

Susceptibility locus for clinical and subclinical coronary artery disease at chromosome 9p21 in the multi-ethnic ADVANCE study

Themistocles L. Assimes^{1,*†}, Joshua W. Knowles^{1,†}, Analabha Basu⁴, Carlos Iribarren⁵, Audrey Southwick³, Hua Tang³, Devin Absher³, Jun Li⁶, Joan M. Fair¹, Geoffrey D. Rubin², Stephen Sidney⁵, Stephen P. Fortmann¹, Alan S. Go^{5,7,8}, Mark A. Hlatky¹, Richard M. Myers³, Neil Risch^{4,5,7} and Thomas Quertermous¹

¹Department of Medicine, ²Department of Radiology, and ³Department of Genetics, Stanford University School of Medicine, Stanford, CA 94305, USA, ⁴Institute for Human Genetics, University of California San Francisco, San Francisco, CA 94143, USA, ⁵Division of Research, Kaiser Permanente of Northern California, Oakland, CA 94612, USA, ⁶Department of Human Genetics, University of Michigan, Ann Arbor, MI 48109, USA and ⁷Department of Epidemiology and Biostatistics and ⁸Department of Medicine, University of California, San Francisco, CA 94107, USA

Received November 20, 2008; Revised and Accepted April 18, 2008

A susceptibility locus for coronary artery disease (CAD) at chromosome 9p21 has recently been reported, which may influence the age of onset of CAD. We sought to replicate these findings among white subjects and to examine whether these results are consistent with other racial/ethnic groups by genotyping three single nucleotide polymorphisms (SNPs) in the risk interval in the Atherosclerotic Disease, Vascular Function, and Genetic Epidemiology (ADVANCE) study. One or more of these SNPs was associated with clinical CAD in whites, U.S. Hispanics and U.S. East Asians. None of the SNPs were associated with CAD in African Americans although the power to detect an odds ratio (OR) in this group equivalent to that seen in whites was only 24–30%. ORs were higher in Hispanics and East Asians and lower in African Americans, but in all groups the 95% confidence intervals overlapped with ORs observed in whites. High-risk alleles were also associated with increased coronary artery calcification in controls and the magnitude of these associations by racial/ethnic group closely mirrored the magnitude observed for clinical CAD. Unexpectedly, we noted significant genotype frequency differences between male and female cases ($P = 0.003$ – 0.05). Consequently, men tended towards a recessive and women tended towards a dominant mode of inheritance. Finally, an effect of genotype on the age of onset of CAD was detected but only in men carrying two versus one or no copy of the high-risk allele and presenting with CAD at age >50 years. Further investigations in other populations are needed to confirm or refute our findings.

INTRODUCTION

Four separate genome-wide association (GWA) studies have recently identified a susceptibility locus for coronary artery disease (CAD) at chromosome 9p21 in subjects of white/European ancestry (1–4). The risk interval has been narrowed down to a block approximately 58 kb wide,

which does not appear to contain any annotated genes (4). In one study, single nucleotide polymorphisms (SNPs) in this interval were also found to be associated with subclinical CAD as estimated by coronary artery calcification (CAC) (4).

The potential for association of these SNPs with clinical and subclinical CAD has not been adequately explored in other

*To whom correspondence should be addressed at: Falk Cardiovascular Research Building, Stanford, CA 94305-5406, USA. Tel: +1 6504984154; Fax: +1 6507251599; Email: tassimes@cvmed.stanford.edu

†The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors.

racial/ethnic groups. In African Americans, the only other racial/ethnic group that has been studied for both outcomes, two SNPs in the risk interval (rs10757274 and rs2383206) were not associated with either outcome (4). However, the statistical power to detect an association in blacks was limited because of smaller sample sizes and appreciably lower frequencies of the risk alleles compared with whites.

Only one study to date has explored the effects of SNPs in the risk interval on the age of onset of CAD (3). In that study, the high-risk allele of one SNP (rs10757278) conferred a significantly higher genotype-specific OR in the subset of subjects with early onset myocardial infarction (MI) compared with all cases combined. Furthermore, a linear regression analysis within cases revealed that each copy of the high-risk allele reduced the age of onset of acute myocardial infarction (AMI) by approximately 1 year.

Within the multi-ethnic Atherosclerotic Disease, Vascular Function, and Genetic Epidemiology (ADVANCE) study, we sought to replicate associations between three SNPs (rs10757274, rs2383206 and rs10757278) in the risk interval with clinical and subclinical CAD among whites and African Americans and to explore whether these associations extend to other racial/ethnic groups, specifically Hispanics and East Asians. The ADVANCE study also provided the opportunity to examine sex differences and to further examine the effect of genotype on the age of onset of clinical CAD, because subjects with a broad range of age of first presentation of CAD were included in the study.

RESULTS

Study cohort

The overall study population included 451 cases with early onset CAD, 716 young controls, 1337 cases with later onset CAD and 992 older controls (Table 1). As expected, traditional risk factors were more common in cases compared with controls and also more prominent as risk factors for the early onset group. The majority of study cases had white/European ancestry ($n = 1201$, 66%), whereas the remainder were of African Americans ($n = 96$, 5.3%), Hispanics ($n = 108$, 6.0%), East Asians ($n = 117$, 6.5%) or mixed ($n = 287$, 15.9%) ancestry.

Association of SNPs with clinical CAD

All SNPs were found to be in Hardy-Weinberg equilibrium (HWE) in all racial/ethnic groups and in all case/control strata ($P > 0.05$, details not shown).

The three SNPs were correlated to each other in all race/ethnic groups studied with pair wise correlations being highest in whites ($r^2 = 0.92$ – 0.95) and East Asians ($r^2 = 0.93$ – 0.94), followed by admixed non-blacks ($r^2 = 0.88$ – 0.93), Hispanics ($r^2 = 0.87$ – 0.93), admixed blacks ($r^2 = 0.72$ – 0.91) and black/African Americans ($r^2 = 0.61$ – 0.85) (Supplementary Material, Table S1). The SNPs were all in the same haplotype block in the white, Chinese and Japanese HapMap samples, but not in the Yoruban samples (Supplementary Material, Fig. S1).

The high-risk allele ('G' for all three SNPs) was strongly associated with CAD in whites ($n = 1196$ – 1200 cases, 1031–1036 controls, Tables 2 and 3). The strongest association was found with rs10757278, which was present at an allele frequency of 54% in cases versus 47% in control subjects (age- and gender-adjusted OR per 'G' allele: 1.28, $P = 7 \times 10^{-5}$). None of the SNPs were associated with CAD in African American subjects (strongest association rs1075278 with OR: 1.17, $P = 0.46$), but the power to detect an OR of 1.25 for these SNPs was only 24–30% given the small sample size ($n = 93$ – 96 cases, 324–325 controls) and the lower frequencies of the high-risk allele (22.4–45.7% in controls). In Hispanics ($n = 80$ – 82 cases, 107–108 controls), all three SNPs were statistically significantly associated with disease. The strongest association was with rs2383206, in which the risk allele was present in 63% of cases but only 51% in controls, yielding an OR of 2.12 ($P = 0.002$). In East Asians ($n = 116$ cases, 103 controls), the ORs for the three SNPs varied from 1.42 to 1.55 with the strongest signal associated with rs2383206 ($P = 0.04$). Compared with whites, the point estimate of the OR in both Hispanics and East Asians was higher but there was still considerable overlap of the 95% confidence intervals (95% CI).

For the total (younger + older) control groups, the risk allele frequency in whites, Hispanics and East Asians were virtually identical for all three SNPs. Specifically, for rs10757274, the frequencies are 0.485, 0.488 and 0.495 in whites, Hispanics and East Asians, respectively. The corresponding frequencies for rs2383206 and rs10757278 are 0.507, 0.506, 0.490 and 0.473, 0.482, 0.490, respectively. Therefore, for the subject group of mixed ancestry without African ancestry (composed primarily of European, Native American and Asian ancestry), there is no stratification bias in a case–control comparison and these groups can be compared without adjustment. For this group ($n = 212$ – 215 cases, 109–110 controls), we also found significant associations for rs10757274 and rs10757278, and suggestive evidence for rs2383206 (Table 3), providing an additional support for the positive association of these SNPs with CAD in these racial/ethnic groups. In contrast, in the admixed group with black/African ancestry ($n = 71$ – 72 cases, 60 controls), there was no significant evidence of association, similar to what we observed for the African Americans. In this group, African was the predominant ancestry for the majority of subjects.

Adjustment for other traditional risk factors (smoking, hypertension, high cholesterol, diabetes and body mass index) had no appreciable effects on the ORs in any of the racial/ethnic groups (details not shown). Furthermore, the ORs were virtually unchanged when cases were restricted to patients presenting with AMI (Supplementary Material, Table S2). For instance, in whites, the ORs for presentation with clinically significant CAD ranged from 1.27 to 1.28 whereas the OR for presentation with AMI ranged from 1.28 to 1.30.

In African Americans ($n = 93$ – 96 cases, 324–325 controls), a haplotype association (case–control) test was not significant (P -value over all haplotypes was 0.59, and for the high-risk GGG haplotype was 0.25) although the frequency of the GGG haplotype was slightly higher in cases (24.3%) compared with controls (21.4%). Among African American

Table 1. Non-genetic characteristics of ADVANCE study participants according to case/control status

	Young cases (<i>n</i> = 472)	Young controls (<i>n</i> = 742)	Older cases (<i>n</i> = 1337)	Older controls (<i>n</i> = 992)
Age (years) ^{a,c,e}	45.3 ± 6.5	44.3 ± 5.5	62 ± 8.4	65.8 ± 2.9
Time from first ever event to study visit (months) ^{b,d}	21.3 (2.7–222.1)	–	3.5 (1.4–26.4)	–
Cardiovascular risk factors				
Body mass index ^b	31.1 (17.3–61.2)	26.8 (15.8–66.2)	28.4 (16.9–66.1)	27.5 (17.3–52.9)
Current/former smoker	276 (58.5)	266 (35.8)	842 (63)	569 (57.4)
Hypertension	154 (32.6)	143 (19.3)	641 (47.9)	407 (41)
Diabetes mellitus	101 (21.4)	51 (6.9)	274 (20.5)	147 (14.8)
High cholesterol	129 (27.3)	148 (19.9)	604 (45.2)	356 (35.9)
Ancestry and sex				
White/Europeans				
Males	103 (21.8)	156 (21.0)	705 (52.7)	424 (42.7)
Females	151 (32.0)	213 (28.7)	242 (18.1)	253 (25.5)
Black/African Americans				
Males	11 (2.3)	125 (16.9)	34 (2.5)	55 (5.5)
Females	35 (7.4)	129 (17.4)	16 (1.2)	24 (2.4)
Male Hispanics				
Males	13 (2.8)	12 (1.6)	65 (4.9)	36 (3.6)
Females	12 (2.5)	10 (1.4)	18 (1.4)	24 (2.4)
Male East Asians				
Males	28 (5.9)	23 (3.1)	57 (4.3)	42 (4.2)
Females	16 (3.4)	12 (1.6)	16 (1.2)	26 (2.6)
Male admixed non-blacks				
Males	22 (4.7)	12 (1.6)	86 (6.4)	39 (3.9)
Females	60 (12.7)	24 (3.2)	47 (3.5)	35 (3.5)
Male admixed blacks				
Males	7 (1.5)	0 (0)	28 (2.1)	22 (2.2)
Females	14 (3.0)	26 (3.5)	23 (1.7)	12 (1.2)

^aMean and standard deviation are presented.^bMedian and range are presented, for all other variables counts and percentages are presented.^cThe age cutoff for a 'young case' was not the same for men (≤45 years) and women (≤55 years).^dYoung cases may have had their qualifying coronary event as early as 1 January 1999, whereas all older cases had their qualifying coronary event after the start of recruitment in October 2001.^eAt event for cases, at study visit date for controls.

carriers of either the AAA and/or the GGG haplotype, a haplotype trend regression demonstrated that each additional copy of the GGG haplotype increased risk of CAD by 8%, but this result was not significant ($P = 0.74$). Haplotype association tests for all other race/ethnicity groups were no more revealing than the individual SNP association tests (details not shown).

Genotype-specific ORs adjusted for gender were generally consistent with an additive model of inheritance suggested in prior studies (Table 3). However, when we calculated genotypic ORs stratified by sex, we noted a trend towards higher ORs in women, especially in the heterozygotes (Supplementary Material, Table S3). Furthermore, the ORs in women tended more towards a dominant mode of inheritance, whereas in men the mode of inheritance appeared closer to recessive. We compared genotype counts for the three SNPs between the white, Hispanic and East Asian males combined ($n = 967$ – 970) and the white, Hispanic and East Asian females combined ($n = 452$ – 454), and found significant differences for rs2383206 ($\chi^2 = 8.92$, $P = .011$), rs10757278 ($\chi^2 = 11.44$, $P = .003$) and rs10757274 ($\chi^2 = 6.07$, $P = .048$). The differences were primarily a result of a higher frequency of the GG genotype and lower frequency of the AG

genotype in men compared with women for all three SNPs (Supplementary Material, Table S3).

Association of SNPs with CAC

The risk allele for all three SNPs was strongly associated with higher CAC scores in whites. The minimally adjusted OR based on ordinal logistic regression ranged from 1.37 to 1.40, $P = 1.8 \times 10^{-5}$ – 7.0×10^{-4} (Table 4). As was also observed for clinical CAD, the magnitude of this relationship was even stronger in Hispanic (OR, 1.61–1.94; $P = 0.10$ – 0.20 ; $n = 58$ – 60) and East Asian subjects (OR, 1.58–2.21; $P = 0.02$ – 0.17 ; $n = 67$), but once again there was considerable overlap of the confidence intervals between these race/ethnicity groups as a result of small numbers of non-white subjects (details not shown). Conversely, ORs were near one and there was no significant association of these SNPs with CAC scores in African-American control subjects (OR: 0.85–1.18, $P > 0.32$, $n = 259$), similar to our observations for CAD in this group. We also analyzed the two admixed control groups, as described in the 'Association of SNPs with clinical CAD' section. Again, as identically seen for clinical CAD, we observed a positive association between these SNPs and CAC score in the admixed controls without African ancestry (OR,

Table 2. Genotype counts in the ADVANCE study for three SNPs in the risk interval at the chromosome 9p21 CAD susceptibility locus, stratified by race/ethnicity, case/control status and source of cases and controls (young onset versus older onset)

	Genotype count (%)			Cases	AG	GG
	Controls	AA	AG	AA	AG	GG
Young						
White						
rs10757274	84 (23.4)	203 (56.5)	72 (20.1)	51 (20.2)	129 (51)	73 (28.9)
rs2383206	78 (21.7)	201 (56.0)	80 (22.3)	48 (19.0)	126 (50.0)	78 (31.0)
rs10757278	84 (23.3)	208 (57.6)	69 (19.1)	54 (21.3)	129 (50.8)	71 (28.0)
Black						
rs10757274	141 (57.6)	87 (35.5)	17 (6.9)	25 (55.6)	15 (33.3)	5 (11.1)
rs2383206	73 (29.8)	120 (49)	52 (21.2)	14 (30.4)	21 (45.7)	11 (23.9)
rs10757278	149 (60.6)	84 (34.1)	13 (5.3)	25 (54.3)	17 (37.0)	4 (8.7)
Hispanic						
rs10757274	4 (18.2)	10 (45.5)	8 (36.4)	3 (12.0)	12 (48.0)	10 (40.0)
rs2383206	5 (22.7)	9 (40.9)	8 (36.4)	2 (8.0)	11 (44.0)	12 (48.0)
rs10757278	6 (27.3)	9 (40.9)	7 (31.8)	2 (8.0)	13 (52.0)	10 (40.0)
East Asian						
rs10757274	7 (20.0)	20 (57.1)	8 (22.9)	8 (18.6)	17 (39.5)	18 (41.9)
rs2383206	8 (22.9)	19 (54.3)	8 (22.9)	7 (16.3)	19 (44.2)	17 (39.5)
rs10757278	8 (22.9)	19 (54.3)	8 (22.9)	8 (18.6)	17 (39.5)	18 (41.9)
Admixed non-black						
rs10757274	15 (41.7)	12 (33.3)	9 (25)	15 (18.3)	39 (47.6)	28 (34.1)
rs2383206	9 (25.0)	18 (50)	9 (25)	13 (16.0)	39 (48.1)	29 (35.8)
rs10757278	15 (41.7)	14 (38.9)	7 (19.4)	17 (21.0)	39 (48.1)	25 (30.9)
Admixed black						
rs10757274	10 (38.5)	11 (42.3)	5 (19.2)	12 (57.1)	7 (33.3)	2 (9.5)
rs2383206	6 (23.1)	13 (50.0)	7 (26.9)	7 (33.3)	11 (52.4)	3 (14.3)
rs10757278	11 (42.3)	12 (46.2)	3 (11.5)	11 (52.4)	9 (42.9)	1 (4.8)
Older						
White						
rs10757274	183 (27.1)	329 (48.7)	163 (24.1)	193 (20.5)	448 (47.5)	302 (32.0)
rs2383206	167 (24.7)	330 (48.8)	179 (26.5)	170 (18.0)	449 (47.6)	324 (34.4)
rs10757278	194 (28.7)	328 (48.6)	153 (22.7)	203 (21.5)	458 (48.4)	285 (30.1)
Black						
rs10757274	46 (58.2)	29 (36.7)	4 (5.1)	25 (52.1)	21 (43.8)	2 (4.2)
rs2383206	29 (36.7)	39 (49.4)	11 (13.9)	16 (32.7)	24 (49.0)	9 (18.4)
rs10757278	49 (62.0)	28 (35.4)	2 (2.5)	27 (54.0)	20 (40.0)	3 (6.0)
Hispanic						
rs10757274	16 (27.6)	32 (55.2)	10 (17.2)	11 (13.4)	47 (57.3)	24 (29.3)
rs2383206	15 (25.0)	32 (53.3)	13 (21.7)	8 (9.6)	48 (57.8)	27 (32.5)
rs10757278	17 (28.3)	30 (50.0)	13 (21.7)	12 (14.5)	47 (56.6)	24 (28.9)
East Asian						
rs10757274	19 (27.9)	32 (47.1)	17 (25.0)	8 (11.0)	40 (54.8)	25 (34.2)
rs2383206	18 (26.5)	34 (50.0)	16 (23.5)	9 (12.3)	39 (53.4)	25 (34.2)
rs10757278	18 (26.5)	34 (50.0)	16 (23.5)	9 (12.3)	43 (58.9)	21 (28.8)
Admixed non-black						
rs10757274	19 (26.0)	36 (49.3)	18 (24.7)	31 (23.3)	75 (56.4)	27 (20.3)
rs2383206	15 (20.3)	37 (50)	22 (29.7)	27 (20.5)	72 (54.5)	33 (25.0)
rs10757278	21 (28.4)	34 (45.9)	19 (25.7)	33 (25.2)	74 (56.5)	24 (18.3)
Admixed black						
rs10757274	20 (58.8)	9 (26.5)	5 (14.7)	24 (47.1)	22 (43.1)	5 (9.8)
rs2383206	15 (44.1)	11 (32.4)	8 (23.5)	15 (30.0)	26 (52.0)	9 (18.0)
rs10757278	21 (61.8)	11 (32.4)	2 (5.9)	25 (49.0)	21 (41.2)	5 (9.8)

1.47–2.18; lowest $P = 0.03$; $n = 72$). In contrast, again as we observed previously for CAD, we saw no association in the admixed group with African ancestry (OR, 0.64–0.84; $P > 0.39$; $n = 34$).

Further adjustment for all traditional risk factors of CAD had no appreciable effect on OR in whites, African Americans, admixed non-blacks and admixed blacks, but the ORs in Hispanics decreased (adjusted OR range from 1.3 to 1.52) while ORs in East Asians increased (adjusted OR range from 1.86 to 2.63).

Association of SNPs with the age of onset of clinical CAD

We did not observe an appreciably higher OR for the risk alleles in the set of young onset cases (≤ 45 years for men and ≤ 55 years for women) and controls compared with the set of older onset cases and controls in whites, Hispanics and East Asians (Table 3). However, we noted a younger mean age of presentation of CAD in non-black subjects ($n = 1419$ – 1424) with two copies versus zero or one copy of any of the risk alleles. When this analysis was stratified

Table 3. Risk of developing clinical CAD in carriers of the high-risk alleles compared with non-carriers in the ADVANCE study, stratified by race/ethnicity

	Young OR ^a	CI	Older OR ^a	CI	Young and older combined OR ^{a,b}	CI	OR ^c AG versus AA	CI	OR ^c GG versus AA	CI
White										
rs10757274	1.29*	1.02–1.65	1.27**	1.10–1.47	1.28***	1.13–1.45	1.23	0.99–1.52	1.72***	1.35–2.20
rs2383206	1.26	0.99–1.61	1.27**	1.10–1.47	1.27***	1.12–1.44	1.25*	1.00–1.56	1.69***	1.32–2.16
rs10757278	1.26	0.99–1.61	1.29**	1.12–1.49	1.28***	1.13–1.45	1.24**	1.01–1.53	1.72***	1.35–2.20
Black										
rs10757274	1.04	0.62–1.70	0.94	0.48–1.83	1.00	0.67–1.49	1.10	0.66–1.83	1.22	0.48–3.11
rs2383206	0.96	0.60–1.51	1.17	0.66–2.05	1.03	0.72–1.47	0.98	0.57–1.69	1.17	0.59–2.32
rs10757278	1.13	0.67–1.88	1.22	0.62–2.41	1.17	0.77–1.75	1.21	0.73–2.00	1.78	0.66–4.81
Hispanic										
rs10757274	1.31	0.55–3.22	1.95*	1.10–3.60	1.73*	1.07–2.85	2.02	0.92–4.42	2.70*	1.09–6.66
rs2383206	1.84	0.78–4.62	2.27*	1.27–4.22	2.12*	1.31–3.53	2.88*	1.22–6.77	3.96*	1.49–10.5
rs10757278	1.86	0.80–4.65	2.02*	1.15–3.66	1.97*	1.23–3.22	2.58*	1.18–5.62	3.04*	1.22–7.53
East Asian										
rs10757274	1.49	0.79–2.88	1.50	0.89–2.57	1.50*	1.0–2.26	1.66	0.80–3.44	2.57*	1.12–5.86
rs2383206	1.59	0.84–3.13	1.52	0.90–2.61	1.55*	1.03–2.35	1.69	0.81–3.51	2.80*	1.23–6.41
rs10757278	1.55	0.83–2.99	1.34	0.78–2.30	1.42	0.95–2.16	1.62	0.79–3.32	2.21	0.97–5.07
Admixed non-black										
rs10757274	1.78*	1.05–3.10	0.97	0.61–1.55	1.27	0.89–1.8	1.78*	1.02–3.11	1.54 ^d	0.80–2.95
rs2383206	1.49	0.84–2.60	0.86	0.54–1.38	1.08	0.75–1.54	1.24	0.68–2.27	1.26	0.64–2.47
rs10757278	1.81*	1.05–3.20	0.94	0.59–1.50	1.23	0.87–1.76	1.74*	1.01–3.01	1.36	0.71–2.58
Admixed black ^c										
rs10757274	–	–	1.22	0.63–2.44	–	–	1.43 ^d	0.66–3.10	0.44	0.13–1.51
rs2383206	–	–	1.28	0.68–2.44	–	–	1.79	0.77–4.15	0.88	0.32–2.41
rs10757278	–	–	1.70	0.82–3.78	–	–	1.35	0.63–2.89	0.98	0.24–4.10

^aDerived by logistic regression assuming a log-additive mode of inheritance and adjusted for age and gender. For combined analyses, it is further adjusted for source of cases/controls (young versus older), source \times age, source \times gender.

^bCombined logistic regression OR presented only if no significant heterogeneity of the OR detected between the two sources of cases/controls ($P > 0.05$ for interaction SNP \times source of cases/controls).

^cDerived using Cochran–Mantel–Haenszel statistics and adjusted for source of cases/controls and sex.

^dP for Breslow day test of heterogeneity of the genotypic OR across the two sources of cases/controls < 0.05 .

^eThe logistic regression model did not converge in the young admixed blacks due to small numbers.

* $P \leq 0.05$, ** $P \leq 1.0 \times 10^{-3}$, *** $P \leq 1.0 \times 10^{-4}$.

by gender, it became apparent that this trend was largely driven by the men ($n = 967$ – 970 , Supplementary Material, Table S4). Overall, non-black men with two copies of any of the risk alleles presented with CAD about 1.5–1.8 years earlier than individuals with zero or one copy of the risk allele (Wilcoxon $P = 0.04$ – 0.08). This was not the case in women ($n = 452$ – 454). Figure 1 graphically illustrates these observations for rs10757278 in white, Hispanic and East Asian cases combined. It shows that SNP genotype is associated with age of onset of CAD in men presenting primarily over the age of 50 years, but is not associated with age of onset of CAD in women. Similar findings were observed for the other two SNPs (details not shown) with the P -value for differences in the age of onset distributions between genotypes for men and women being between 0.004 and 0.05 for the three SNPs using a Kolmogorov–Smirnov test.

DISCUSSION

With the tidal wave of genetic information that is emerging from GWA studies, it is crucial to replicate findings in multiple studies as well as extend the findings to multiple racial/ethnic groups (5). The results of our study confirm the previously reported associations between these SNPs and both

clinical and subclinical CAD in white/European subjects. In addition, we extend these findings, for the first time, to U.S. Hispanics and East Asians. On the other hand, these SNPs were not associated with CAD in African-American subjects. Our analyses of the two ‘admixed’ groups supported our conclusions in our four other racial/ethnic groups *in toto*. The group of admixed subjects without African ancestry showed a level of association comparable with the other non-African groups, whereas the admixed group with African ancestry showed no association.

Our findings of ethnic variation in risks, with higher ORs in Latinos and East Asians and lower ORs in African Americans when compared with whites, were consistent with our independent case–control analysis of CAD and CAC analysis among controls only. Furthermore, the lack of association in African Americans seen in our study is consistent with the observations of the single other study that examined this ethnic group (4). However, in all settings, the sample sizes for the non-white subjects in our study were not large, and hence additional independent investigation of these risks in other studies of these groups is required to definitively determine whether the risks are different or not. For example, two recent case–control association studies of clinical CAD in indigenous East Asians obtained genetic risks similar to

Table 4. Risk of higher coronary artery calcification scores in controls carrying the high-risk allele compared with non-carriers

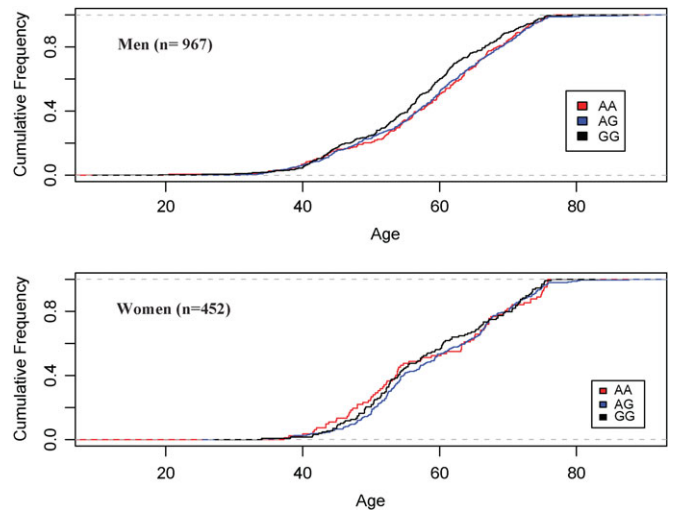
	CAC score (<i>n</i>)			OR ^a	<i>P</i> -value
	0	1–100	>100		
White					
rs10757274	334	267	277	1.40	0.0008
rs2383206	335	267	277	1.38	0.0013
rs10757278	335	267	277	1.37	0.0018
Black ^b					
rs10757274	192	34	33	0.85	0.59
rs2383206	193	34	33	0.78	0.32
rs10757278	193	34	33	1.18	0.61
Hispanic					
rs10757274	17	21	20	1.94	0.10
rs2383206	17	22	21	1.67	0.18
rs10757278	17	22	21	1.61	0.20
East Asian					
rs10757274	16	26	25	2.01	0.033
rs2383206	16	26	25	2.21	0.02
rs10757278	16	26	25	1.58	0.17
Admixed non-Black					
rs10757274	16	29	27	2.18	0.03
rs2383206	16	30	27	1.48	0.25
rs10757278	16	30	27	1.47	0.24
Admixed Black					
rs10757274	9	12	13	0.67	0.39
rs2383206	9	12	13	0.84	0.68
rs10757278	9	12	13	0.64	0.44

^aOR is derived by ordinal logistic regression assuming a log-additive mode of inheritance and adjusting for source of controls (CARDIA versus ADVANCE), age and gender.

^bBlacks have overall lower CAC scores because the majority came from the CARDIA study and were young.

those found in whites from other studies (6,7). Hence, the excess risks we observed for this group may also simply represent chance statistical fluctuation, or an effect of local environment. Differential risks, if confirmed, could be explained by (i) incomplete linkage disequilibrium (LD) among these and other SNPs on chromosome 9 in different ethnic groups and/or differential frequency of the actual (untested) causal SNP in this region; (ii) interactions with other unlinked genes whose frequencies differ across ethnic groups or (iii) interactions with varying environmental risk factors.

Examination of SNP associations in different racial/ethnic groups which have distinct patterns of LD can sometimes help resolve which SNP (or SNPs) is most likely to be causative. In this case, all three SNPs we studied were in very strong and similar LD in all the non-black populations, making discrimination of functionality among the three SNPs in these populations impossible. The LD was weaker in the African-American population (as is often the case), making such an analysis possible in this group. Although associations were overall non-significant for any of the SNPs for either CAD or CAC in African Americans, we did note that SNP rs10757278 showed the largest OR independently for both CAD and CAC. Hence, it may be more likely that this SNP is either a functional variant or in strongest LD with the functional variant in this region. Additional studies of African Americans would be quite valuable in helping to resolve this question.

**Figure 1.** Distribution of age of onset of clinical coronary artery disease by genotype for rs10757278 combining young and old white, Hispanic, and East Asian cases, stratified by sex.

We detected a strong association with subclinical CAD as estimated by CAC in all non-African race/ethnicity groups. This finding has not been previously reported. Furthermore, the results of the CAC analysis in the two admixed groups mirrored the conclusions from our CAD analysis. Because CAC is highly correlated with the volume of coronary atherosclerosis, is not influenced by plaque rupture and is one of the strongest predictors of all clinical complications of CAD (8), our results support the hypothesis, first put forth by McPherson *et al.* (4), that high-risk alleles in this region predispose individuals to forming atherosclerotic plaque rather than directly contributing to plaque instability and rupture. The fact that we observed a similar magnitude of association of the risk allele with CAD among cases presenting with AMI versus cases not presenting with AMI also supports this hypothesis although it is possible that many of the subjects not presenting with AMI may have suffered from subclinical ruptures prior to presentation. Once the causal SNP(s) are identified, mechanistic studies should help prove or disprove this hypothesis.

Helgadóttir *et al.* (3) reported an allelic OR of 1.49 (95% CI, 1.31–1.69) in early onset disease (men, <50 years; women, <60 years) compared with an OR of 1.26 (95% CI, 1.16–1.36) among all cases (men, <70 years; women, <75 years). In contrast, we did not detect a significant difference in the magnitude of the OR between our early onset cases and controls (men, ≤45 years; women, ≤55 years) and our older onset cases and controls. However, we did observe a significant difference in the age of presentation in white, Hispanic and East Asian men carrying two copies of the risk allele versus men carrying zero or one copy of the risk allele, but only after the age of about 50 years. Therefore, an attenuated age effect in our data may be because of a larger representation of very early onset subjects. These results suggest that these SNPs likely play a role in determining the age of onset of CAD, but not at very young ages.

We found a significant difference in genotype frequencies between male and female cases, with relatively more men

homozygous for the G allele and more women heterozygous for the G allele. Whether these differences exist in other cohorts should be explored before firm conclusions are made on the mode of inheritance for these SNPs in men compared with women.

In conclusion, we confirmed the association of SNPs at the 9p21 locus with *both* clinical and subclinical CAD in a population of white/European subjects. We also confirmed a lesser association in African Americans and extended these associations, for the first time, to U.S. Hispanics and East Asians in whom the relative risks appeared to be increased relative to whites, although confidence intervals were wide. Contrary to findings in other studies to date (3), in our study these SNPs did not appear to influence the age of onset of CAD at very young ages in men and at all ages in women. We noted significant genotype frequency differences between male and female cases. Consequently, men tended towards a recessive and women tended towards a dominant mode of inheritance. Further investigations in other populations are needed to confirm or refute our findings.

MATERIALS AND METHODS

Between October 28, 2001 and December 31, 2003, a total of 3179 subjects was recruited from Kaiser Permanente of Northern California, a large integrated healthcare delivery system in the San Francisco and greater Bay area, and enrolled into three case groups and two control groups. Detailed description of the eligibility criteria and the source population for all groups can be found elsewhere (9–11). Briefly, case groups included early onset CAD (AMI, angina pectoris with positive angiogram or revascularization procedure in men ≤ 45 or women ≤ 55 years old), incident stable exertional angina at an older age and incident AMI at an older age. Older controls included men and women aged 60–69 years at the time of identification in June 2001. The young control group included subjects between the ages of 30 and 45 for men or 30 and 55 for women as well as a subset of 479 participants from the Coronary Artery Risk Development in Young Adults (CARDIA) study (12) originally recruited at the Oakland field center and attending the study's Year 15 examination in 2000–2001. All controls were free of clinical CAD, cerebrovascular disease (CVD) and peripheral arterial disease (PAD) at the time of recruitment. Thus, the total study sample included 3658 subjects.

Through a phone interview, an extensive self-administered questionnaire, and/or the use of the Kaiser electronic clinical databases, we documented the presence or absence and age of onset of clinically significant CAD, CVD and PAD, as well as all traditional cardiovascular risk factors in all participants. For this study, selected risk factors (smoking, hypertension, high cholesterol and diabetes) were defined based on self-report. Among cases, these risk factors were considered to be present only if subjects reported an age of onset of the risk factor that was younger than the age of onset of clinically significant CAD. All subjects provided information on their own race/ethnicity while non-CARDIA participants also provided information on the race/ethnicity of their parents and grandparents as well as their place of birth. This information

was used to classify participants into one of the following racial/ethnic groups: white/European, black/African American, Hispanic, South Asian, East Asian, Pacific Islander, Native American or admixed among at least two of the above categories.

At the clinic visit, we measured the height and weight of all participants and collected whole blood for DNA extraction and quantification of various serum biomarkers. By design, CAC was measured among control participants only. In the CARDIA young controls, CAC was measured using electron-beam computed tomography (EBCT) (Imatron C-150 scanner, Imatron Inc., San Francisco, CA, USA) as part of the Year 15 (2000–2001) examination protocol (13). CAC was not determined in non-CARDIA young controls. In the older, ADVANCE control subjects, CAC was measured using 4- or 16-row multidetector computed tomography (Siemens Medical Solutions, Erlangen, Germany) and a protocol derived from the Multi-Ethnic Study of Atherosclerosis study (14). Details of the ADVANCE CAC protocol can be found elsewhere (15). CAC scores generated with these two methods are highly correlated and can be combined for analytic purposes (13–16).

We genotyped three SNPs (rs10757274, rs2383206 and rs10757278) in the risk interval on chromosome 9p21 using the TaqMan® assay in all participants. The Applied Biosystems pre designed assay IDs used to genotype these SNPs were C_26505812_10, C_1754669_10 and C_11841860_10.

We excluded from the analysis subjects who did not provide blood for DNA ($n = 40$) or who did not fill out the study questionnaire ($n = 9$). Because of small numbers of either cases or controls, we excluded South Asians ($n = 55$), Pacific Islanders ($n = 9$) and Native Americans ($n = 2$).

The ADVANCE Study was approved by the Institutional Review Board at Stanford and the Kaiser Foundation Research Institute. All subjects gave written informed consent.

Statistical analyses

We first downloaded the latest publicly available HapMap dataset of genotypes (build 36) and created LD plots using Haploview (17) for a 76 kb region on chromosome 9 encompassing the 58 kb high-risk interval and highlighting the location of the three SNPs we genotyped. LD plots were created separately for each of the four Hapmap populations (Europeans, Yorubans, Chinese and Japanese), and haplotype blocks were inferred using the method of Gabriel *et al.* (18).

We then calculated allele frequencies of all SNPs stratified by racial/ethnic group in the ADVANCE cases and controls separately. Initial analyses showed marked differences in the allele frequencies for all SNPs between black/African-American controls and non-black controls. Because such differences would increase the probability of confounding within our 'admixed Hispanic' and 'admixed non-Hispanic groups', we re-classified these two groups into two new groups ('admixed black' and 'admixed non-black') based on self-reported black/African-American ancestry before proceeding with our association analyses. We tested for HWE of the SNPs among controls in each racial/ethnic group with the permutation version of the exact test (19) and calculated pair wise

LD measures (D' and r^2) between the three SNPs among cases and controls combined.

Next, we calculated age- and gender-adjusted OR for the presence of clinical CAD using multivariate unconditional logistic regression stratified by racial/ethnic group. ORs were first calculated for the comparison of the young onset cases to young controls, the older onset cases to the older controls separately and then across both sets of cases and controls if no heterogeneity between the two ORs was detected. For these regression analyses, we present only an additive model of inheritance (on the logit scale) because findings to date are most consistent with this model (2–4,20) but we also calculated genotypic odds ratios (ORs) for individuals with one (or two) copies of the risk alleles versus those with no copies of the risk alleles across both sets of cases and controls using Cochran–Mantel–Haenszel statistics.

We used the online genetic power calculator (21) to calculate, *post hoc*, the power to detect an OR of 1.25 in a specific race/ethnic group if an association was not observed with any of the SNPs. These calculations assumed a log additive model of inheritance, a population prevalence of symptomatic CAD of 6.2% (22) and a Type 1 error of 0.05.

We used multivariate ordinal logistic regression to model the association between SNPs and CAC scores among controls stratified by racial/ethnic group. The CAC outcome was expressed as ordinal categories of Agatston scores of 0, 1–100 and >100.

We explored the influence of these SNPs on the age of onset of CAD by three means. First, we compared the OR derived for the younger onset cases and controls to the older onset cases and controls. Secondly, we determined the mean age of onset of disease by genotype within cases and tested for differences between groups with a Kruskal–Wallis test. Finally, we plotted the age of onset distribution by genotype.

Logistic regression analyses for clinical CAD were repeated after removing cases whose qualifying event was not their first manifestation of CAD (by history) or was not an AMI. Some analyses were also repeated stratified by gender to explore differences between men and women. The effect of further adjusting ORs for all other traditional risk factors was also explored. Finally, haplotypes were inferred (23) and association tests were performed (24–26) for clinical CAD stratified by race/ethnicity group.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

ACKNOWLEDGEMENTS

The ADVANCE investigators wish to thank the study participants and the staff who contributed to the ADVANCE study. We especially acknowledge Shannon Brady and Amita Aggarwal for genotyping assistance.

Conflict of Interest statement. None declared.

FUNDING

ADVANCE study: Donald W. Reynolds Foundation, Las Vegas, NV, USA; National Institutes of Health (RO1 HL087647-01 from the National Heart, Lung, and Blood Institute). CARDIA study: National Institutes of Health (N01-HC-48047, N01-HC-48048, N01-HC-48049, N01-HC-48050 and N01-HC-95095 from the National Heart, Lung, and Blood Institute).

REFERENCES

- Samani, N.J., Erdmann, J., Hall, A.S., Hengstenberg, C., Mangino, M., Mayer, B., Dixon, R.J., Meitinger, T., Braund, P., Wichmann, H.E. *et al.* (2007) Genome-wide association analysis of coronary artery disease. *N. Engl. J. Med.*, **357**, 443–453.
- Wellcome Trust Case Control Consortium. (2007) Genome-wide association study of 14,000 cases of seven common diseases and 3,000 shared controls. *Nature*, **447**, 661–678.
- Helgadottir, A., Thorleifsson, G., Manolescu, A., Gretarsdottir, S., Blondal, T., Jonasdottir, A., Jonasdottir, A., Sigurdsson, A., Baker, A., Palsson, A. *et al.* (2007) A Common Variant on Chromosome 9p21 Affects the Risk of Myocardial Infarction. *Science*, **316**, 1491–1493.
- McPherson, R., Pertsemliadis, A., Kavaslar, N., Stewart, A., Roberts, R., Cox, D.R., Hinds, D.A., Pennacchio, L.A., Tybjaerg-Hansen, A., Folsom, A.R. *et al.* (2007) A common allele on chromosome 9 associated with coronary heart disease. *Science*, **316**, 1488–1491.
- Chanock, S.J., Manolio, T., Boehnke, M., Boerwinkle, E., Hunter, D.J., Thomas, G., Hirschhorn, J.N., Abecasis, G., Altshuler, D., Bailey-Wilson, J.E. *et al.* (2007) Replicating genotype–phenotype associations. *Nature*, **447**, 655–660.
- Hinohara, K., Nakajima, T., Takahashi, M., Hohda, S., Sasaoka, T., Nakahara, K.I., Chida, K., Sawabe, M., Arimura, T., Sato, A. *et al.* (2008) Replication of the association between a chromosome 9p21 polymorphism and coronary artery disease in Japanese and Korean populations. *J. Hum. Genet.*, **53**, 357–359.
- Shen, G.Q., Li, L., Rao, S., Abdullah, K.G., Ban, J.M., Lee, B.S., Park, J.E. and Wang, Q.K. (2007) Four SNPs on chromosome 9p21 in a South Korean population implicate a genetic locus that confers high cross-race risk for development of coronary artery disease. *Arterioscler. Thromb. Vasc. Biol.*, **28**, 360–365.
- Ardehali, R., Nasir, K., Kolandaivelu, A., Budoff, M.J. and Blumenthal, R.S. (2007) Screening patients for subclinical atherosclerosis with non-contrast cardiac CT. *Atherosclerosis*, **192**, 235–242.
- Go, A.S., Iribarren, C., Chandra, M., Lathon, P.V., Fortmann, S.P., Quertermous, T. and Hlatky, M.A. (2006) Statin and beta-blocker therapy and the initial presentation of coronary heart disease. *Ann. Intern. Med.*, **144**, 229–238.
- Taylor-Piliae, R.E., Norton, L.C., Haskell, W.L., Mahbouda, M.H., Fair, J.M., Iribarren, C., Hlatky, M.A., Go, A.S. and Fortmann, S.P. (2006) Validation of a new brief physical activity survey among men and women aged 60–69 years. *Am. J. Epidemiol.*, **164**, 598–606.
- Iribarren, C., Go, A.S., Husson, G., Sidney, S., Fair, J.M., Quertermous, T., Hlatky, M.A. and Fortmann, S.P. (2006) Metabolic syndrome and early-onset coronary artery disease: is the whole greater than its parts? *J. Am. Coll. Cardiol.*, **48**, 1800–1807.
- Hughes, G.H., Cutter, G., Donahue, R., Friedman, G.D., Hulley, S., Hunkeler, E., Jacobs, D.R., Jr, Liu, K., Orden, S., Pirie, P. *et al.* (1987) Recruitment in the coronary artery disease risk development in young adults (CARDIA) study. *Control. Clin. Trials*, **8**, 68S–73S.
- Carr, J.J., Nelson, J.C., Wong, N.D., McNitt-Gray, M., Arad, Y., Jacobs, D.R. Jr, Sidney, S., Bild, D.E., Williams, O.D. and Detrano, R.C. (2005) Calcified coronary artery plaque measurement with cardiac CT in population-based studies: standardized protocol of Multi-Ethnic Study of Atherosclerosis (MESA) and Coronary Artery Risk Development in Young Adults (CARDIA) study. *Radiology*, **234**, 35–43.
- Detrano, R.C., Anderson, M., Nelson, J., Wong, N.D., Carr, J.J., McNitt-Gray, M. and Bild, D.E. (2005) Coronary calcium measurements: effect of CT scanner type and calcium measure on rescan reproducibility—MESA study. *Radiology*, **236**, 477–484.

15. Fair, J.M., Kiazand, A., Varady, A., Mahboub, M., Norton, L., Rubin, G.D., Iribarren, C., Go, A.S., Hlatky, M.A. and Fortmann, S.P. (2007) Ethnic differences in coronary artery calcium in a healthy cohort aged 60 to 69 years. *Am. J. Cardiol.*, **100**, 981–985.
16. Stanford, W., Thompson, B.H., Burns, T.L., Heery, S.D. and Burr, M.C. (2004) Coronary artery calcium quantification at multi-detector row helical CT versus electron-beam CT. *Radiology*, **230**, 397–402.
17. Barrett, J.C., Fry, B., Maller, J. and Daly, M.J. (2005) Haploview: analysis and visualization of LD and haplotype maps. *Bioinformatics*, **21**, 263–265.
18. Gabriel, S.B., Schaffner, S.F., Nguyen, H., Moore, J.M., Roy, J., Blumenstiel, B., Higgins, J., DeFelice, M., Lochner, A., Faggart, M. *et al.* (2002) The structure of haplotype blocks in the human genome. *Science*, **296**, 2225–2229.
19. Guo, S.W. and Thompson, E.A. (1992) Performing the exact test of Hardy–Weinberg proportion for multiple alleles. *Biometrics*, **48**, 361–372.
20. Samani, N.J., Erdmann, J., Hall, A.S., Hengstenberg, C., Mangino, M., Mayer, B., Dixon, R.J., Meitinger, T., Braund, P., Wichmann, H.E. *et al.* (2007) Genomewide association analysis of coronary artery disease. *N. Engl. J. Med.*, **357**, 443–453.
21. Purcell, S., Cherny, S.S. and Sham, P.C. (2003) Genetic Power Calculator: design of linkage and association genetic mapping studies of complex traits. *Bioinformatics*, **19**, 149–150.
22. Holtby, S., Zahnd, E., Yen, W., Lordi, N., McCain, C. and SDiSogra, C. (2004) Health of California's adults, adolescents and children: findings from CHIS 2001. UCLA Center for Health Policy Research, Los Angeles.
23. Excoffier, L. and Slatkin, M. (1995) Maximum-likelihood estimation of molecular haplotype frequencies in a diploid population. *Mol. Biol. Evol.*, **12**, 921–927.
24. Zhao, J.H., Curtis, D. and Sham, P.C. (2000) Model-free analysis and permutation tests for allelic associations. *Hum. Hered.*, **50**, 133–139.
25. Zaykin, D.V., Westfall, P.H., Young, S.S., Karnoub, M.A., Wagner, M.J. and Ehm, M.G. (2002) Testing association of statistically inferred haplotypes with discrete and continuous traits in samples of unrelated individuals. *Hum. Hered.*, **53**, 79–91.
26. Schaid, D.J., Rowland, C.M., Tines, D.E., Jacobson, R.M. and Poland, G.A. (2002) Score tests for association between traits and haplotypes when linkage phase is ambiguous. *Am. J. Hum. Genet.*, **70**, 425–434.