

Title page

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The association between polymorphism of NLRP3 rs10754558 and susceptibility to arteriosclerosis obliterans in Chinese Han male population

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Abstract

Background and aims: Cholesterol crystals have been proved to be able to cause inflammation. NLRP3 inflammasome is activated in response to the accumulation of cholesterol crystals to produce IL-1 β , which is associated with atherosclerotic lesions. NLRP3, as part of innate immunity, is involved in the regulation of inflammatory activity described above. The main objective of this study was to investigate the relationship between the polymorphism of NLRP3 rs10754558 and the susceptibility to arteriosclerosis obliterans (ASO) in Chinese Han males.

Methods: The NLRP3 rs10754558 genotype was detected by the TaqMan allele assays in 758 male patients suffered from arteriosclerosis obliteration and 793 male controls. Blood glucose, total cholesterol (TC), triglyceride (TG), urea nitrogen, creatinine, serum uric acid, high-density lipoprotein, low-density lipoprotein, alanine aminotransferase (ALT), aspartate aminotransferase (AST) and IL-1 β were detected in all subjects. Clinical data were recorded and genotype-phenotype was analyzed. Independent sample T test was used for comparison between the two groups. The odds ratio (ORs) with 95% confidence interval (CIs) was calculated to show the strength of the relationship between the genotype distribution or allele frequencies and ASO. Genotype-phenotype analysis was performed in ASO patients by ANOVA.

Results: The frequencies of genotype and allele in ASO group were significantly different from that in control group ($p = 0.022$ by genotype, $p = 0.003$ by allele). People who were carrying genotype GG had a higher risk for ASO than those carrying genotype CC (OR=1.574,95%CI 1.161-2.135,P=0.003), which was still significant after the adjustment of the history of smoking, TC, LDL, fasting blood glucose, systolic blood pressure and BMI (OR=1.448,95%CI 1.046-1.461,P=0.004). Moreover, there was an interaction between rs10754558 of NLRP3 and rs2043211 of CARD8 gene. Under the premise of carrying the T allele of CARD8 rs2043211, the G allele of NLRP3 rs10754558 increases the susceptibility to ASO. This gene-gene interaction is consistent with IL-1 β levels.

Conclusion: Our finding suggests the polymorphisms of NLRP3 rs10754558 are probably associated with the development of ASO in Chinese Han male population. And there may be an interaction between rs10754558 of NLRP3 and rs2043211 of CARD8 gene in the development of ASO.

Key Words

polymorphism, NLRP3, CARD8, arteriosclerosis obliterans, IL-1 β

Introduction

Lower extremity arteriosclerosis obliterans characterized by ischemic symptom in leg is caused by stenosis or occlusion of lower extremity artery due to atherosclerotic lesion. Atherosclerosis is caused by a combination of environmental and genetic factors acting on the body. In recent years, inflammatory factors have been shown to be involved in the process of atherosclerosis^[1-4]. Cholesterol crystals have been proved to be able to cause inflammation^[5-7]. NLRP3 inflammasomes are activated in response to deposition of cholesterol crystals and then produce IL-1 β , which is associated with atherosclerotic lesions^[8, 9]. The NLRP3 inflammasome was composed of NLRP3 protein, apoptosis-associated speck (ASC) like protein, and caspase-1 protein^[10, 11]. CARD8 protein regulates this process by inhibiting NF- κ B activation and caspase-1^[12-14]. NLRP3 inflammasome abnormalities are a contributing factor to a variety of inflammatory and autoimmune diseases, such as Muckle-Wells autoimmune disorder^[15, 16], Parkinson's disease (PD), Alzheimer's disease (AD), multiple sclerosis (MS), autism spectrum disorder (ASD)^[17] and gout^[18, 19]. Because of the important role of NLRP3 in inflammatory activities, we speculated that the polymorphism of NLRP3 rs10754558 might be related to the occurrence of arteriosclerosis obliterans. To test this hypothesis, we designed a case-control study to examine the possible association between NLRP3 rs10754558 polymorphism and arteriosclerosis obliterans in Chinese Han males.

Study subjects, ethics and consent

From March 2010 to January 2016, a total of 758 male patients with lower extremity arteriosclerosis obliterans and 793 healthy, age-matched male subjects participated in the case-control study. The basic demographic and clinical characteristics of the study participants are listed in Tables 1 and 2. All subjects were recruited continuously from Qingdao Municipal Hospital. The diagnosis of lower extremity arteriosclerosis obliterans is based on the standards published by the ministry of health of China in 2011 (WS 339-2011) and the relevant guidelines issued by ACCF/AHA in 2011 and 2012^[20, 21]. None of the samples in the control group had a personal or family history of arteriosclerosis obliterans or other serious diseases. Blood glucose, total cholesterol (TC), triglyceride (TG), urea nitrogen, creatinine, serum uric acid, high-density lipoprotein, low-density lipoprotein, alanine aminotransferase (ALT), aspartate aminotransferase (AST) and IL-1 β were detected in all subjects. Phenotypic characteristics, including demographic data and disease duration, were recorded using a standardized questionnaire. Each participant in the study received a statement about the study and signed a written informed consent prior to enrollment. The research plan was approved by the Ethics Committee of Qingdao Municipal Hospital.

Genotyping

Genomic DNA was extracted from peripheral venous blood using GenElute™ Blood Genomic DNA Kit (Sigma). The SNP of NLRP3 rs10754558 was detected by using TaqMan SNP Genotyping Assays which contained a pair of primers, forward, 5'-CCTGAGCTGACCGTCGTCTT-3', and reverse, 5'-ATGAGGTCACCAAGAGGAACATC-3' (Applied Biosystem). PCR was performed in the ABI 7900HT Fast Real-Time PCR System, following the manufacturer's instructions. 10% of the samples were analyzed repeatedly to confirm the accuracy of the TaqMan analysis. The consistency rate of two genotype calls was 100%.

Statistical analysis

The Quanto 1.2.4 software was used to assess statistical power. Assuming that the ORs of these loci in Chinese are 1.30-1.50, we obtained the estimated powers greater than 90% for the correlation between SNP and ASO risk. Statistical analysis was done by SPSS version 20.0 software (IBM Corporation, USA). Independent samples t-test was used for comparisons between the two groups. The deviations from Hardy - Weinberg equilibrium were tested by Pearson's χ^2 test. Odds ratios (ORs) . The odds ratio (ORs) with 95% confidence interval (CIs) was calculated to show the strength of the relationship between the genotype distribution or allele frequencies and ASO. Genotype-phenotype analysis was performed in ASO patients by ANOVA. The analysis took into account smoking history. $p < 0.05$ was considered statistically significant.

Results

Clinical characteristics of subjects

Table 1 and Table 2 respectively showed the basic characteristics and clinical characteristics of the enrolled subjects in this study. Table 1 showed that, except for age, the differences between the two groups were statistically significant ($p < 0.05$). As can be seen from Table 2, the TC, LDL, IL-1 β , fasting blood glucose, systolic blood pressure and BMI of the ASO group were significantly higher than those of the control group ($p < 0.05$). Other characteristics of ASO patients were not significantly different from those of the control group.

The polymorphism of NLRP3 rs10754558 and ASO

No deviations from Hardy - Weinberg equilibrium were found for the genotype distribution of NLRP3 rs10754558 polymorphism in either ASO patients or control group ($p > 0.05$). Table 3 showed the correlation between NLRP3 rs10754558 polymorphism and ASO. The genotype distribution of NLRP3 rs10754558 in ASO patients was significantly different from that in the control group ($p = 0.022$). Those carrying genotype GG had a higher risk for ASO than those carrying genotype CC (OR=1.574, 95%CI 1.161-2.135, $P=0.003$), which was also significant after the adjustment of the history of smoking, TC, LDL, fasting blood glucose, systolic blood pressure and BMI (OR=1.448, 95%CI 1.046-1.911, $P=0.004$).

Genotype-phenotype analysis

The results of genotype-phenotype analysis were shown in Table 4. It was obviously different in the TG and TC levels among those three genotypes. There was also significant difference in the levels of IL-1 β between individuals carrying the GG, GC and CC genotype of rs10754558 ($p = 0.011$). Patients carrying genotype GG of NLRP3 rs10754558 polymorphism had higher TG (3.16 ± 1.27 mmol/L vs. 1.69 ± 1.57 mmol/L, $p = 0.009$) and TC (6.91 ± 1.91 mmol/L vs. 5.33 ± 0.97 mmol/L, $p = 0.010$) levels compared to those carrying the CC genotype. Patients carrying the GG genotype also had an increased IL-1 β

level compared with those carrying the CC genotype (8.41 ± 1.01 ng/L vs. 6.97 ± 1.06 ng/L, $p = 0.003$).

Discussion

Arteriosclerosis obliterans is a kind of autoinflammatory disorder of artery, which is characterized by atherosclerotic thickening, medial calcification of the arterial walls and loss of elasticity^[22]. Some studies have proved that cholesterol crystals can activate the NLRP3 inflammasome in phagocytes in a process involving phagolysosomes damage^[8]. The NLRP3 inflammasome is involved in cholesterol crystals mediated arteriosclerosis by controlling the inflammatory caspase-1-mediated IL-1 β release^[8, 23].

The case-control study was performed to detect the possible genetic association between NLRP3 rs10754558 polymorphism and ASO in Chinese Han male population. The results showed that there was a significant difference in the genotype distribution of NLRP3 rs10754558 polymorphism between patients and controls ($p = 0.022$). Those carrying genotype GG had a higher risk for ASO than those carrying genotype CC (OR=1.448, 95%CI 1.046-1.461, $P=0.004$) after the adjustment of the history of smoking, fasting blood glucose, systolic blood pressure, TC, LDL, and BMI. So far to know, this is the first study that reports the relationship between polymorphisms of NLRP3 and ASO. In the following genotype-phenotype analysis, we found that TG and TC levels showed an obviously different distribution among the three different genotypes. Patients with genotype GG showed higher levels of TG and TC compared to those with genotype CC. As we all know, TG and TC can raise the risk of ASO^[24-29]. The genotype-phenotype analysis indicates that NLRP3 rs10754558 polymorphism is associated with TG and TC levels, which further proves the association between NLRP3 rs10754558 polymorphism and susceptibility to ASO.

The secretion of the IL-1 β by macrophages is a major driver of pathogenesis in atherosclerosis^[30]. As a response to cholesterol crystal accumulation, the NLRP3 inflammasome is activated to produce IL-1 β ^[8, 9]. It has been confirmed that the mRNA level of NLRP3 in human peripheral blood mononuclear cells is positively correlated with serum IL-1 β concentration, which is involved in the atherosclerosis process. Knockout the gene of NLRP3 significantly reduced the production of IL-1 β . Therefore, NLRP3 plays an important role in accelerating the process of atherosclerosis, causing plaque formation and promoting the onset of ASO. The risk allele G of rs10754558 contributed to the mRNA stability more than the other allele. The allele specific construct of rs10754558 containing the G allele showed 1.3-fold higher activity than the other constructs containing the C allele^[31]. Therefore, we believe that the polymorphism of NLRP3 rs10754558 can affect the function of NLRP3 inflammasome. Individuals with different genotypes show different genetic susceptibility to ASO. Although there were significant differences in the concentrations of IL-1 β between GG genotypes and CC genotypes in the control group, as their overall IL-1 β levels were much lower than those of the case group, ASO did not occur. This is possibly because of a state of proinflammatory and anti-inflammatory homeostasis balance in vivo, in which the level of IL-1 β was not sufficient to reach the pathogenic level. In addition, in the gene-phenotypic correlation analysis, we found that the levels of TC and TG of the three genotypes were significantly different. ASO patients with GG gene had higher levels of TC and TG than those with CC gene. Both TC and TG have been shown to increase the risk of ASO^[32-35]. The results of gene phenotype analysis showed that the polymorphism of NLRP3 rs10754558 was correlated with the

level of TC and TG, which further proved that the polymorphism of NLRP3 rs10754558 was correlated with ASO susceptibility.

ASO is the result of multiple signaling pathways affecting the occurrence and development of atherosclerosis. In this process, the effect of a single gene is very difficult to play a major pathogenic effect, so there could be multiple loci involved. The candidate genes probably interact with other genes or interact with other risk factors (such as smoking) to play their pathogenic effect. At present, it is a difficult task to explore the association between multi-locus genotype combinations and a disease. Thanks to clever statistical and geneticist experts, logistic regression analysis is one of several statistical methods to evaluate gene-gene interactions^[36]. There have been many reports on the interactions between CARD8 gene and NLRP3 gene in other autoimmune related diseases^[37, 38]. In this study (the part of our study about the association between polymorphism of CARD8 rs2043211 and susceptibility to ASO had been published before^[39]), we analyzed whether there was a biological interaction between different genotypes of these two polymorphic sites in the pathogenesis of lower extremity arteriosclerosis obliteration. The results showed that there was an interaction between rs10754558 of NLRP3 and rs2043211 of CARD8 gene. After the adjustment of body mass index (BMI), systolic blood pressure, fasting blood sugar, low density lipoprotein cholesterol (LDL), total cholesterol and smoking index, $\chi^2 = 14.114$, $P_{\text{interaction}} = 0.038$. But when the concentration of IL-1 β was put into adjustment, $\chi^2 = 3.812$, $P_{\text{interaction}} = 0.114$, this suggests that there are interactions between genes at these two loci, and this kind of interactions are independent from body mass index (BMI), systolic blood pressure, fasting blood sugar, low density lipoprotein cholesterol, total cholesterol and smoking index, but consistent with IL-1 β levels. This fully suggests that under the premise of carrying the T allele of CARD8 rs2043211, the G allele of NLRP3 rs10754558 increases the susceptibility to ASO. This gene-gene interaction is consistent with IL-1 β levels. The IL-1 β level of GG/TT genotype patients was higher than that of the CC/AA genotype patients, ($P < 0.01$), suggesting the synergistic effect of G allele at NLRP3 rs10754558 and T allele at CARD8 rs2043211 can increase the production of IL-1 β in vivo and promote the onset and progression of ASO. The study of R.L. Roberts et al showed that the interaction between NLRP3 and CARD8 gene was correlated with the pathogenesis of abdominal aortic aneurysm^[37]. The difference was that their study found that the CARD8/NLRP3 AT/CC genotype combination (in which the CARD8 gene took rs2043211 as the study object, and the NLRP3 gene took rs35829419 as the study object) had an obvious protective effect on the pathogenesis of abdominal aortic aneurysm. R.L. Roberts et al believed that the mechanism of this protective effect was consistent with the presumed interaction between different SNP combinations. The T allele at SNP rs2043211 introduces a stop codon polymorphism (Cys10Stop), which results in the expression of a severely truncated protein. The variant protein can't suppress NF- κ B activity, which leads to high constitutive levels of pro-IL-1 β . However, in individuals who carrying the NLRP3 rs35829419 CC genotype, these IL-1 precursors were not converted to IL-1 β with biological activity in large quantities, thus reduced the risk of abdominal aortic aneurysm^[37]. In our study we found that G allele of NLRP3 rs10754558 increased the susceptibility of gene carriers to ASO under the condition of carrying T allele of CARD8 rs2043211. This is not inconsistent with the R.L. Roberts's findings. At CARD8 rs2043211 sites T allele gene box shifts and results in the translation of a protein with

abnormal function, and this protein cannot inhibit the activity of NF - κ B, and a large number of IL-1 β precursors are secreted. In individuals with GG genotype of NLRP3 rs10754558, these IL-1 β precursors are converted to a large number of bioactive IL-1 β , leading to the occurrence of ASO, and the combination of these two genes plays a synergistic pathogenic role.

However, there were some limitations in our study. In the first, we only selected the SNP rs10754558 of NLRP3 and didn't cover all the SNPs of the whole sequence of NLRP3 gene. In the second, the size of the sample in our study wasn't big enough, and all the subjects were chosen from Chinese Han male population. Because of these reasons above mentioned, the results should be interpreted carefully, and the results should be verified in other ethnic populations and in more studies with larger sample. In the third, the mechanisms about the association between NLRP3 rs10754558 polymorphism and susceptibility to ASO are still unclear. Further studies are necessary to explore the mechanisms underlying NLRP3 rs10754558 polymorphism and susceptibility to ASO.

In conclusion, our finding suggests the polymorphisms of NLRP3 rs10754558 are probably associated with the development of ASO in Chinese Han male population. And there may be an interaction between rs10754558 of NLRP3 and rs2043211 of CARD8 gene in the development of ASO. However, our findings need to be verified in more studies with expanding sample and in other ethnic populations.

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Authors' Contributions

Kun Zhang and Wei Song participated in the design of the study, carried out genotyping and data analysis, and drafted the manuscript. Haoshan Qi has been involved in the acquisition and interpretation of data. Mingjin Guo and Jiaxuan Feng have made substantial contributions to conception and design of the study, coordination, and has given final approval of the article to be published.

Disclosure Statement

All authors declare that they have no conflicts of interest.

Table 1

Basic characteristics of controls and patients with ASO.

Characteristic	ASO (n=758)	Controls (n=793)	P values
Age (years)	61.68 ± 10.57	62.05 ± 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

Footnote: Values are shown as percentages, means ± 1 standard deviation, or median (interquartile range).

Table 2

Clinical characteristics of controls and patients with ASO (Values are shown as mean ± standard deviation).

	ASO (n=758)	Controls (n=793)	P values
BMI (kg/m ²)	26.92 ± 3.05	25.63 ± 3.14	0.018
systolic pressure (mmHg)	130.75 ± 9.22	122.33 ± 10.24	0.001
diastolic pressure (mmHg)	80.24 ± 8.21	79.89 ± 9.02	0.278
FBG (mmol/L)	6.49 ± 0.50	4.94 ± 0.47	0.011
Serum uric acid (µmol/L)	308.07 ± 50.06	299.62 ± 48.27	0.073
TG (mmol/L)	1.53 ± 0.25	1.29 ± 0.31	0.178
TC (mmol/L)	5.63 ± 0.52	4.81 ± 0.65	0.023
HDL (mmol/L)	1.40 ± 0.24	1.48 ± 0.22	0.098
LDL (mmol/L)	3.27 ± 0.31	2.58 ± 0.29	0.029
BUN (mmol/L)	5.12 ± 1.08	4.95 ± 1.16	0.472
Cr (µmol/L)	77.13 ± 10.52	76.87 ± 9.87	0.133
ALT (U/L)	38.83 ± 8.72	38.75 ± 7.97	0.429
AST (U/L)	28.81 ± 6.54	29.16 ± 6.08	0.564
IL-1 β (ng/L)	8.21 ± 1.23	4.97 ± 1.07	0.009

Table 3. Comparison of NLRP3 rs10754558 polymorphism between ASO and controls.

	ASO (n=758)	Controls (n=793)	<i>P</i> value	OR 95% CI	<i>p</i> value ^a	OR ^a 95% CI ^a
Genotypes						
CC ^Δ (%)	245 (32.32%)	308 (38.84%)	-	1.000	-	1.000
CG (%)	379 (50.00%)	378 (47.67%)	0.039	1.260 1.011-1.571	0.045	1.244 1.006-1.300
GG (%)	134 (17.68%)	107 (13.49%)	0.003	1.574 1.161-2.135	0.004	1.448 1.046-1.461
Alleles						
C ^Δ (%)	869 (57.32%)	994 (62.67%)	-	1.000	-	1.000
G (%)	647 (42.68%)	592 (37.33%)	0.002	1.250 1.083-1.444	0.003	1.193 1.032-1.358

Footnote: ^ΔBased on CC genotype or C allele; a. After the adjustment of SI, TC, LDL, FBG, SBP, and BMI by logistic regression.

Table 4 Genotype-phenotype analysis of NLRP3 rs10754558 in patients with ASO(BMI, Body mass index; TG, Triglycerides; TC, Total cholesterol, IL-1 β , etc.) ($\bar{x} \pm S$)

	CC A n=245	CG B n=379	GG C n=134	a vs. b vs. c p value	a vs. b p value	a vs. c p value
Age (years)	61.39 \pm 9.82	60.73 \pm 12.72	61.91 \pm 11.43	0.431	0.223	0.128
Age at diagnosis (years)	58.51 \pm 14.48	56.31 \pm 12.31	58.08 \pm 13.71	0.413	0.201	0.136
Disease duration (years)	3.54 \pm 4.03	3.87 \pm 5.46	3.98 \pm 4.66	0.064	0.058	0.404
BMI (kg/m ²)	26.47 \pm 3.31	26.44 \pm 3.10	27.08 \pm 2.06	0.707	0.345	0.256
systolic pressure (mmHg)	141.11 \pm 11.24	140.13 \pm 19.06	137.24 \pm 13.99	0.134	0.331	0.227
diastolic pressure (mmHg)	93.25 \pm 10.92	91.95 \pm 12.78	89.77 \pm 11.16	0.224	0.368	0.116
FBG (mmol/L)	6.57 \pm 1.45	6.44 \pm 1.71	6.22 \pm 1.59	0.653	0.234	0.435
Serum uric acid (μ mol/L)	433.52 \pm 118.11	451.23 \pm 109.54	466.41 \pm 111.52	0.345	0.433	0.333
TG (mmol/L)	1.69 \pm 1.57	2.59 \pm 1.34	3.16 \pm 1.27	0.019	0.023	0.009
TC (mmol/L)	5.33 \pm 0.97	6.45 \pm 1.11	6.91 \pm 1.91	0.031	0.088	0.010
HDL (mmol/L)	1.37 \pm 0.55	1.42 \pm 0.17	1.29 \pm 0.38	0.162	0.233	0.175
LDL (mmol/L)	2.97 \pm 0.59	3.01 \pm 0.46	3.03 \pm 0.36	0.131	0.146	0.223
BUN (mmol/L)	5.68 \pm 1.27	5.48 \pm 2.12	6.09 \pm 3.12	0.812	0.227	0.633
Cr (μ mol/L)	79.47 \pm 26.11	80.41 \pm 23.42	88.71 \pm 24.48	0.677	0.102	0.335
IL-1 β (ng/L)	6.97 \pm 1.06	7.91 \pm 0.92	8.41 \pm 1.01	0.011	0.022	0.003

Table 5 Analysis of gene interaction between NLRP3 rs10754558 locus and CARD8 rs2043211

locus.

Gene type	ASO	Control	OR(95%CI)	P
NLRP3/ CARD8	例数 (%)	例数 (%)		
CC/AA	65 (8.6)	115 (14.5)	1	
CC/AT	121 (16)	143 (18)	1.197 (0.965-1.328)	0.141
CC/TT	59 (7.8)	50 (6.3)	1.288 (0.986-1.388)	0.068
CG/AA	84 (11.1)	101 (12.7)	1.471(0.967-2.239)	0.071
CG/AT	196 (25.9)	179 (22.6)	1.937 (1.344-2.792)	0.000357
CG/TT	99 (13.1)	98 (12.4)	1.787 (1.183-2.701)	0.006
GG/AA	42 (5.5)	33 (4.2)	1.252 (0.892-1.895)	0.333
GG/AT	54 (7.1)	51 (6.4)	1.873 (1.149-3.054)	0.011
GG/TT	38 (5)	23 (2.9)	2.923 (1.603-5.329)	0.000353
Gene-gene interaction				
	$\chi^2=12.787$	P=0.016		
	$\chi^2=14.114^a$	P=0.038 ^a		

Footnote: a. After the adjustment of BMI, SBP, FBG, LDL, TC and SI during the analysis of logistic regression.

P < 0.05 indicates that there is interaction between genes.

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