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Coronary Artery Disease–Associated Locus on Chromosome 9p21 and Early Markers of Atherosclerosis

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Background—Genome-wide association studies have recently identified a locus on chromosome 9p21 that influences risk of coronary artery disease (CAD). The effect of the locus on early markers of atherosclerosis is unknown. We examined its association with carotid intima-media thickness (CIMT) and brachial flow-mediated dilatation (FMD).

Methods and Results—We genotyped 2277 individuals, age 24 to 39 years, from the Cardiovascular Risk in Young Finns Study with CIMT and FMD measurements and 1295 individuals, age 46 to 76 years, from the Health 2000 Survey with CIMT for rs1333049, the chromosome 9p21 variant showing the strongest association with CAD. Both mean and maximum CIMT were significantly higher ($P<0.001$) in the older subjects of the Health 2000 Survey compared with the Young Finns Study. However, there was no association of the rs1333049 genotype with either mean or maximum CIMT at either age ($P=0.959$ and 0.977 for the 2 phenotypes in the Young Finns Study and $P=0.714$ and 0.725 in the Health 2000 Survey). Similarly, there was no association of the locus with variation in FMD in the Young Finns cohort ($P=0.521$).

Conclusions—The chromosome 9p21 locus does not influence CAD risk through a mechanism that also affects CIMT or induces early changes in FMD. (*Arterioscler Thromb Vasc Biol.* 2008;28:1679-1683)

Key Words: genetics ■ coronary artery diseases ■ atherosclerosis ■ carotid-intima media thickness ■ endothelial dysfunction

Coronary artery disease (CAD) has a significant genetic determination that has hitherto been poorly characterized. However, recent genome-wide association studies have identified several novel loci that are strongly associated with CAD. Specifically, a common variant located in a region adjacent to the cyclin dependent kinase inhibitors, *CDKN2A* (encoding p16INK4a) and *CDKN2B* (p15INK4b) on chromosome 9p21.3 has been associated with increased risk in 4 separate genome-wide association and follow-up studies.¹⁻⁵

Clinical manifestations of CAD represent the end stage of a chronic process. As genetic variants are present from birth, their effects on markers of atherosclerosis may be discernible at an earlier stage. Carotid intima-media thickness (CIMT) is an accurately quantifiable and reproducible marker of atherosclerotic risk and predicts future cardiovascular events.^{6,7}

Similarly, impaired brachial artery flow-mediated dilatation (FMD) is another marker of atherosclerotic risk and predicts cardiovascular events.⁸ In this study, we investigated the association of the CAD associated variant on chromosome 9p21.3 on CIMT in 2 population based cohorts of different ages, in combination spanning the age range from 24 to 76 years. We also examined its association with variation in FMD in young healthy subjects.

Materials and Methods

Subjects

We studied subjects from 2 population based cohorts—the Cardiovascular Risk in Young Finns Study and the Health 2000 Survey.^{9,10}

The Cardiovascular Risk in Young Finns Study is a multi-center study of atherosclerotic risk factors of children and young adults (<http://vanha.med.utu.fi/cardio/youngfinnsstudy/>). The first cross-

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sectional study was conducted in 1980 and included 3596 healthy children and adolescents, age 3, 6, 9, 12, 15, and 18 years. Details of the study design have been presented elsewhere.¹¹ Thereafter, these subjects have been followed with periodic examinations. In 2001, 2620 individuals, who had then reached the age of between 24 to 39 years, were studied.⁹ In addition to detailed risk factor assessments, ultrasound examination of CIMT and brachial endothelial function were carried out.^{12,13}

The Health 2000 Survey was a large Finnish cross-sectional health examination survey carried out in 2000 to 2001. The overall study cohort was a 2-stage stratified cluster sample (8028 persons) representing the entire Finnish population age 30 years and above.¹⁰ To study cardiovascular disease risk factors and diabetes more thoroughly, a supplemental study was carried out (sample size 1867 and participation rate 82%). The subjects in the supplemental study were 45 years and older, and the study was executed in the catchments areas of the 5 Finnish University Hospitals because specialized equipment was required. Carotid ultrasound examination was part of this supplemental study.¹⁴ There were 1295 subjects (595 men and 700 women; mean age, 58 years; range, 46 to 76 years) with available carotid ultrasound data. These individuals were selected to be our study group for the present analysis.

Clinical Characteristics

Body mass index (BMI) was calculated as weight in kilograms divided by the square of height in meters. Blood pressure (BP) was measured using a random zero sphygmomanometer in the Young Finns Study and the automatic Omron M4 sphygmomanometer (Omron Healthcare Europe B.V.) in the supplemental Health 2000 Study. Values for systolic and diastolic blood pressure were defined by Korotkoff phases I and V, respectively. The averages of 3 measurements obtained after 5 minutes of sitting with 1 to 2 minutes between readings were used in analyses. Smoking habits were inquired with a questionnaire.

Laboratory Tests

In both studies, venous blood samples were taken after an overnight fast. Total cholesterol, HDL cholesterol, triglyceride, and glucose concentrations were determined enzymatically (Roche Diagnostics, GmbH for HDL; Olympus System Reagent for total cholesterol, triglyceride, and glucose) with a clinical chemistry analyser (Olympus, AU400). LDL cholesterol was calculated with the Friedewald formula.

Ultrasound Imaging

In the Young Finns Study, carotid ultrasound studies were performed using a high-resolution ultrasound system (Sequoia 512, Acuson) with 13.0 MHz linear array transducer. CIMT was measured about 10 mm from the bifurcation on the left common carotid artery focusing the image on the posterior wall and recording images from the angle showing the greatest distance between the lumen-intima interface and the media-adventitia interface.¹² At least 4 measurements were taken at each scan of the common carotid artery incident with the R-wave of the continuously monitored ECG to derive mean and maximum CIMT. The scans were analyzed by 1 reader blinded to subjects' details. The between visit (2 visits 3 months apart) coefficient of variation of mean CIMT measurements for a subset of the subjects was 6.4%.¹²

To assess brachial artery FMD, the left brachial artery diameter was measured both at rest and during the reactive hyperemia.¹³ Increased flow was induced by inflation of a pneumatic tourniquet placed around the forearm to a pressure of 250 mm Hg for 4.5 minutes, followed by release. Three measurements of arterial diameter were performed at end-diastole at a fixed distance from an anatomic marker at rest and at 40, 60, and 80 seconds after cuff release. The vessel diameter after reactive hyperemia was expressed as the percentage relative to the resting scan. The between-visit CV for brachial diameter was 3.2% and for FMD 26.0%.¹³

In the Health 2000 supplemental study carotid ultrasound examination of the right carotid artery was performed according to a

standardized protocol using a 7.5 MHz linear array transducer. The examinations were performed by centrally trained and certified sonographers at 5 study locations around Finland.¹⁴ CIMT measurements were performed off-line with the use of automated imaging processing software. One reader was responsible for reading all ultrasound images. Mean and maximum CIMT were again calculated. The intrareader reproducibility of the CIMT measurements was assessed by calculation of the CIMT twice from 571 randomly selected images of 108 study subjects several weeks apart. The mean difference of the 2 measurements was 0.001 mm (SD 0.123), and the intraclass correlation was 0.934 ($P < 0.001$).¹⁴

Genotyping

Genomic DNA was extracted from peripheral blood leukocytes using a commercially available kit (Qiagen Inc). rs1333049 was genotyped by allelic discrimination using a standard TaqMan assay (further details available on request). Fluorescence was detected post polymerase chain reaction (PCR) using the ABI Prism 7900HT Sequence Detector System and genotypes called using ABI Prism SDS software version 2.1 (ABI). For reference, in the genome-wide association studies,^{1,2} the CAD associated (risk) allele for rs133309 was C.

Statistical Analysis

Univariate data comparisons between genotype groups (and between subjects in the 2 studies) were based on analysis of variance for continuous variables and Chi-square test for categorical variables. Because of skewed distributions, the values for triglycerides were log transformed. Multiple logistic regression analysis was used to identify clinical and laboratory variables that were independently associated with mean and maximum CIMT and to assess the effect of genotype on CIMT taking these variables into account. The statistical tests were performed with SPSS (version 14.00) for the Young Finns Study and SAS (version 8.1) for the Health 2000 cohort. 95% CI for allele frequencies were calculated using confidence interval calculation program, CIA (version 2.1.2). Power calculations were undertaken using the P/S (Power and Sample size calculator) program.

Results

The age range of the subjects was 24 to 39 years (55% female) in the Young Finns Study and 46 to 76 years (55% female) in the Health 2000 cohort. The frequency of the C allele for rs1333049 was 0.41 (95% CI: 0.40 to 0.43) in the Young Finns Study and 0.42 (95% CI: 0.40 to 0.44) in the Health 2000 subjects and the genotypes were in Hardy Weinberg equilibrium in both cohorts. The characteristics of the subjects in each study partitioned by genotype for rs1333049 are shown in Table 1. Overall, the older subjects from the Health 2000 survey had higher BMI, LDL, and HDL cholesterol levels and higher average systolic and diastolic blood pressures compared with the subjects in the Young Finns Study ($P < 0.001$). However, there was no significant difference in any of these traits according to rs1333049 genotype in either age group (Table 1).

Both mean and maximum CIMT were higher in the Health 2000 cohort compared with the Young Finns subjects (Table 1). However, there was no effect of the rs1333049 genotype on either phenotype in either the Young Finns study ($P = 0.959$ and $P = 0.977$, respectively) or in the Health 2000 cohort ($P = 0.714$ and $P = 0.729$). Specifically we did not see a higher CIMT in subjects carrying the CAD-risk associated allele (C) in either cohort. Brachial FMD responses (available in Young Finns) were likewise similar across the genotypes (Table 1, $P = 0.521$).

Table 1. Demographic and Phenotypic Characteristics of Subjects in the Cardiovascular Risk in Young Finns Study and in the Health 2000 Cohort Partitioned by Genotype for the Coronary Artery Disease Risk Variant on Chromosomes 9p21.3

Phenotype	Cardiovascular Risk in Young Finns				Health 2000 Cohort			
	GG	GC	CC	P Value	GG	GC	CC	P Value
Age, y	31.6 (5.0)	31.8 (5.0)	31.8 (5.0)	0.522	58.0 (8.1)	58.6 (8.2)	57.8 (7.5)	0.292
	790	1093	394		454	599	242	
Males, %	44	45	46	0.506	48	44	43	0.394
	790	1093	394		454	599	242	
BMI, kg/m ²	25.1 (4.3)	25.0 (4.4)	25.2 (4.5)	0.742	27.2 (4.6)	27.1 (4.3)	27.4 (4.5)	0.618
	784	1084	391		450	597	241	
LDL chol, mmol/l	3.27 (0.83)	3.30 (0.86)	3.24 (0.82)	0.470	3.39 (0.87)	3.40 (0.86)	3.43 (0.91)	0.839
	782	1074	389		450	586	237	
HDL chol, mmol/l	1.30 (0.32)	1.30 (0.32)	1.26 (0.30)	0.084	1.59 (0.43)	1.57 (0.42)	1.57 (0.45)	0.798
	790	1091	394		453	598	242	
Triglycerides, mmol/l	1.31 (0.73)	1.34 (0.92)	1.38 (0.89)	0.643	1.32 (0.66)	1.43 (1.14)	1.42 (0.82)	0.382
	790	1093	394		453	598	242	
Systolic BP, mm Hg	116.7 (12.8)	116.8 (13.4)	116.0 (12.7)	0.788	138.0 (22.2)	139.0 (21.3)	137.6 (21.3)	0.599
	785	1075	393		454	598	241	
Diastolic BP, mm Hg	70.7 (10.3)	70.8 (11.1)	70.9 (10.8)	0.980	84.5 (11.2)	84.3 (10.0)	84.3 (10.2)	0.932
	785	1075	393		454	598	241	
Smoking, %	25	23	22	0.506	24	22	22	0.485
	785	1093	394		454	599	242	
Mean CIMT, mm	0.58 (0.09)	0.58 (0.09)	0.58 (0.10)	0.959	0.93 (0.23)	0.93 (0.23)	0.94 (0.22)	0.714
	779	1082	390		454	599	242	
Max CIMT, mm	0.62 (0.10)	0.62 (0.10)	0.62 (0.10)	0.977	0.99 (0.21)	1.00 (0.22)	1.00 (0.21)	0.729
	779	1082	390		454	599	242	
FMD, %	7.84 (4.34)	8.04 (4.47)	8.12 (4.39)	0.521	NA	NA	NA	
	718	1015	362					

Values are mean (SD) or prevalence in %. The numbers below each value is the No. of subjects for which the data were available. CIMT indicates carotid intima media thickness; FMD, Brachial artery flow-mediated dilatation; NA, not available. *P* values are unadjusted values based on analysis of variance for continuous variables and Chi-square test for smoking and gender. The CAD-risk associated allele is the C allele.

The results of multivariate logistic regression analysis of mean CIMT in the 2 studies are shown in Table 2. In the Young Finns Study, there were highly significant independent effects of age, gender, BMI, and BP on CIMT and borderline significant effect of smoking. In the Health 2000 cohort, there were similarly significant independent effects of age, gender, and SBP on CIMT. HDL-cholesterol, and smoking but not BMI were also independently associated with CIMT in this cohort. Taking these factors into account there was no independent effect of the rs1333049 genotype on mean CIMT (Table 2). The results for maximum CIMT were similar (not shown).

Discussion

Within a short period of its identification, the chromosome 9p21 locus has been shown to have a robust association with CAD in a wide range of populations.^{1-5,15} The risk-associated allele, defined by the C allele of rs1333049, or alleles of other SNPs in strong linkage disequilibrium with it examined in some studies, have consistently shown an increased risk of 25% to 40% per copy of allele. The region of association with CAD on chromosome 9p21 spans ≈50 to 60 kb¹⁻⁴ and is located adjacent to genes coding for the cyclin-dependent

kinases p16/CDKN2A and p15/CDKN2B as well as p14/ARF. These genes play a central role in the regulation of the cell cycle and may be implicated in the pathogenesis of atherosclerosis through their role in transforming growth factor (TGF)- β -induced growth inhibition.^{16,17} Interestingly, although the 9p21 locus itself does not contain a protein coding gene, recent studies have shown that it codes a large noncoding RNA, ANRIL, which is expressed in atherosclerotic tissue.^{15,18} Furthermore, expression of ANRIL is coordinated with that of p14/ARF and possibly also p16/CDKN2A and p15/CDKN2B, in both physiological and pathological conditions,¹⁸ suggesting that it may regulate the expression of these genes. Further studies are required, but this could provide a potential mechanism by which the locus affects CAD risk.

In this context, it is relevant to examine the association of the 9p21 locus with other forms of atherosclerotic and vascular disease as well as markers of atherosclerosis. Indeed, a recent study has also shown an association of the locus with abdominal aortic aneurysms as well as with intracranial aneurysms.¹⁹ Among atherosclerosis-related phenotypes, CIMT has gained particular prominence, both because of the ease, accuracy, and reproducibility of its measurement

Table 2. Determinants of Mean CIMT in the Young Finns Study and the Health 2000 Cohort: Results From Multivariable Regression Analysis

	Young Finns Study		Health 2000 Cohort	
	Beta (error)	P	Beta (error)	P
Gender	0.0101 (0.0043)	0.017	0.0247 (0.0094)	0.009
Age	0.0050 (0.0004)	<0.001	0.0097 (0.0006)	<0.001
BMI	0.0024 (0.0005)	<0.001	0.0015 (0.0011)	0.190
SBP	0.0054 (0.0020)	0.010	0.0096 (0.0024)	<0.001
DBP	0.0060 (0.0025)	0.015	0.0036 (0.0050)	0.472
LDL cholesterol	0.0036 (0.0023)	0.121	0.0009 (0.0051)	0.863
HDL cholesterol	0.0004 (0.0066)	0.947	-0.0264 (0.0131)	0.045
Triglycerides	-0.0062 (0.0045)	0.161	0.0337 (0.0296)	0.255
Smoking	0.0086 (0.0048)	0.050	0.0242 (0.0107)	0.024
rs1333049 GG/GC vs CC	-0.0009 (0.0048)	0.845	0.0107 (0.0112)	0.337

BMI indicates body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure. The beta values are based on the following: age, per year increase; BMI, per 1 kg/m² increase; for SBP and DBP, per 10 mm Hg increase; for LDL, HDL, and triglycerides, per 1 mmol/l increase. The beta for triglycerides is for log-transformed values. The beta coefficients for the comparison of GG/GC vs CC genotypes is shown. The results were similar when modelling GG vs GC vs CC or GG vs CC. The CAD risk associated allele is the C allele.

as well as the evidence for its correlation with atherosclerotic burden and future cardiovascular events.^{6,7,20,21} Furthermore, because genetic determinants could presumably be active from a young age, assessment of any association with CIMT in a young predisease cohort provides a useful means of investigating the early impact of such determinants on atherosclerosis. Therefore, we examined the association of the chromosome 9p21 locus in CIMT in 2 cohorts, including a cohort of young adult subjects as well as a cohort with an age-range similar to cohorts which demonstrated the association of the locus with CAD. Somewhat surprisingly, we found no evidence of an association of the locus with either mean or maximum CIMT in either age group.

A number of possible explanations for the lack of association of the 9p21 locus with CIMT need to be considered. It is unlikely that selection bias is a factor. Both cohorts were population based, ethnically homogeneous, and of Caucasoid origin where the association with 9p21 has been robustly demonstrated.¹⁻⁵ Similarly, it is unlikely that the lack of association reflects either imprecision of measurement of CIMT or adequate power to detect an effect. Prospective studies have shown that every 0.1-mm increase in CIMT is associated with a 20% to 30% higher risk of subsequent CAD.^{7,22,23} In a recent meta-analysis of the association between rs1333049 and coronary artery disease, each copy of the risk allele (C) was associated with a 24% (95% CI: 20% to 29%) increased risk of coronary artery disease.⁵ These estimates suggest that the expected effect of the rs1333049 on CIMT would be approximately 0.1 mm per allele if the association with CAD was mediated through a similar mechanism. Posthoc power calculations in our data showed that we had 80% power at an alpha of 0.05 to detect a 0.02-mm difference in mean CIMT between CC and GG subjects in the Young Finns Study and 0.06 mm in the Health 2000 cohort. Hence we had >99% power at an alpha of 0.01 to detect a 0.2 mm difference in CIMT between CC and GG subjects in

both cohorts. Furthermore, we easily detected several previously reported effects of other cardiovascular risk factors on CIMT in both cohorts. Therefore, a plausible, and perhaps mechanistically more interesting, explanation is that the chromosome 9p21 locus affects risk of CAD through mechanisms that are not manifested in the carotid wall and reflected by changes in CIMT.

Endothelial dysfunction is believed to be an early event in atherosclerosis and may predate the development of clinical disease by several decades.^{8,24,25} Reduction in FMD is a validated marker of endothelial dysfunction and predicts future cardiovascular events, at least in older adults.⁸ Several traditional risk factors for atherosclerosis such as hypercholesterolaemia, diabetes, and hypertension correlate with reductions in FMD.²⁵ Although the lack of a significant association between the 9p21 locus and FMD in the Young Finns study does not exclude the possibility that such an effect will be observed in older subjects, our finding again suggests that this genetic locus does not enhance risk of CAD by itself primarily causing endothelial dysfunction at a young age.

The recent finding that the 9p21 locus is also associated with the development of intracranial aneurysms¹⁹ suggests that the mechanism of its effect on the vascular wall is perhaps more complex than simply promoting the development of atherosclerosis. If the mechanism relates to cell growth and turnover as discussed earlier, it is possible that this affects coronary plaque stability or vulnerability rather than its development per se. Further studies are necessary to understand the mechanism(s) by which the chromosome 9p21 locus affects risk of CAD. In this regard, our finding of a lack of association of the locus with CIMT and with FMD at a young age provides valuable information in directing this search.

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Disclosures

None.

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