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Original Article

The relationship between the polymorphism of SG13S114 A/T in *ALOX5AP* gene and the vulnerability of carotid atherosclerosis in Chinese Han population

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Abstract: The purpose of this study was to determine the relationship between the polymorphism of SG13S114 A/T in *ALOX5AP* gene and the vulnerability of carotid atherosclerosis in Chinese Han population. Polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP) of SG13S114 A/T in *ALOX5AP* gene was performed for 284 patients with atherothrombotic cerebral infarction, of which 152 cases were diagnosed with stable plaque and the other 132 cases had vulnerable plaques. We found that the frequency of SG13S114 AA genotype and A allele of *ALOX5AP* gene in the cases with vulnerable plaques was significantly higher than those in the cases with stable plaques. These results suggested that high expression of SG13S114 AA genotype was related to the vulnerability of carotid atherosclerosis. SG13S114 A allele may be a risk factor of vulnerable plaques.

Keywords: 5-lipoxygenase activating protein, atherosclerosis, gene polymorphism, polymerase chain reaction

Introduction

Atherosclerosis (AS) is a chronic inflammatory disease. Multiple environmental and genetic factors facilitate the inflammatory processes of the artery wall. Abrupt rupture of the vulnerable plaques in the carotid atherosclerosis, activation of platelet and thrombus formation are one of the most important mechanisms of the cerebral infarction. Recent clinical and animal studies revealed that 5-lipoxygenase activating protein (FLAP) and leukotriene produced in the pathway of 5-lipoxygenase (5-LO) played an important role in the development and advancement of AS [1]. Thus, FLAP and leukotriene are considered as risk factors of AS. FLAP is encoded by *ALOX5AP* gene. The genotype of *ALOX5AP* gene that is most closely related to the cerebrovascular disease is SG13S114. However, the genetic data on the relationship between this genotype and the risk factors of plaque stability in carotid atherosclerosis in Chinese Han population is not available. Therefore, in this study, we used PCR-RFLP to determine the genotype of 132 cases with vulnerable plaques as diagnosed by carotid artery ultrasound and 152 cases with stable plaques. The SG13S114 polymorphism of *ALOX5AP* gene was compared between these two groups of cases to predict the genetic risk genotypes for the formation of vulnerable plaques.

Materials and Methods

Study subjects

The subjects included in this study were hospitalized patients in the Department of Neurology from August 2006 to May 2009. Carotid artery ultrasound was performed within 48 hours of cerebral infarction in the patients that met the requirement. Based on the ultrasonic criteria for the classification of carotid plaque [2], the carotid plaques with hypoechoes were classified into vulnerable group (n=132, male, 92 and female, 40) and the plaques with hyperechoes. were classified into stable group (n=152, male, 100 and female, 52). The age of the patients in the vulnerable group was between 52 and 88 with an average of 72.2±6.1. The age of the

patients in the stable group was between 45 and 88 with an average of 70.5±10.5. The diagnosis of the atherothrombotic cerebral infarction was in accordance with the new subtype classification of ischemic stroke by Sang Won Han[3].Cases were confirmed with head CT and/or MRI. The patients with non-symptom cerebral infarction, hemorrhage after infarction and cerebral hemorrhage were excluded from the study. All the cases were Han Chinese without a history of cerebral apoplexy. There was no consanguinity relationship among the cases in the study. None of the cases had liver/kidney disease, thrombolytic therapy, hematologic diseases, auto-immune disease, thyroid disease, cardiovascular disease, peripheral vascular diseases, vascular embolization disease, tuberculosis, malignant tumor, pregnant nor medication with any type of antihypercholesterolemic or anti-aggregation of platelet drugs. All the experiments were conducted with the informed consent.

PCR-RFLP analysis

Blood samples (2 ml) were collected from the peripheral vein of the patients on the second day after hospitalization and were stored at -80℃ for DNA extraction. EDTA-anticoagulated blood samples were lyzed in a low osmotic condition. Genomic DNA was extracted from the leukocytes using a routine phenol-chloroform method. Extracted DNA was dissolved in the appropriate volume of double distilled water. DNA concentration was measured with a nuclear acid analyzing instrument before preserving in -80℃. The specific primer for detecting SG13S114 polymorphism in *ALOX5AP* gene was 5'-GTG TTC AGG AAG GGA GTT TCT GT-3' (forward) and 5'-GTC TAT GGT TGC AAC ATT GAG ATT A-3'(reverse) synthesized by Shanghai GeneCore Biotechnologies Co., Ltd (Shanghai, China). PCR was performed in a 25 μl volume containing 0.1 μg of DNA, 2.5 μl of 10×Buffer, 1 μl of 25 mM • L^{-1} $MgCl_2$, 0.5 μl of 10 mM • L^{-1} dNTP, 0.5 μl of 20 μM • L^{-1} primers (forward and reverse), 0.5 μl Taq DNA polymerase (Promega, 5U/μl) and appropriate volume of sterile water. The parameter for amplification of *ALOX5AP* gene was predenaturing at 95℃ for 2min followed by 35 cycles of 94℃ for 45 s, 53℃ for 45 s and 72 ℃ for 1 min and a final extension of 72 ℃ for 10 min [4]. PCR was performed with a Thermo PX2 instrument. PCR products were purified (Takara, Agarose Gel DNA Fragment Recovery Kit) and digested with VspI restriction enzyme (Ferments, Glen Burnie, MD) for 16 h at 37℃. The digested products were electrophoresed in 2.5% agarose gel.

Statistical analysis

Statistical analysis was performed with SPSS12.0 software. ANOVA analysis was performed to compare the measurement data from each group. Chi square analysis was performed for the frequencies in each group. $P<0.05$ was considered as significant difference (two-sided test). Gene calculating method was used to determine the frequency of the genotype in each group.

Results

Comparison of clinical feature between the group with vulnerable plaques and that with stable plaques

The clinical feature for the patients with vulnerable plaques and those with stable plaques were shown in **Table 1**.

Table 1. Clinical characteristics of the cases in this study.

	Vulnerable plaque group (n=132)	Stable plaque group (n=152)	p
Age,y	72.2±6.1	70.6±10.5	0.16
Gender,male	92	100	0.48
Body mass index,kg/m^2	21.2±2.25	21.11±2.06	0.66
Smokes	72	80	0.74
Total cholesterol,mmol/L	4.83±1.26	4.65±1.31	0.14
HDL-cholesterol,mmol/L	1.45±0.57	1.53±0.29	0.07
Triglyceride,mmol/L	1.86±1.13	1.74 ±1.07	0.25
Hypertension	88	104	0.75
Type 2 diabetes	16	22	0.56

Table 2. Frequency of ALOX5AP SG13S114 genotype in each group.

Group	No of cases	Frequency of genotype (n, %)			Frequency of alleles (%)	
		TT	TA	AA	A	T
Vulnerable plaque	132	36(27.3)[1)]	72(54.5)	24(18.2)	120(45.5)[2)]	144(54.5)
Stable plaque	152	76(50.0)	68(44.7)	8(5.3)	84(27.6)	220(72.4)

Note: Comparison of genotype frequency between vulnerable plaque and stable plaque groups (χ^2=21.096, *P*<0.01). Comparison of allele frequency between vulnerable plaque and stable plaque groups (χ^2=19.50, *P*<0.01).

Statistical analysis showed that there was no significant difference between the clinical features of vulnerable plaque group and those of stable plaque group (*P*>0.05).

PCR-RFLP detection of SG13S114 in the ALOX5AP gene

The size of the PCR products amplified with the *ALOX5AP* gene specific primers was 212 bp. The PCR products were digested with vspI restriction enzyme and the results showed that genotype TT of SG13S114 in the *ALOX5AP* gene produced a single band (212 bp), genotype TA produced three bands (25 bp, 187 bp and 212 bp) and genotype AA produced two bands (25 bp and 187 bp) (**Figure 1**).

Analysis of the genotype and allele frequencies in each group

The distribution of the SG13S114 polymorphism in the vulnerable plaque and stable plaque groups was in accordance with the Hardy-Weinberg Equilibrium (χ^2=1.427, *P*=0.232), suggesting a population representativeness. The frequency of genotype AA in the group with vulnerable plaques was 18.2%, which was significantly higher than that in the group with stable plaques (5.3%) (χ^2=21.096, *P*<0.01) (**Table 2**). The frequency of A allele in the group with vulnerable plaques was 45.5%, which was significantly higher than that in the group with stable plaques (27.6%) (χ^2=19.50, *P*<0.01) (**Table 2**).

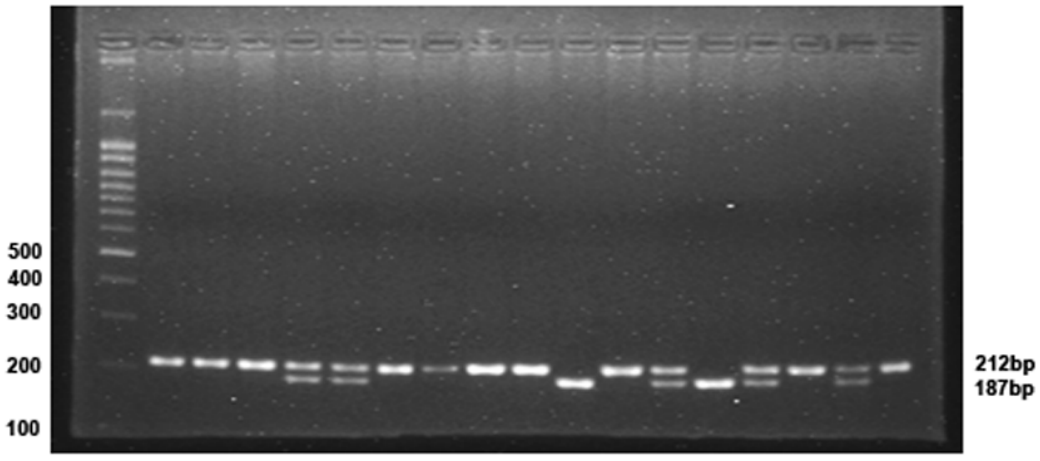

Figure 1. Electrophoresis of SG13S114 PCR product digested with vspI restriction enzyme. Lane 1 represented 100 bp DNA ladder. Lane 2, 3, 4, 7, 8, 9, 10, 12, 16 and 17 represented the genotype TT (a single band of 212 bp). Lane 5, 6, 13, 15 and 17 represented genotype TA (three bands of 212 bp, 187 bp and 25 bp). Lane 11 and 14 represented genotype AA (two bands of 187 bp and 25 bp).

Discussion

More and more attention has been paid to the role of inflammation and immune response in the development and advancement of AS [5,6]. Recent studies have suggested that AS is an inflammatory disease. Inflammation plays a critical role in the formation and destabilization of AS plaques [7]. High level of inflammatory factors represents the presence of the vulnerable plaques. Spanbroek et al. [8] found that the expression of FLAP and 5-LO in the lesions of AS plaques of the aorta coronary and carotid arteries was significantly increased in comparison with the normal controls [8]. Leukotriene is a strong proinflammatory factor and FLAP is the main regulator for the synthesis of leukotriene. Studies have demonstrated that polymorphism of *ALOX5AP* gene is a risk factor of myocardial infarction in Iceland[9], Scotland [10]. and other European countries [11]. Studies conducted in China showed that genotype and alleles of *ALOX5AP* gene were different in different ethnics of Eastern populations. Increased frequency of SG13S114AA genotype indicates the high susceptibility to cerebral infarction [12]. However, the association between the genotype of SG13S114 and carotid atherosclerosis has not been determined. Presence of plaques indicates a tendency of rupture, formation of thrombus and rapid advancement. The internal portion of these plaques contains large amount of lipid, hemorrhage and ulceration, which displayed hypoechoes in the ultrasound test [2,4]. Studies have shown that atherothrombotic cerebral infarction is mainly related to the rupture of the vulnerable plaques in the carotid artery. Identification and characterization of the genetic marker for the patients with vulnerable plaques has become the research focus in the areas of cerebral cardiovascular diseases recently [5,6].

ALOX5AP gene is located in the chromosome13q12-13, containing 5 exons and 4 introns with a size of 31 kb. *ALOX5AP* gene is a haploid with 4 single nucleotide polymorphisms (SNPs). *ALOX5AP* gene encodes FLAP, a critical regulator for the synthesis of leukotriene. The FLAP/5-LO pathway catalyzes the arachidonic acid to produce unstable leukotriene (A4), which is further transformed into leukotriene (B4) or other types of cysteinyl-leukotriene (C4, D4 and E4) [15,16]. Leukotriene is an important proinflammatory factor, which can activate leukocytes, facilitate the adhesion of monocytets on the endothelium of blood vessels and increase the permeability of blood vessels [15]. The FLAP/5-LO pathway can facilitate the formation of fatty streak in atherosclerosis, development of stable and vulnerable plaques and rupture of the plaques, which ultimately causes cerebral and cardiovascular disease and apoplexia.

We showed that the frequency of SG13S114AA genotype in the vulnerable plaque group was significantly higher than that in the stable plaque group. These results suggested that mutation of *ALOX5AP* gene could increase the production of chemokines, proinflammatory cytokines in the macrophages and vascular wall cells and the secretion of these inflammatory factors could deteriorate the inflammation, formation of atherosclerosis, stenosis, damage and rupture of the blood vessels [16]. Expression of FLAP and 5-LO encoded by *ALOX5AP* gene is influenced by external conditions. For example, long-wave UV, cerebral ischemia reperfusion injury (CIRI) and TGF-β can induce the synthesis of 5-LO. IL-4 does not have effect on the expression of 5-LO in the macrophage of mouse peritoneal cavity, but upregulates the expression of FLAP [17].

High expression of SG13S114AA genotype of *ALOX5AP* gene may be related to the vulnerability of the carotid atherosclerosis plaques. Allele A of SG13S114 might be the risk gene of vulnerable plaques. Therefore, the detection of the polymorphism of SG13S114A/T could be used for early diagnosis and risk predictions of the atherothrombotic cerebral infarction. The early identification of susceptible populations based on the genotype of SG13S114 and early intervention will greatly improve the results of the prevention and treatment of ACI.

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