.3)	115 (76.7)	-	-	Reference
CT	55 (38.2)	31 (20.7)	11.362	0.001	2.43 (1.44-4.10)
TT	5 (3.5)	4 (2.7)	0.626	0.429	1.71 (0.45-6.26)
C	223 (77.4)	261 (87.0)	-	-	Reference
T	65 (22.6)	39 (13.0)	9.242	0.002	1.95 (1.26-3.02)

Table 4. Association of haplotypes and CHD susceptibility

Haplotype			CHD n (%)	Control n (%)	P	OR (95% CI)
-460T/C	-634G/C	+936C/T				
T	G	C	131	191	-	Reference
T	C	T	65	39	0.000	2.43 (1.54-3.83)
C	C	C	19	10	0.006	2.77 (1.25-6.15)
C	G	C	56	35	0.001	2.33 (1.45-3.76)
C	C	C	17	25	0.980	0.99 (0.52-1.91)

been reported to associate with some malignant tumors such as breast cancer, lung cancer, gastric cancer, colorectal cancer and thyroid cancer [16-20]. *VEGF* +936C/T polymorphism may be associated with the occurrence and development of diabetic nephropathy and C allele may be the susceptibility factor for diabetic nephropathy [21]. In addition, *VEGF* -460C/T and +405C/G polymorphisms were reported to be related to osteoarthritis [22]. Nevertheless, few studies analyzed the relationship of *VEGF* polymorphisms and CHD susceptibility. Our study indicated that *VEGF* polymorphisms (-460C/T, -634G/C and +936C/T) were significantly associated with CHD susceptibility. For genetic susceptibility factors for diseases, SNPs not only had an isolated effect on the occurrence and development of diseases,

but there were some certain internal associations and effects among them [23]. Haplotype is a reflection of genetic correlation thus the study of haplotype can easily reveal the correlation between multi-SNPs and disease susceptibility [24, 25]. -460T/C and -634G/C in promoter region of *VEGF* were adjacent to each other and +936C/T in untranslated region 3 is near the promoter region. The haplotypes analysis indicated that T-C-T, C-C-C and C-G-C haplotypes all could increase the risk for CHD (OR: 2.43, 2.77 and 2.33).

In conclusion, *VEGF* polymorphisms (-460C/T, -634G/C and +936C/T) were significantly associated with CHD susceptibility. Moreover, the haplotypes composed by -460T/C, -634G/C and 936C/T polymorphisms located in *VEGF* gene promoter are considered as the susceptibility factors for CHD. Nevertheless, further studies are needed to clarify how the polymorphisms and haplotypes control the plasma

levels of *VEGF*, and participate in the occurrence and development of CHD.

Disclosure of conflict of interest

None.

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