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Linkage of large vessel carotid atherosclerotic stroke to inflammatory genes via a systematic screen

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Abstract

Background—Inflammatory cytokines including the IL-1 family, TNF-α and IL-6 mediate the formation of thrombosis on the luminal surface of atherosclerotic plaques. Gene polymorphisms that regulate these cytokines' expression may explain part of the variation in susceptibility to stroke in patients with carotid atherosclerosis. The aim of this study is to evaluate the role of single-nucleotide polymorphisms (SNPs) and haplotypes in inflammatory genes as they relate to symptomatic carotid atherosclerosis.

Methods—The study included 95 subjects with symptomatic (transient ischemic attacks (TIA) or stroke) and 113 subjects with asymptomatic carotid atherosclerotic disease. A panel of evenly spaced SNPs including previously reported functionally significant polymorphisms were genotyped for *IL-1β* (10 SNPs), *IL-1α* (9 SNPs), *IL-1RN* (11 SNPs), *IL-6* (7 SNPs) and *TNF-α* and *TNF-β* (7 SNPs).

Results—Using single SNP analysis, *IL-1RN* rs315934 (p=0.025), *IL-1RN* rs315946 (p=0.042), IL-1RN rs315921 (p=0.035), *IL-6* rs1180243 (p=0.018), and *IL-1α* rs2071373 (p=0.025) were associated with decreased odds of symptomatic carotid disease. Additionally, two diplotypes of the *IL-1RN* gene (p=0.023 and p=0.0064) and one diplotype in the *IL-1α* gene (p=0.02) were associated with a protective affect from cerebral ischemic events. Logistic analysis for interaction of the protective SNPs reveal an additive effect of all SNP pair combinations.

Conclusion—These results suggest genetic polymorphisms in pro-inflammatory genes may contribute to inter-individual differences in the development of symptomatic carotid atherosclerotic disease.

Keywords

stroke; atherosclerosis; inflammatory; immune genes; single nucleotide polymorphism; haplotype

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Introduction

Twin and family history studies support the role of genetic influence as a risk factor for stroke[1,2,3]. Studies reveal a two to four fold increased risk of stroke in monozygotic versus dizygotic twin pairs[1,2]. Further, a family history of stroke before the age of 65 conveys an increased risk of stroke compared to those without a family history (OR of 1.38; 95% CI, 1.01-1.90) [4]. Although some genetic polymorphisms have been associated with ischemic stroke in multiple studies [5,6], others have failed to replicate [7,8,9]. This is believed in part to be due to the complexities of ischemic stroke phenotypes, including lack of separating ischemic stroke subtypes for analysis, variability in genetic influences of risk factors and vascular response to risk factors, population heterogeneity and interaction effects of a multigenic process.

These and other concerns have prompted a call for guidelines for reporting genetic association in stroke [10]. Paramount among the recommendations is the accurate determination of ischemic stroke subtypes using a standardized classification system and well-defined case and control subjects [4,10,11]. Jerrard-Dunne, et al, in a case controlled study found family related risk for stroke was influenced by stroke subtype, with large vessel disease carrying an OR of 2.24 (1.49-3.36) compared to a lower risk in subjects with ischemic strokes from all causes (OR=1.69; 1.25-2.29) [4]. No familial association was noted for cardioembolic or cryptogenic strokes in this study. Studies in the cardiology literature and more recently in the stroke literature have begun to identify genes associated with large vessel atherosclerosis formation and subsequent myocardial and cerebral ischemic events [12,13,14,15]. Atherosclerosis has been consistently postulated to be a chronic inflammatory process and has prompted the study of numerous immune mediated pathways with regards to initiation, progression and activation of atherosclerotic plaque [16,17]. Whereas comparing subjects with and without carotid atherosclerotic disease provides the opportunity to examine genetic influence on plaque initiation and development [18], the study of symptomatic versus asymptomatic subjects with similar degrees of stenosis and risk factor exposure provides an opportunity to evaluate the potential influence of inflammatory mediator genes on the conversion of the quiescent asymptomatic plaque to an active thromboembolic state [19,20].

Therefore, we have examined a group of inflammatory genes that are reported to convert the endothelial surface over the atherosclerotic plaque to a pro-thrombotic and procoagulant state [21,22,23] in a cohort of symptomatic (case) compared to asymptomatic (control) subjects with high grade carotid atherosclerotic disease. We utilized marker panels for the *IL-1* family, *IL-6* and *TNF* that include both known functional markers and other markers evenly spaced within the genes with sufficient density to identify haplotype block structure and moderately abundant haplotypes [24].

Materials and Methods

Participants

The study population was made up of 95 subjects diagnosed with symptomatic carotid atherosclerotic stroke and a control population of 113 subjects with asymptomatic carotid atherosclerotic disease seen in the Cerebrovascular Clinic and Vascular Surgery Clinic at the National Naval Medical Center in Bethesda, Maryland. Informed consent was obtained according to human research protocols approved by the human research committees of the National Naval Medical Center and Uniformed Services University, Bethesda, Maryland. Patients with stroke were classified as atherothrombotic stroke utilizing the Trial of Org 10172 in Acute Stroke Treatment (TOAST) classification [25]. TOAST classification was adjudicated by a single cerebrovascular neurologist within the first 30 days of the index

event following full work up for etiology. Patients with concomitant atrial fibrillation in the symptomatic group were excluded. Inclusion for enrollment required a carotid ultrasound finding of > 50% stenosis in all patients. Carotid Doppler stenosis utilized PSV >125mm/sec and B-mode cross sectional analysis, confirmed by CT Angiography or conventional angiography using NASCET criteria. All subjects were identified to have large vessel extracranial atherosclerotic disease (>50 %) scheduled for carotid endarterectomy. Ischemic events were confirmed clinically by neurologists from the Stroke Clinic and by neuro-imaging with CT and/ or MRI. Asymptomatic subjects with extra-cranial carotid plaque had CT scans of the head to confirm no evidence of silent infarcts consistent with thrombo-embolic stroke. Clinical history was obtained on all asymptomatic subjects to assess for symptom report that would be consistent with a prior TIA or stroke by study neurologist from the stroke clinic. All participants were U.S. Caucasians of Western European descent based on self report.

SNP markers

The physical position and frequency of minor alleles (> 0.05) from a commercial database (Celera Discovery System, CDS, February, 2005) were used to select SNPs. 5′ nuclease assays (*vide infra)* were designed for 9 *IL 1*α, 10 *IL 1*β, 11 *IL 1RN*, 7 *IL 6*, and 7 *TNF*α/*β* SNPs. (data provided online at www.stroke.com). SNP panels for *IL-1*β, *IL-6* and *TNFα and β* were described previously [(24)].

Genomic DNA

Genomic DNA was extracted from lymphoblastoid cell lines and diluted to a concentration of 5 ng/μL. 2 μL aliquots were dried in 384-well plates.

Polymerase Chain Reaction (PCR) amplification

Genotyping was performed by the 5′ nuclease method [(26)] using fluorogenic allele-specific probes. Oligonucleotide primer and probe sets were designed based on gene sequence from the CDS, February 2005. Primers and detection probes used for each gene polymorphism are listed in Table 2.

In each reaction well, 2.5 μL of PCR Master Mix (Applied Biosystems, CA), containing AmpliTaq Gold® DNA Polymerase, dNTPs, Gold Buffer and MgCl2, were mixed with 900 nM of each forward and reverse primer and 100 nM of each reporter and quencher probe. DNA was incubated at 50°C for 2 min and at 95°C for 10 min, and amplified on an ABI 9700 GeneAmp PCR system for 40 cycles at 92°C (Assays on Demand) or 95°C (Assays by Design) for 15 s and 60°C for 1 min (Applied Biosystems).

Allele-specific signals were distinguished by measuring endpoint 6-FAM or VIC fluorescence intensities at 508 nm and 560 nm, respectively, and genotypes were generated using Sequence Detection System Software Version 1.7 (Applied Biosystems, CA). Genotyping error rate was directly determined by re-genotyping 25% of the samples, randomly chosen, for each locus. The overall error rate was <0.005. Genotype completion rate was 0.98.

Single SNP analysis and haplotype analysis—Association analysis was performed using 1) single marker analysis, and 2) haplotypes (constructed using SNPs in the same haplotype block). For single marker analysis, logistic regression was applied with atherosclerosis type, asymptomatic vs. symptomatic, as the dependent variable, SNP genotype (1,1 vs 1,2 vs 2,2) as the independent variable and gender, smoking, hypertension, hypercholesterolemia, diabetes, peripheral vascular disease, cardiovascular disease, white blood cell count and age as covariates.

Haplotype frequencies were estimated using a Bayesian approach implemented with PHASE [27]. These frequencies closely agreed with results from a maximum likelihood method implemented via an expectation-maximization (EM) algorithm [28]. Haplotype analysis was conducted as follows. Step 1: haplotype phases were inferred using expectation-maximization (EM) algorithm [29], where diplotype probabilities were assigned for each subject. Step 2: stepwise regression [30] was applied to select haplotypes which were associated with atherosclerosis type, at this step haplotypes with frequency < 5% were excluded, and the effects of nine covariates were fixed in the model. Step 3: the odds ratio for each haplotype, using the most frequent haplotype as reference, was calculated by adjusting for the nine covariates and weighting for the haplotype's probability. Step 4: if the haplotype was significant at p=0.1 for stepwise regression at step 2 and had the highest or lowest odds ratio (such haplotypes were denoted as candidate haplotypes), a multi-locus diplotype for each patient was created from the combination of haplotype pairs, and the candidate haplotype was treated as one allele and all others were pooled and treated as another allele. The multi-locus diplotypes were analyzed similarly to single locus SNP, except that the haplotype's probability was used as a weighting factor. SAS v9.1 was used for all statistical analyses [29].

Haploview version 2.03 Software (Whitehead Institute for Biomedical Research, USA) was used to compute Linkage Disequilibrium (D') matrices, and to generate haplotype blocks using the algorithm of confidence intervals [31]. Haplotype block structure was ascertained by looking for regions in which the D' values between neighboring markers were consistently above 80%. Haplotype block structure boundaries were supported by high mean and median values within each block

Results

Demographic profiles, including age, gender, and risk factor exposure were similar between subjects with symptomatic and asymptomatic atherosclerosis (See Table 1). Genotypes were successfully obtained for all 44 SNPs studied in the 5 genes. SNP location in relation to gene structure is shown in Figure 1a-e. All genotype frequencies conformed to Hardy-Weinberg equilibrium. Within *IL 1α, IL-1β, IL-6 and TNF-α*, single conserved LD blocks (16 kb, 17 kb, 14 kb and 7 kb, respectively) were observed. A 12 kb block (block 1) and a 3 kb block (block 2) was observed for the *IL-1RN* gene. Some disruptions of D' (a measure of LD) occurring within blocks are clearly attributable to low allele frequencies that lead to increased variance in estimation of LD. We compared our results for each gene to LD structure based on data from four populations (European, Chinese, Japanese and African origins) available in the HapMap project (http://www.hapmap.org/) and found that the block structures we observed are consistent with the exception of *IL-1β*. We found one haplotype block for *IL-1β* in Caucasians but the HapMap data indicate no strong LD across *IL-1β* region in any population. This could relate to a limited sample size used in HapMap.

Using single marker analysis, *IL-1RN* rs315934 (p=0.025), *IL-1RN* rs315946 (p=0.042), IL-1RN rs315921 (p=0.035), *IL-6* rs1180243 (p=0.018), and *IL-1α* rs2071373 (p=0.025) were significantly associated with odds of symptomatic carotid disease. All p values are unadjusted for multiple comparisons. *IL-1RN* rs315934 (p=0.015), *IL-6* rs1180243 (p=0.024), and *IL-1α* rs2071373 (p=0.031) remained independently associated with a decrease in atherothrombotic stroke when adjusted for the covariates of gender, coronary artery disease, peripheral vascular disease, smoking, hypertension, hypercholesterolemia, diabetes, white blood cell count and age (Table 2).

Table 3 showed that two haplotypes were significantly associated with atherothrombotic stroke based on stepwise regression [30]. *IL1RN* 1212122 was shown to be a susceptibility

haplotype ($p < 0.05$), whereas the *IL-1RN* 1211122 haplotype was associated with reduced ischemic risk compared with the other three observed haplotypes. The *IL-1α* 111211212 showed a trend toward being a protective haplotype compared with other two observed haplotypes in the *IL-1α*. Diplotypes were constructed based on the above three haplotypes, by treating the haplotype with significant association as one allele and combining the other haplotypes as another allele.

Diplotype analysis (Table 4) demonstrated that the haplotype of *IL-1RN* 1212122 was associated with increase risk of atherothrombotic stroke, and there was about a three fold (OR= 2.80, p =0.02) greater odds of atherothrombotic stroke for the subjects with homozygous diplotypes of the haplotype than those without the haplotype. Haplotype *IL-1RN* 1211122 and *IL-1α* 111211212 were associated with decrease risk of atherothrombotic stroke, the subjects with the haplotype had about half (*IL-1RN* 1211122: OR=0.39, p=0.0064; *IL-1α 111211212*: OR=0.51, p =0.02) odds of atherothrombotic stroke compared with those without the haplotype.

Logistic analysis using pairs of markers at different genes suggested that several combinations of SNPs may have greater effects on the risk of symptomatic carotid atherosclerosis than the individual SNPs (See Table 5). Analysis of SNP pair combinations revealed that subjects homozygous for the G allele in the *IL-1α* rs2071373 SNP paired with homozygous for G in the *IL-1RN* rs315921 SNP (O.R.=4.1, p=0.01), or homozygous for A in the *IL1RN* rs315934 SNP (O.R.=4.7, p=0.003), or homozygous for A in the *IL-1RN* rs315946 SNP (O.R.=3.9, p=0.02) had the highest adjusted odds ratio for symptomatic carotid atherosclerotic disease.

Discussion

This study suggests that in patients with carotid atherothrombotic disease polymorphisms in genes that mediate inflammation are associated with ischemic events. . Single SNP analysis demonstrated that three *IL-1RN* polymorphisms, one *IL-1α* polymorphism and one *IL-6* polymorphism were associated with a protective effect. Conversely, combinations of allele pairs that lack these protective SNPs were associated with symptomatic carotid disease. Further, our data suggests that specific haplotype and diplotype combinations in the *IL1-RN* and *IL-1α* genes may be associated with fewer strokes in patients with atherosclerotic disease. Caution should be taken in interpretation of these results, since the study of multiple genes increases the risk of false positive and even the haplotypes that had a significant p-value with correction for 6 genes need confirmation. As stated in the Results, all p-values in this study were adjusted to the significant effect of covariates (gender, coronary artery disease, peripheral vascular disease, smoking, hypertension, hypercholesterolemia, diabetes, white blood cell count and age) but unadjusted for multiple comparisons). Ultimately, future prospective studies that evaluate the contribution of these and other provisional susceptibility and protective genes as well as gene combinations will need to be performed in large cohorts. In case of replication of our findings these studies would provide validation and confirmation of the contribution of polymorphic inflammatory genes to the development of large-vessel carotid atherosclerotic stroke. By publishing the results of our study that are important but preliminary, we anticipate and encourage other research groups to conduct similar study in their patient populations of larger size and different ethnicities, as well as molecular functional studies on markers used in our study. Having this additional data available we can further investigate biological plausibility of the association of polymorphic inflammatory genes with stroke or other cardiovascular events.

Recent reviews recommend that genetic studies of stroke risk consider stroke subtypes because atherothrombotic stroke has been shown to have a greater genetic association than

cardioembolic stroke. Further distinction may be needed between genes that cause vascular pathology and those contributing directly to vascular events. For example, studies have identified gene polymorphisms that are associated with the development of atherosclerotic disease in the coronary and cerebral vessels without association with MI or stroke [13,32]. Conversely, genes that are involved in the hemostatic factor pathways, and intuitively linked to potential ischemic event risk, have not been found to be associated with the early atherosclerotic development [33].

To reduce the confounding effects of multiple stroke subtypes and clinical variances with intermediate vascular phenotypes we used a case control paradigm in which we studied genes of interest in a population of patients with high grade atherosclerotic disease. Patients with carotid atherosclerosis were stratified for genotype analysis based on the presence of large vessel atherothrombotic cerebral ischemic events as defined by the TOAST criteria [25]. Identification of risk factor burden, as previously described [34,35] were comparable between the symptomatic and asymptomatic patients (table 1), thus making classic risk factors a less likely cause to explain the clinical differences. Genetic variation in the *IL-1* family, *TNF-α* and *IL-6* were chosen for study based on their biological plausibility given that these inflammatory mediators are implicated in the conversion of the endothelial surface from an anti-coagulant/anti-aggregate state to a pro-thrombotic and pro-aggregate state [17,21,22].

Limitations: this study followed many of the guidelines suggested in recent position statements in the field of cerebrovascular genetics [4,10,11]; however, the small population size increases the chance of type I error. Also, the restriction of our population to Caucasians of Western European descent may limit the applicability to other populations. On the other hand, this reduces the confounder of subpopulation variance in haplotype blocks and increases reliability that difference in SNPs and haplotypes were not confounded by uncertain LD in mixed populations.

In summary, polymorphisms in genes that mediate inflammatory and ultimately thrombotic pathways on the endothelial surface of carotid atherosclerotic plaques are associated with cerebral ischemic event occurrence in an at risk population. These SNPs represent good candidates for validation studies in the determination of genetic predisposition for stroke in patient with risk for carotid atherothrombotic stroke subtype.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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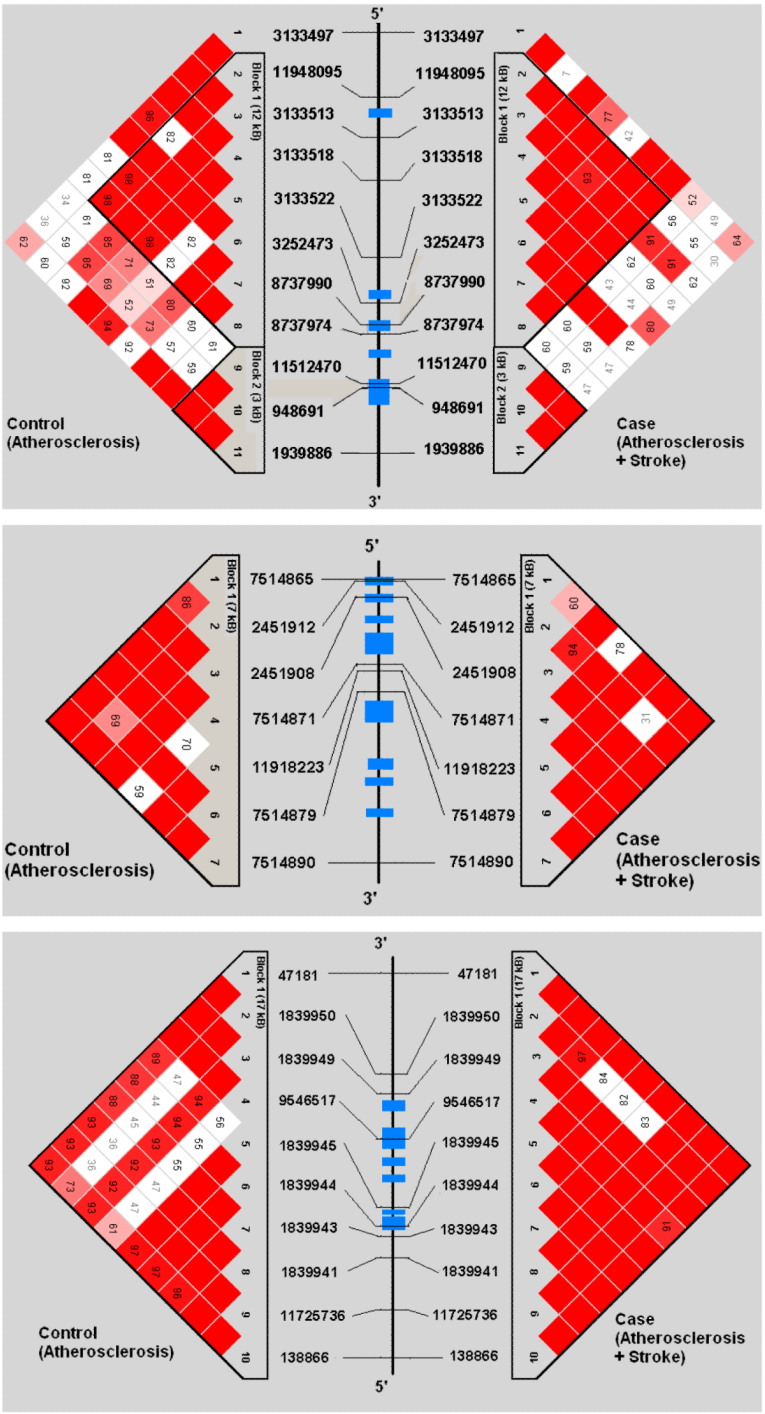

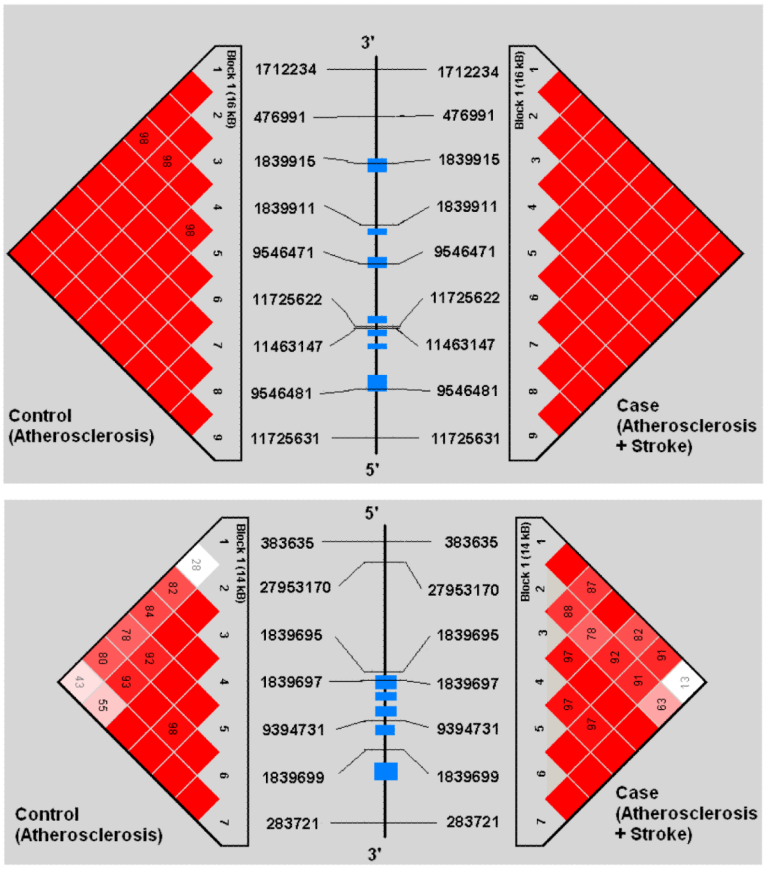

Figures 1a-1e.

represent a schematic of the study genes with an LD Matrix for subjects with symptomatic carotid atherosclerosis (Case) compared to subjects with asymptomatic carotid atherosclerosis. Horizontal cross lines indicate the approximate positions of each SNP. The blue boxes represent exons. Each box in the matrices represent D', % linkage disequilibrium (LD) between SNP pairs, as generated by Haploview. Boxes without numbers represent complete LD, D' = 1. SNP ID numbers are from the public database rs numbers.

Table 1

Demographic and clinical data in patients with carotid atherosclerosis

	Asymptomatic Atherosclerosis (n=113)	**Symptomatic Atherosclerosis (n=95)**	**p-value**
	%	%	Fisher's exact
Gender (male)	73	82	0.1371
Smoking	77	72	0.3722
Hypertension	89	83	0.1683
Hypercholesterolemia	75	69	0.3546
Diabetes	22	27	0.3811
Peripheral Vascular Disease	27	27	0.9916
Cardiovascular Disease	44	48	0.5476
	Mean (std)	Mean (std)	t-test
Age	70 (9.1)	70(8.4)	0.5831
White Blood Cell Count	7.4 (2.0)	7.6 (2.3)	0.9981

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Table 2

Single SNP analysis

SNP	HW test	Genotype	Number of subjects for		Crude OR		Adjusted OR*	
			Asymptomatic Atherosclerosis	Symptomatic Atherosclerosis	Estimate	p-value	Estimate	p-value
IL-1α	0.138	2/2	43	50	1.0		1.0	
rs2071373		½	51	29	0.5	**0.022**	0.5	**0.028**
		1/1	9	7	0.7	0.461	0.7	0.453
		1/1+1/2			0.5	**0.025**	0.5	**0.031**
IL1-RN	0.894	1/1	64	60	1.0		1.0	
rs315921		½	37	17	0.5	**0.038**	0.6	0.110
		2/2	2	1	0.5	0.612	0.5	0.544
		2/2+1/2			0.5	**0.035**	0.5	0.096
IL1-RN	0.981	2/2	56	62	1.0		1.0	
rs315934		½	35	14	0.4	**0.005**	0.4	**0.017**
		1/1	2	1	0.5	0.521	0.4	0.474
		1/1+1/2			0.4	**0.005**	0.4	**0.015**
IL-1RN	0.230	1/1	65	62	1.0		1.0	
rs315946		½	31	14	0.5	**0.042**	0.5	0.072
IL-6	0.189	1/1	50	56	1.0		1.0	
rs1180243		½	44	23	0.5	**0.018**	0.5	**0.024**
		2/2	4	8	1.8	0.367	1.9	0.319

Table 3

Haplotype frequencies and Odds ratio for gene *IL-1RN* and *IL-1α*

			Number of haplotype for		Crude OR	Adjusted OR*
SNP	**Haplotype**	**p-value****	**Asymptomatic Atherosclerosis**	**Symptomatic Atherosclerosis**	**Estimate**	**Estimate**
IL-1RN	1212122	0.05	77	85	1.00	
	1112222		22	14	0.58	0.59
	1211122	0.01	41	18	0.39	0.40
	2222111		69	57	0.74	0.72
IL-1α	111212212		82	79	1.00	
	111211212	0.07	71	45	0.66	0.64
	222122121		54	51	0.98	0.94

*Adjusted for all 9 variables in table 1

**the haplotypes showing significant association (p<0.1) in stepwise regression (Zaykin 2002).

Table 4

Diplotype (combination of haplotype pair) frequencies and Odds ratio for gene *IL-1RN* and *IL-1α*

	Number of subjects		Crude Odds Ratio			Adjusted Odds Ratio*		
No. of the haplotype copies	**Asymptomatic Atherosclerosis**	**Symptomatic Atherosclerosis**	**Estimate**	**95%CI**	**p-value**	**Estimate**	**95%CI**	**p-value**
***IL-1α*:111211212**								
0	41	49	1.0 (ref)			1.0 (ref)		
1	52	30	0.48	0.26 - 0.89	**0.0197**	0.47	0.25 - 0.89	**0.0213**
2	9	7	0.65	0.23 - 1.85	0.4185	0.64	0.22 - 1.88	0.4171
1 or 2			0.51	0.28 - 0.91	**0.0225**	0.50	0.27 - 0.91	**0.0241**
***IL-1RN*: 1211122**				-			-	
0	64	68	1.0 (ref)	-		1.0 (ref)	-	
1	37	15	0.39	0.2 - 0.77	**0.0071**	0.42	0.2 - 0.85	**0.0169**
2	2	1	0.48	0.05 - 4.82	0.5316	0.37	0.03 - 4.03	0.4167
1 or 2			0.39	0.2 - 0.77	**0.0064**	0.41	0.2 - 0.84	**0.0139**
***IL-1RN*: 1212122**								
0	40	24	1.0 (ref)			1.0 (ref)		
1	52	42	1.36	0.71 - 2.61	0.3599	1.43	0.72 - 2.85	0.3050
2	12	19	2.80	1.15 - 6.8	**0.0234**	2.85	1.11 - 7.33	**0.0292**

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Table 5

Logistic analysis for two SNPs combined

Genotype		Number of subjects for		Crude OR		Adjusted OR*	
SNP1 genotypes	**SNP2 genotypes**	**Asymptomatic Atherosclerosis**	**Symptomatic Atherosclerosis**	**Estimate**	**p-value**	**Estimate**	**p-value**
***IL-1α*rs2071373**	***IL-1RN* rs315921**						
11 or 12	12 or 22	23	6	1.0 (ref)		1.0 (ref)	
22 or 22	11	36	28	**3.0**	**0.0366**	2.7	0.0690
22	12 or 22	16	12	2.9	0.0767	2.9	0.0804
11	11	17	32	**4.5**	**0.0041**	**4.1**	**0.0104**
***IL-1α* rs2071373**	***IL-1RN* rs315934**						
11 or 12	11 or 12	25	7	1.0 (ref)		1.0 (ref)	
12 or 22	22	31	27	3.1	**0.0238**	2.7	0.0558
22	11 or 12	12	8	2.4	0.1654	2.2	0.2170
11	22	24	35	5.2	**0.0010**	4.7	**0.0032**
***IL-1α* rs2071373**	***IL-1RN* rs315946**						
11 or 12	12 or 22	15	5	1.0 (ref)		1.0 (ref)	
12 or 22	11	40	29	2.2	0.1736	2.1	0.2054
22	12 or 22	16	9	1.7	0.4304	1.8	0.4068
11	11	25	33	**4.0**	**0.0177**	**3.9**	**0.0256**

*Adjusted for all 9 variables in table 1