Int J Clin Exp Med 2016;9(3):6693-6698
www.ijcem.com /ISSN:1940-5901/IJCEM0020106

Original Article

Lack of an independent relationship between CXCL16 gene rs2277680 polymorphism and instability of carotid plaque in the Chinese Han population

Yuanlin Zhou[1], Yun Chen[1], Min Zhu[2], Huihua Jiang[1], Feng Zhu[1], Zhou Zheng[1], Xiaofei Hu[1]

[1]Department of Neurology, Taizhou Hospital, Affiliated Hospital of Wenzhou Medical University, Taizhou, Zhejiang, PR China; [2]Department of Public Laboratory, Taizhou Hospital, Affiliated Hospital of Wenzhou Medical University, Taizhou, Zhejiang, PR China

Received November 19, 2015; Accepted January 25, 2016; Epub March 15, 2016; Published March 30, 2016

Abstract: Background: Chemokine CXC ligand 16 (CXCL16) may have an important role in the development of vulnerable carotid plaques. The rs2277680 polymorphism located in exon 4 of CXCL16 has been shown to accelerate inflammation, which increases the instability of carotid plaques. The aim of this study was to investigate the association between the CXCL16 gene rs2277680 polymorphism and the instability of carotid plaques in the Chinese Han population. Methods: For this study, 554 patients were enrolled and divided into three groups according to the results of carotid ultrasound examination: vulnerable plaque group (n = 129), stable plaque group (n = 189) and no plaque group (n = 236). Additionally, the CXCL16 gene rs2277680 polymorphism was genotyped by polymerase chain reaction-restriction fragment length polymorphism. Results: The frequency of the GA+AA genotypes was not significantly different between the three groups (82.9% vs 87.8% vs 85.2%, $x^2 = 1.531$, P = 0.465). The frequency of the A allele was also not significantly different between the three groups (59.69% vs 61.38% vs 61.65%, $x^2 = 0.771$, P = 0.374). Conclusions: There is no correlation between the CXCL16 gene rs2277680 polymorphism and the instability of carotid plaques in the Chinese Han population.

Keywords: Chemokine CXC ligand 16, atherosclerosis, polymorphism, genetic

Introduction

Cerebrovascular disease has become the most common cause of death and disability in China, and 70% of cerebrovascular disease cases are ischemic stroke [1]. Atherosclerosis (AS) is the most common cause of cerebral infarction. In the presence of inflammatory cytokines, atherosclerosis develops in the carotid intima, and when the atherosclerotic plaque ruptures, hemorrhage and thrombosis occur and result in cerebral infraction. Inflammation is the most important pathophysiological mechanism of this process [2]. Infiltration of inflammatory cells is a key to the pathological process of atherosclerosis, and chemokines play an important role in the accumulation and infiltration of inflammatory cells to the lesion site [3]. CXCL16 (chemokine CXC ligand 16), a serum chemokine, mediates the inflammatory reaction to promote atherosclerosis [4, 5]. CXCL16 exists in two forms: a membrane-bound form and a serum-soluble form. The rs2277680 polymorphism is a single base mutation in exon 4 of the CXCL16 gene. This mutation alters the membrane-bound form of CXCL16 into the serum-soluble form and is correlated with serum hs-CRP level [6]. Therefore, investigating whether the rs2277680 polymorphism is associated with the instability of carotid plaques in the Chinese Han population has merit.

Methods

Study population

All subjects were hospitalized at the Department of Neurology for the first time from September 2013 to March 2015. They underwent carotid artery ultrasound examination, and for some patients, the diagnosis of a carotid plague was confirmed by Computed Tomography

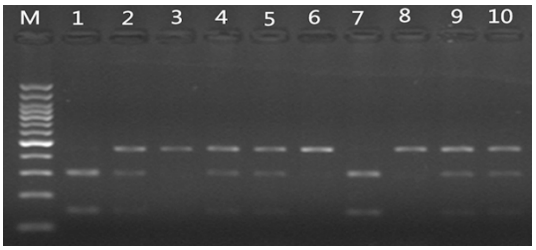

Figure 1. Electrophoresis of the CXCL16 products digested with the PvuII-HF restriction enzyme. Lane M is the 100 bp DNA ladder; lanes 1 and 7 are the GG genotype (two bands of 144 bp and 296 bp); lanes 2, 4, 5, 9 and 10 are the GA genotype (three bands of 144 bp, 296 bp and 440 bp); lanes 3, 6 and 8 are the AA genotype (a single band of 440 bp).

Angiography (CTA) or Magnetic Resonance Angiography (MRA). Based on the ultrasonic criteria [7] for the classification of carotid plaques, patients who had plaques with echolucent or mixed echo characteristics were classified into the vulnerable plaque group (n = 129; age range: 48 to 87 years, with an average age of 69.36). Patients who had plaques with echogenic characteristics were classified into the stable plaque group (n = 189; age range: 47 to 90 years, with an average age of 70.58). Patients with no plaques or plaques with intima that were less than 1 mm thick were classified into the no plaque group (n = 236; age range: 43 to 85 years, with an average age of 68.77). All patients who had no consanguinity were of Han Chinese heritage and did not have a history of stroke. None of the included patients had any of the following conditions: tumors, autoimmune disease, infectious disease, rheumatic disease, blood disease, acute coronary syndrome, atrial fibrillation, thyroid disease, severe liver or kidney dysfunction, or peripheral vascular disease. Additionally, none of the included patients were taking glucocorticoids or immunosuppressants. There were no significant differences in mean age or sex ratio between the three groups. This study was approved by the Medical Ethics Committee of Taizhou Hospital, and all subjects signed informed consent.

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP)

Genomic DNA was extracted from whole blood samples using a peripheral blood genomic DNA extraction kit according to the manufacturer's instructions. The concentration of the extracted DNA concentration was determined by measuring its absorption at 260 nm in a Biophotometer. All DNA samples were frozen at -80°C until use.

The primers used to amplify the CXCL16 polymorphism were synthesized by Generay Biotechnology Co. Ltd in Shanghai China. The forward primer was 5'-TCTTGCACAGCACATAGGAAAG-3', and the reverse primer was 5'-CTGCTTTTTCACCTTGTCTCCT-3'. The PCR reaction was performed in a 25 μl volume containing 1 μl of DNA, 12.5 μl of 2× MasterMix, 0.5 μl of 20 μM primers (forward and reverse) and 10.5 μl distilled water. The cycling parameters for the amplification of the CXCL16 gene were as follows: pre-denaturation at 95°C for 5 min followed by 35 cycles of denaturation at 94°C for 45 s, annealing at 56°C for 30 s and extension at 72°C for 45 s and a final extension step at 72°C for 10 min. Following amplification, the amplified PCR fragments were confirmed to be 440 bp in length by electrophoresis on a 1.5% agarose gel. An 8 ml aliquot of the PCR product was digested with 0.5 U PvuII-HF #R3151V restriction enzyme (New England Biolabs) at 37°C for 2 h. The digested products were electrophoresed on a 2.0% agarose gel. As shown in **Figure 1**, digestion of the AA homozygote DNA with PvuII-HF, which does not digest the A allele, resulted in a single fragment of 440 bp. The GG homozygote, which included a recognition site for the PvuII-HF restriction enzyme, was digested into two fragments of 144 bp and 296 bp in length. The AG heterozygote was digested into three fragments of 144 bp, 296 bp and 440 bp in length. To verify the results of the PCR-RFLP assay, 10 μl of each PCR sample was sequenced using a Prism 3100 DNA Genetic Analyzer (Sunny Biotechnology Co., Ltd, Shanghai, China).

Statistical analysis

The statistical analysis was performed using SPSS 21.0 software. Clinical data are presented as means ± standard deviation (SD). We used ANOVA with a LSD post-hoc test to compare the data between the three groups. Countable data were assessed using the R×C chi-squared test (x^2). The genotype and allele frequencies were determined using gene counting method: allele frequency = (2× homozygote

Table 1. Demographic and clinical characteristics of the study subjects (sample size = 554)

Characteristics	Vulnerable plaque (n = 129)	Stable plaque (n = 189)	No plaque (n = 236)	Test value	P
Age (years)	69.36±8.94	70.58±9.41	68.77±10.87	1.925	0.147
Male/Female (%)	57.4/42.6	55.0/45.0	53.4/46.6	0.675[a]	0.714
BMI (kg/m^2)	22.56±2.81	21.41±2.26	21.86±2.03	1.061	0.230
Hypertension (Yes%)	71.3	70.4	61.5	5.329[a]	0.070
Diabetes (Yes%)	21.7	20.1	21.6	0.177[a]	0.915
Smoking (%)	36.4	30.2	29.2	2.164[a]	0.339
Drinking (%)	24.8	23.3	20.8	0.865[a]	0.649
SBP ($\bar{x}$±s, mmHg)	151.94±23.73	153.70±24.79	146.96±22.20	3.688	0.026
DBP ($\bar{x}$±s, mmHg)	85.54±12.42	84.88±11.67	87.06±12.86	1.404	0.247
FBG ($\bar{x}$±s, mmol/L)	6.082±2.22	6.01±2.12	5.81±1.83	0.696	0.499
TG (mmol/L)	1.98±1.74	1.70±1.33	1.59±1.07	3.342	0.036
TC (mmol/L)	4.77±1.08	4.66±1.07	4.56±0.96	1.451	0.235
HDL-C (mmol/L)	1.25±0.25	1.27±0.30	1.31±0.30	1.618	0.199
LDL-C (mmol/L)	2.48±0.72	2.52±0.79	2.57±0.86	0.474	0.623
FG (g/L)	3.57±0.92	3.57±1.08	3.31±1.51	2.273	0.104
Hcy (μmol/L)	12.82±5.99	13.66±7.48	14.09±7.97	0.919	0.400

BMI: body mass index; SBP: systolic blood pressure; DBP: diastolic blood pressure; FBG: fasting blood glucose; TG: triglycerides; TC: total cholesterol; HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol; FG: fibrinogen; Hcy: homocysteine. [a]Determined using partition analysis. All other data values were determined using ANOVA analysis. The level of significance was defined as P<0.05.

+ heterozygote number)/(2× number of subjects). Hardy-Weinberg analysis was performed to compare the observed and expected genotype frequencies using the x^2 test. The odds ratio (OR) and 95% confidence interval (CI) of the OR were calculated using a multiple logistic regression model with adjustment for the clinical data. A P<0.05 was considered statistically significant.

Results

The clinical characteristics of the study population are summarized in **Table 1**. The study population included 129 patients with vulnerable carotid plaques, 189 patients with stable carotid plaques and 236 patients with no carotid plaques. There were no differences in age, gender, or body mass index (BMI); the incidence of hypertension, diabetes, smoking, or drinking; diastolic blood pressure (DBP); or fasting blood glucose (FBG), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), fibrinogen (FG) or homocysteine (Hcy) levels between the three groups (P>0.05). There were significant differences in systolic blood pressure (SBP) and triglycerides (TG) levels between the three groups (P<0.05).

The genotype and allele frequencies of CXCL16 gene rs2277680 polymorphism in the three groups are displayed in **Table 2**. The genotype frequencies of the three groups were in accordance with the Hardy-Weinberg equilibrium (x^2 = 0.067, P = 0.967 for the vulnerable group; x^2 = 1.199, P = 0.549 for the stable group; x^2 = 0.000, P = 1.000 for the no plaque group), suggesting that our study object had group representation. Compared to the AA genotype, the frequency of the GA+AA genotypes was not significantly different in the three groups (82.9% vs 87.8% vs 85.2%, x^2 = 1.531, P = 0.465). The frequency of the A allele was also not significantly different in the three groups (59.69% vs 61.38% vs 61.65%, x^2 = 0.771, P = 0.374).

The logistic regression analysis was estimating the risk factors for carotid artery plaque formation in **Table 3**. The AG+AA genotype had no association with the formation of carotid artery plaque (OR = 0.952, P = 0.908). Hypertension, TG, TC and FG were risk factors for the

Table 2. Genotype and allele frequencies of the CXCL16 rs2277680 polymorphism

Group	Vulnerable plaque (n = 129)	Stable plaque (n = 189)	No plaque (n = 236)	χ^2	*P*-value
CXCL16 genotype					
GG (%)	22 (17.1)	23 (12.2)	35 (14.8)	1.531	0.465
GA+AA (%)	60+47 (82.9)	100+66 (87.8)	111+90 (85.2)		
CXCL16 allele					
G (%)	104 (40.3)	146 (38.6)	181 (38.3)	0.289	0.866
A (%)	154 (59.7)	232 (61.4)	291 (61.7)		

The level of significance was defined as P<0.05.

Table 3. Binary logistic regression estimating the risk factors for vulnerable plaque

Independent variables	β	*P*-value	*OR*	95% *CI*
Hypertension	0.693	0.012	2.000	1.166-3.430
HDL-C (mmol/L)	-0.518	0.074	0.595	0.337-1.052
TG (mmol/L)	0.910	0.001	2.483	1.429-4.316
TC (mmol/L)	1.145	0.012	3.143	1.289-7.666
FG (g/L)	0.782	0.007	2.186	1.244-3.840
Constant	-0.305	0.405	0.737	
CXCL16 AG+AA	-0.050	0.908	0.952	0.410-2.207

Divide HDL-C by 1.15 mmol/L, divide TG by 1.7 mmol/L, divide TC by 5.68 mmol/L, divide FG by 3.50 g/L. The logistic regression model: $\ln[p/(1-p)] = \alpha + \beta X + e$, β is the estimated logit coefficient.

formation of carotid artery plaque. In contrast, HDL-C was protective against carotid plaque (OR = 0.595, P = 0.074).

Discussion

CXCL16 (chemokine CXC ligand 16), a serum chemokine of 254 amino acids, is involved in inflammatory reactions and immune responses and is activated by consumption of oxidized low-density lipoproteins. This chemokine plays an important role in the occurrence and development of atherosclerosis [8-11]. CXCL16 is a 254 amino acid-long protein with four domains: a trans-membrane domain, cytoplasmic domain, chemotactic function domain and mucin domain, which is a component of the extracellular matrix. The rs2277680 polymorphism is a single base mutation in exon 4 of the CXCL16 gene of guanosine (G) to adenosine (A) that results in the encoded amino acid being changed from alanine to valine. This amino acid residue is located in the mucin domain, which localizes close to the cell membrane. CXCL16 exists in two forms: a membrane-bound form and a serum-soluble form. After cleavage by a metalloproteinase (ADAM-10), membrane-bound CXCL16 is released extracellularly in a soluble form [12] that causes inflammatory cells to aggregate and infiltrate the lesion, promotes angiogenesis and the formation of atherosclerosis and impacts the stability of artery plaques [10, 13, 14]. However, the cleavage site that generates the serum-soluble form of CXCL16 is located in the mucin domain, which is also where the rs2277680 polymorphism is located. Chandrasekar found that CXCL16 promotes the proliferation of smooth muscle cells and adhesion between cells, reduces muscle cell damage and impedes the migration of monocytes to sub-endothelial tissues through the P13K pathway. Thus, it can prevent the formation of foam cells and inhibit the process of atherosclerosis [15].

Yi GW found that the concentration of serum-soluble CXCL16 was closely related to the severity of coronary atherosclerotic heart disease and the extent of coronary artery stenosis [16]. Lundberg found that the rs2277680 polymorphism was not associated with the risk of coronary atherosclerotic heart disease but was related to the extent of coronary artery stenosis [17]. Luo Yongbai researched the relationship between acute coronary syndrome (ACS) and the rs2277680 polymorphism and found that the CXCL16 gene rs2277680 polymorphism was closely related to ACS susceptibility in a Chinese Han population in Xian province. ACS susceptibility was significantly higher for individuals with the AA genotype than for those with the GG genotype, and the A allele was associated with a significantly higher risk of ACS than the G allele [18]. Although there are some differences between the results of these studies, overall, they suggested that the rs2277680 polymorphism is related to coronary atherosclerosis.

Song Shuang found that the CXCL16 gene rs2277680 polymorphism was independently associated with the serum level of CXCL16, and the serum level of CXCL16 was significantly higher for individuals with the AA genotype than for those with the GG+GA genotypes. Additionally, the AA genotype was closely related to atherosclerotic stroke, and the A allele was an important marker of the severity of atherosclerosis [19]. Wang Kedi also found that the serum level of CXCL16 was closely related to atherosclerotic cerebral infarction; however, neither the frequency of the CXCL16 gene rs2277680 polymorphism nor the allele frequencies were significantly different between the case and control groups. Therefore, the CXCL16 gene rs2277680 polymorphism is not related to the incidence of atherosclerotic cerebral infarction, but it is related to the degree of the inflammatory reaction [20].

These studies indicated that the rs2277680 polymorphism was associated with the inflammatory response, but there were some differences in the findings of these studies regarding atherosclerotic stroke, which may have been due to the following reasons: First, the distribution of the CXCL16 gene rs2277680 polymorphism differs by race and region. Second, some CXCL16 gene polymorphisms may be in linkage disequilibrium with adjacent gene SNPs. Third, Chandrasekar [15] found that membrane-bound CXCL16, which belongs to the scavenger receptor family, is involved in macrophage phagocytosis of oxidized low density lipoprotein and has an anti-atherosclerotic role. In contrast, serum-soluble CXCL16 has roles in chemotaxis, the inflammatory reaction and the promotion of atherosclerosis. If a mutation caused the function of serum-soluble CXCL16 to be significantly greater than that of membrane-bound CXCL16, it would promote the formation of atherosclerosis and impact the stability of the plaque, causing plaque rupture and bleeding and resulting in cerebral infarction. However, if the mutation elevated the level of serum-soluble CXCL16 while also stimulating inflammation, the phagocytic function of phagocytes containing membrane-bound CXCL16 would be enhanced resulting in the effects of both forms of the CXCL16 protein being equal and canceling each other out; this would result in no overall effect of this mutation on atherosclerosis. CXCL16 is a molecule with two forms, and the mechanisms of some of the functions of CXCL16 are very complex. Therefore, because the levels of the serum-soluble and membrane-bound forms of CXCL16 were not measured in this study, further in-depth studies are necessary.

There are several limitations of this study. First, currently available data suggest that the CXCL16 gene rs2277680 polymorphism increases the level of the serum-soluble form relative to the level of the membrane-bound form, but we did not measure the serum level of CXCL16. Second, the formation and rupture of atherosclerotic plaques is a pathological process caused by multiple genes and multiple factors, but our study only examined one polymorphic site of one gene. These deficiencies require further research. However, the results of this study no obvious correlation between the CXCL16 gene rs2277680 polymorphism and carotid plaque vulnerability in the Chinese Han population.

Acknowledgements

Medical and health research project of Zhejiang province (2013KYB289).

Disclosure of conflict of interest

None.

Address correspondence to: Yuanlin Zhou, Department of Neurology, Taizhou Hospital, Affiliated Hospital of Wenzhou Medical University, Taizhou 317000, Zhejiang, PR China. E-mail: zyl@enzemed.com

References

[1] Wang YJ, Zhang SM, Zhang L, Wang CX, Dong Q, Gao S, Huang RX, Huang YN, Lv CZ, Liu M, Qin HQ, Rao ML, Xiao Y, Xu YM, Yang ZH, Wang YJ, Wang CX, Wang JZ, Wang WZ, Wang J, Wang WJ, Wu J, Wu SP, Zeng JS, Zhang SM, Zhang L, Zhao XQ, Zhong LY. Chinese guidelines for the secondary prevention of ischemic stroke and transient ischemic attack 2010. CNS Neurosci Ther 2012; 18: 93-101.

[2] Hansson GK. Inflammation,atherosclerosis, and coronary artery disease. N Engl J Med 2005; 352: 1685-1695.

[3] Galkina E, Ley K. Leukocyte influx in atherosclerosis. Curr Drug Targets 2007; 8: 1239-1248.

[4] Shimaoka T, Nakayama T, Kume N, Takahashi S, Yamaguchi J, Minami M, Hayashida K, Kita

T, Ohsumi J, Yoshie O, Yonehara S. Cutting edge: SR-PSOX/CXC chemokine ligand 16 mediates bacterial phagocytosis by APCs through its chemokine domain. J Immunol 2003; 171: 1647-1651.

[5] Jiang X, Shimaoka T, Kojo S, Harada M, Watarai H, Wakao H, Ohkohchi N, Yonehara S, Taniguchi M, Seino K. Cutting edge: critical role of CXCL16/CXCR6 in NKT cell trafficking in allograft tolerance. J Immunol 2005; 175: 2051-2055.

[6] Wang KD, Liu ZZ, Wang RM, et al. Study on the relationship between CXCL16 gene polymorphism and CRP. Laboratory Medicine 2011; 26: 1-4.

[7] Mathiesen EB, Bonaa KH, Joakimsen O. Echolucent plaques are associated with high risk of ischemic cerebrovascular events in carotid stenosis: the tromso study. Circulation 2001; 103: 2171-2175.

[8] Hammad MA, Abdel-Bakky MS, Walker LA, Ashfaq MK. Oxidized low-density lipoprotein and tissue factor are involved in monocrotaline/lipopolysaccharide-induced hepatotoxicity. Arch Toxicol 2011; 85: 1079-1089.

[9] Seizer P, Stellos K, Selhorst G, Krämer BF, Lang MR, Gawaz M, May AE. CXCL16 is a novel scavenger receptor on platelets and is associated with acute coronary syndrome. Thromb Haemost 2011; 105: 1112-1114.

[10] Shimaoka T, Nakayama T, Fukumoto N, Kume N, Takahashi S, Yamaguchi J, Minami M, Hayashida K, Kita T, Ohsumi J, Yoshie O, Yonehara S. Cell surface-anchored SR-PSOX/CXC chemokine ligand 16 mediates firm adhesion of CXC chemokine receptor 6-expressing cells. J Leukoc Biol 2004; 75: 267-274.

[11] Sheikine Y, Bang CS, Nilsson L, Samnegård A, Hamsten A, Jonasson L, Eriksson P, Sirsjö A. Decreased plasma CXCL16 /SR-PSOX concentration is associated with coronary artery disease. Atherosclerosis 2006; 188: 462-466.

[12] Hundhausen C, Schulte A, Schulz B, Andrzejewski MG, Schwarz N, von Hundelshausen P, Winter U, Paliga K, Reiss K, Saftig P, Weber C, Ludwig A. Regulated shedding of transmembrane chemokines by the disintegrin and metalloproteinase 10 facilitates detachment of adherent leukocytes. J Immunol 2007; 178: 8064-8072.

[13] Shimaoka T, Kume N, Minami M, Hayashida K, Kataoka H, Kita T, Yonehara S. Molecular cloning of a novel scavenger receptor for oxidized low density lipoprotein, SR-PSOX, on macrophages. J Biol Chem 2000; 275: 40663-40666.

[14] Zhuge X, Murayama T, Arai H, Yamauchi R, Tanaka M, Shimaoka T, Yonehara S, Kume N, Yokode M, Kita T. CXCL16 is a novel angiogenic factor for human umbilical vein endothelial cells. Biochem Biophys Res Commun 2005; 331: 1295-1300.

[15] Chandrasekar B, Bysani S, Mummidi S. CXCL16 signals via Gi, phosphatidylinositol 3-kinase, Akt, I kappa B kinase, and nuclear factor kappa B and induces cell-cell adhesion and aortic smooth muscle cell proliferation. J Biol Chem 2004; 279: 3188-3196.

[16] Yi GW, Zeng QT. Circulating CXCL16 is related to the severity of coronary artery stenosis. Arch Med Res 2008; 39: 531-535.

[17] Lundberg GA, Kellin A, Samnegård A, Lundman P, Tornvall P, Dimmeler S, Zeiher AM, Hamsten A, Hansson GK, Eriksson P. Severity of coronary artery stenosis is associated with a polymorphism in the CXCL16/SR-PSOX gene. J Intern Med 2005; 257: 415-422.

[18] Luo BY, Wang YN, Zhen SH, et al. Association of A181V CXCL16 gene polymorphism and acute coronary syndrome. Chinese Journal of Arteriosclerosis 2009; 12: 112-125.

[19] Song S, Pan XD, Ma AJ, et al. Study on the correlation between A181V polymorphism and TOAST classification. Chinese Journal of Stroke 2012; 7: 91-97.

[20] Wang KD, Liu ZZ, Wang RM, Wang YJ, Zhang GJ, Su JR, Kang XX. Chemokine CXC Ligand 16 serum concentration but not A181 V genotype is associated with atherosclerotic stroke. Clin Chim Acta 2010; 411: 1447-1451.