Original Paper

The Association between Polymorphism of CARD8 rs2043211 and Susceptibility to Arteriosclerosis Obliterans in Chinese Han Male Population

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Key Words

Arteriosclerosis obliterans • Card8 • Polymorphism • IL-1 β

Abstract

Background and aims: Cholesterol crystals have been shown to cause inflammation. As a response to cholesterol crystal accumulation, the NLRP3 inflammasome is activated to produce IL-1 β which eventually leads to atherosclerotic lesions. As a part of innate immunity, CARD8 is involved in the modulation of above mentioned inflammatory activities. The primary objective of this study was to investigate the association between polymorphism of CARD8 rs2043211 and susceptibility to arteriosclerosis obliterans (ASO) in Chinese Han male population. **Methods:** 758 male arteriosclerosis obliterans patients and 793 male controls were genotyped for rs2043211 with the TaqMan allele assays. Fasting blood-glucose (FBG), total cholesterol (TC), triglycerides (TG), urea nitrogen, creatinine, Serum uric acid, high density lipoprotein, low density lipoprotein, ALT, AST, and IL-1 β in the blood were detected for all subjects. Clinical data were recorded to analyze the genotype-phenotype. Independent samples t-test was used to perform the comparisons between two groups. Odds ratios (ORs) with 95% confidence intervals (CIs) were calculated to measure the strength of relationship in the genotype distribution and allele frequencies between patients and controls. The analysis of variance was used for a genotype-phenotype analysis of the ASO patients. **Results:** The genotypic and allelic frequencies in the ASO group were significantly different from that in the control group ($P = 0.014$ by genotype, $P = 0.003$ by allele). Those carrying the genotype TT had a higher risk for ASO than those carrying the genotype AA (OR = 1.494, 95%CI 1.131-1.974, $P = 0.005$). The difference was also significant after the adjustment for the history of smoking, TC, LDL, fasting blood glucose, systolic blood pressure and BMI (OR = 1.525, 95%CI 1.158-2.009, $P = 0.003$). **Conclusion:** Our finding suggests that the polymorphism of CARD8 rs2043211 is probably associated with the development of ASO in Chinese Han male population.

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Introduction

Arteriosclerosis obliterans is characterized by the ischemic symptoms of lower extremity which are due to the stenosis and/or occlusion of artery based on the pathology of atherosclerotic change. This disease is relatively highly prevalent, affecting more than 200 million people worldwide [1]. Arteriosclerosis is caused by environmental and genetic factors [2]. In recent years, inflammatory factor has been proved participating in the atherosclerotic procedure [3-7]. It has been proved that cholesterol crystals can cause inflammation [8-10]. As a response to cholesterol crystal accumulation, the NLRP3 inflammasome is activated to produce IL-1 β and IL-1 β can cause atherosclerotic lesions [11, 12]. NLRP3 inflammasome is consist of NLRP3 protein, apoptosis-associated speck (ASC)-like protein and caspase-1 protein [13, 14]. The CARD8 (caspase recruitment domain family member 8) protein regulates this process by inhibiting the activation of NF- κ B and caspase-1 [15-17]. The CARD8 protein is encoded by the 13 exons of the CARD8 gene on chromosome 19q13. The SNP rs2043211 of CARD8 changes cysteine at codon10 to a premature termination codon, thus yielding a premature, truncated protein, which influences the protein function in inflammasome-mediated processes and NF- κ B suppression [18, 19]. Lots of studies show that the polymorphisms of CARD8 contribute to the development of some inflammatory diseases, such as inflammatory bowel disease [20-22], gout [23], abdominal aortic aneurysm [24], rheumatoid arthritis [17, 25, 26] and Alzheimer's disease [27, 28]. As a part of innate immunity, CARD8 is involved in the modulation of inflammatory activities. Because of the important role of CARD8 in inflammatory activities, we hypothesized that the polymorphism of CARD8 rs2043211 may be associated with the risk of arteriosclerosis obliterans development. To test this hypothesis, we designed the case-control study to validate the possible association between the polymorphism of CARD8 rs2043211 and arteriosclerosis obliterans in a Chinese Han male population.

Materials and Methods

Study subjects, ethics and consent

From March 2010 to January 2016, a total of 758 male patients with arteriosclerosis obliterans and 793 age-matched healthy male subjects were enrolled in the case-control study. Clinical and biological characteristics of the study participants are listed in Table 1. All subjects were consecutively recruited from the Qingdao Municipal hospital. The diagnosis of arteriosclerosis obliterans was based on the guidelines released by ACCF/AHA in 2011 and 2012 [29, 30]. Age over 40 years, the intermittent claudication and ischemic rest pain were used as the main diagnostic criteria, and the Ankle/Brachial Index (ABI $\leq$ 0.9) was used as complementary parameter. All ASO patients had been confirmed by CT angiography (CTA). Samples of controls without a personal or familial history of arteriosclerosis obliterans or other serious illness was collected. Fasting blood-glucose (FBG), total cholesterol (TC), triglycerides (TG), urea nitrogen, creatinine, Serum uric acid, high density lipoprotein, low density lipoprotein, ALT, AST, and IL-1 β in the blood were detected for all subjects. Phenotypic characteristics including demographic data and disease duration were recorded using a standardized questionnaire. Every subject including control subject participating in this study received an explanation about the study before enrollment and had signed a written informed consent. The study protocol was approved by the Ethical Committee of the Qingdao Municipal hospital.

Plasma parameters determination

FBG was tested by glucose oxidase method. Serum uric acid, total cholesterol (TC), and triglycerides (TG) were tested by the oxides method. High density lipoprotein and low density lipoprotein were tested by selective inhibition method. Urea nitrogen, ALT and AST were tested by enzyme coupling method. Creatinine was tested by alkaline picrate method. All the parameters above were measured in an automatic biochemistry instrument (HITACHI 7600-120). And the expression level of IL-1 β in plasma was measured by ELISA kit (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions.

Genotyping

Genomic DNA was extracted from peripheral venous blood using GenElute™ Blood Genomic DNA Kit (Sigma). The SNP of CARD8 rs2043211 was detected by using TaqMan SNP Genotyping Assays which contained a pair of primers, forward, 5'-AGTTGACACTCAGGAACAGCACGG A-3', and reverse, 5'-CAATAA TGGCTCTGCCTCTGTCTC A-3' (Applied Biosystem). The PCR was carried out in the ABI 7900HT Fast Real-Time PCR System, as the manufacturer's instructions. 10% of samples were analyzed repeatedly to confirm the accuracy of the TaqMan assays. Concordance rate between two times genotype calls was 100%.

Statistical analysis

Statistical power was assessed using the Quanto 1.2.4 software. Assuming the ORs from 1.30 to 1.50 for these loci in Chinese, we obtained the estimated powers greater than 90% for the association of the SNP with the risk of ASO. SPSS version 20.0 software (IBM Corporation, USA) was used for the statistical analysis. Independent samples t-test was used to perform the comparisons between two groups. Pearson's χ^2 test was used to test for deviations from Hardy-Weinberg equilibrium. Odds ratios (ORs) with 95% confidence intervals (CIs) were calculated to measure the strength of relationship in the genotype distribution and allele frequencies between patients and controls. The analysis of variance was used for a genotype-phenotype analysis of the ASO patients. Analysis was repeated taking into account the history of smoking. P-values < 0.05 were considered to be statistically significant.

Table 1. Clinical and Biological Characteristics of subjects. Footnote: Values are shown as percentages, means $\pm$ 1 standard deviation, or median (interquartile range)

Characteristic	ASO(n=758)	Controls(n=793)	P values
Age (years)	61.68 $\pm$ 10.57	62.05 $\pm$ 10.91	0.314
Hyperlipidaemia (%)	50.92	29.03	0.0002
Diabetes (%)	20.44	5.62	0.0021
Hypertension (%)	56.75	27.91	0.0002
Ischaemic heart disease (%)	42.82	5.22	0.0013
Smoking status (pack years)	21 (10-45)	2 (0-12)	0.0011
BMI(kg/m ²)	26.92 $\pm$ 3.05	25.63 $\pm$ 3.14	0.018
systolic pressure (mmHg)	130.75 $\pm$ 9.22	122.33 $\pm$ 10.24	0.001
diastolic pressure (mmHg)	80.24 $\pm$ 8.21	79.89 $\pm$ 9.02	0.278
FBG(mmol/L)	6.49 $\pm$ 0.50	4.94 $\pm$ 0.47	0.011
Serum uric acid (μ mol/L)	308.07 $\pm$ 50.06	299.62 $\pm$ 48.27	0.073
TG (mmol/L)	1.53 $\pm$ 0.25	1.29 $\pm$ 0.31	0.178
TC(mmol/L)	5.63 $\pm$ 0.52	4.81 $\pm$ 0.65	0.023
HDL (mmol/L)	1.40 $\pm$ 0.24	1.48 $\pm$ 0.22	0.098
LDL (mmol/L)	3.27 $\pm$ 0.31	2.58 $\pm$ 0.29	0.029
BUN(mmol/L)	5.12 $\pm$ 1.08	4.95 $\pm$ 1.16	0.472
Cr(μ mol/L)	77.13 $\pm$ 10.52	76.87 $\pm$ 9.87	0.133
ALT(U/L)	38.83 $\pm$ 8.72	38.75 $\pm$ 7.97	0.429
AST(U/L)	28.81 $\pm$ 6.54	29.16 $\pm$ 6.08	0.564
IL-1 β (ng/L)	8.21 $\pm$ 1.23	4.97 $\pm$ 1.07	0.009

Results

Clinical and biological characteristics

Table 1 showed the clinical and biological characteristics of the subjects included in the study. It showed that age was significantly different between the two groups (P < 0.05). And the levels of TC, LDL, IL-1 β , fasting blood glucose, systolic blood pressure and BMI in ASO patients group was much more higher than that in the controls (P < 0.05). There was no obvious difference between ASO patients and controls in the other characteristics.

The polymorphism of CARD8 rs2043211 and ASO

No deviations from Hardy-Weinberg equilibrium were detected for the genotype distribution of CARD8 rs2043211 polymorphism in ASO patients or controls (P > 0.05). Table 2 showed the association between the polymorphism of CARD8 rs2043211 and ASO. It was obviously different in the genotype distribution of the polymorphism of CARD8 rs2043211 between ASO patients and controls (P = 0.014). Those carrying genotype TT had a higher risk for ASO than those carrying genotype AA (OR = 1.494, 95%CI1.131-1.974, P = 0.005), which was also significant after the adjustment of the history of smoking, TC, LDL, fasting blood glucose, systolic blood pressure and BMI(OR = 1.525, 95%CI1.158-2.009, P = 0.003).

Table 2. Comparison of CARD8 rs2043211 polymorphism between ASO and controls. Footnote: a. after the adjustment of the history of smoking, TC, LDL, fasting blood glucose, systolic blood pressure, BMI and IL-1 β

	ASO (n=758)	Controls (n=793)	P value	OR 95% CI	P value ^a	OR ^a 95% CI
Genotypes						
AA (%)	191 (25.20)	249 (31.40)	-	1.000	-	1.000
AT (%)	371 (48.94)	373 (47.04)	0.032	1.297 1.023-1.644	0.027	1.322 1.045-1.671
TT (%)	196 (25.86)	171 (21.56)	0.005	1.494 1.131-1.974	0.003	1.525 1.158-2.009
Alleles						
A (%)	753 (49.67)	871 (54.92)	-	1.000	-	1.000
T (%)	763 (50.33)	715 (45.08)	0.003	1.234 1.072-1.421	0.002	1.252 1.088-1.443

Table 3. Genotype-phenotype analysis in our study(BMI, Body mass index; TG, Triglycerides; TC, Total cholesterol, IL-1 β ,etc.) ($\bar{x} \pm s$)

Characteristic	AA (n=191)	AT (n=371)	TT (n=196)	P value for ANOVA analysis	P value for AA vs. TT
Age(years)	61.35 $\pm$ 10.27	61.43 $\pm$ 10.83	61.98 $\pm$ 11.15	0.453	0.314
Age at diagnosis(years)	55.18 $\pm$ 12.16	54.79 $\pm$ 11.24	54.92 $\pm$ 11.58	0.389	0.371
Disease duration(years)	7.85 $\pm$ 3.53	7.03 $\pm$ 3.69	7.68 $\pm$ 3.47	0.114	0.233
BMI(kg/m ²)	26.65 $\pm$ 3.09	27.12 $\pm$ 3.12	26.98 $\pm$ 3.27	0.237	0.238
systolic pressure (mmHg)	130.98 $\pm$ 8.91	130.26 $\pm$ 9.06	130.64 $\pm$ 8.93	0.386	0.415
diastolic pressure (mmHg)	79.83 $\pm$ 9.32	80.58 $\pm$ 8.67	80.29 $\pm$ 8.45	0.423	0.302
FBG(mmol/L)	6.33 $\pm$ 0.41	6.53 $\pm$ 0.37	6.48 $\pm$ 0.45	0.543	0.597
Serum uric acid (μ mol/L)	303.89 $\pm$ 50.45	314.11 $\pm$ 51.02	306.12 $\pm$ 50.71	0.329	0.577
TG (mmol/L)	1.49 $\pm$ 0.37	1.52 $\pm$ 0.31	1.57 $\pm$ 0.29	0.255	0.234
TC(mmol/L)	5.75 $\pm$ 0.48	5.39 $\pm$ 0.57	5.69 $\pm$ 0.54	0.242	0.377
HDL (mmol/L)	1.43 $\pm$ 0.46	1.37 $\pm$ 0.39	1.39 $\pm$ 0.41	0.463	0.474
LDL (mmol/L)	2.85 $\pm$ 0.49	3.22 $\pm$ 0.36	3.63 $\pm$ 0.43	0.037	0.022
BUN(mmol/L)	5.17 $\pm$ 1.34	5.07 $\pm$ 1.19	5.09 $\pm$ 1.41	0.702	0.707
Cr(μ mol/L)	76.92 $\pm$ 8.57	77.08 $\pm$ 9.45	77.54 $\pm$ 9.23	0.426	0.387
ALT(U/L)	39.08 $\pm$ 7.32	38.90 $\pm$ 7.08	38.59 $\pm$ 7.47	0.471	0.396
AST(U/L)	29.13 $\pm$ 6.12	28.92 $\pm$ 5.36	28.55 $\pm$ 5.25	0.216	0.169
IL-1 β (ng/L)	7.94 $\pm$ 0.93	8.31 $\pm$ 1.02	8.75 $\pm$ 1.06	0.014	0.019

Genotype-phenotype analysis

The results of genotype-phenotype analysis were showed in Table 3. It was obviously different in the LDL levels among those three genotypes. There was also significant difference in the levels of IL-1 β between individuals carrying the TT, AT and AA genotype of rs2043211 ($P = 0.014$). Patients carrying genotype TT of CARD8 rs2043211 polymorphism had higher LDL levels compared to those carrying the AA genotype (3.63 $\pm$ 0.43 mmol/L vs. 2.85 $\pm$ 0.49 mmol/L, $P = 0.022$). Patients carrying the TT genotype also had an increased IL-1 β level compared with those carrying the AA genotype ($P = 0.019$).

Discussion

As a kind of autoinflammatory disorder, arteriosclerosis obliterans is histologically typified by atherosclerotic thickening, loss of elasticity, and medial calcification of the arterial walls [31]. Recent studies have showed that cholesterol crystals activate the NLRP3 inflammasome in phagocytes *in vitro* in a process that involves phagolysosomal damage [11]. The NLRP3 inflammasome, including CARD8 can control inflammatory caspase-1-mediated IL-1 β release and is involved in cholesterol crystals mediated arteriosclerosis [11, 32].

We performed the case-control study to examine the possible genetic association between CARD8 rs2043211 polymorphism and ASO in a Chinese Han male population. We found that there was an obvious difference in the genotype distribution of CARD8 rs2043211 polymorphism between cases and controls ($P = 0.014$). After the adjustment of the history of smoking, TC, LDL, fasting blood glucose, systolic blood pressure and BMI, those carrying genotype TT had a higher risk for ASO than those carrying genotype AA (OR = 1.525, 95%CI 1.158-2.009, $P = 0.003$). As far as we know, this is the first study to report the association between polymorphisms of CARD8 and ASO. In contrast to coronary atherosclerotic heart disease, few genetic variants that influence susceptibility to ASO have been discovered. Susceptibility to ASO may be influenced by genetic variants acting independently of those risk factors, such as smoking, diabetes mellitus, hypertension and hyperlipidaemia etc. Identification of such genetic variants will provide insights into underlying pathophysiologic mechanisms of ASO. Our study result will be helpful in increasing our understanding of the molecular genetics of ASO and in facilitating the development of novel diagnostic and therapeutic approaches for this disease.

In the genotype-phenotype analysis, we found that LDL levels showed a significantly different distribution among those three different genotypes. Patients carrying genotype TT showed higher LDL levels compared to those carrying the genotype AA. LDL can raise the risk of ASO [33-35], and the genotype-phenotype analysis indicates that CARD8 rs2043211 polymorphism is associated with LDL levels, which further proves the association between CARD8 rs2043211 polymorphism and susceptibility to ASO.

The secretion of the IL-1 β by macrophages is a major driver of pathogenesis in atherosclerosis [36]. As a response to cholesterol crystal accumulation, the NLRP3 inflammasome is activated to produce IL-1 β [11, 12]. The CARD8 protein regulates this process by inhibiting the activation of NF- κ B and caspase-1 [15-17]. The CARD8 protein can negatively regulate caspase-1-mediated IL-1 β expression and nuclear factor NF- κ B activation [37, 38]. The SNP rs2043211 introduces a stop codon polymorphism (Cys10Stop), which results in the expression of a severely truncated protein. The variant protein can not suppress NF- κ B activity, which leads to high constitutive levels of pro-IL-1 β [37-40]. In this study, the results showed that the prevalence of the TT variant genotype was much higher in ASO patients than in controls. So, it is possible that the over expression of CARD8 due to higher promoter activity of the TT genotype may result in a dramatically inflammatory immune response and then increase the susceptibility to ASO.

However, our study had some limitations. Firstly, we only chose the SNP rs2043211 of CARD8 and did not include all the SNPs to cover the whole sequence of CARD8 gene. Secondly, the sample size in the present study was not big enough, and all subjects were selected from Chinese Han male population. Thus, the results should be interpreted carefully. Our results need to be verified in more studies with larger sample and in other ethnic populations. Thirdly, the mechanism about the association between CARD8 rs2043211 polymorphism and susceptibility to ASO is still unknown. Further studies are needed to explore the mechanism underlying CARD8 rs2043211 polymorphism and susceptibility to ASO.

In conclusion, CARD8 rs2043211 polymorphism is significantly associated with susceptibility to ASO in Chinese Han males. However, our findings need to be verified in more studies with expanding sample and in other ethnic populations.

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Disclosure Statement

All authors declare that they have no conflicts of interest.

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