RESEARCH NOTE

A common variant in chromosome 9p21 associated with coronary artery disease in Asian Indians

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Introduction

Coronary artery disease (CAD) is a complex disorder with a broad pathological spectrum (Topol *et al*. 2006). Although large-scale studies have implicated multiple factors as contributing to the inherited risk of CAD, there is inadequate knowledge on the exact identity of the candidate genes and the quantum of their effect on the disease etiopathology in the predisposed, yet previously untested, populations. Recent studies have reported the association of common variants in the 9p21 genomic region with CAD in Caucasian populations (Helgadottir *et al*. 2007; Wellcome Trust Case Control Consortium 2007; Schunkert *et al*. 2008; Ye *et al*. 2008). To date, the Korean, Japanese and Chinese are the only Asian populations to have been tested for these variants (Hinohara *et al*. 2008; Shen *et al*. 2008; Zhou *et al*. 2008). Beside CAD, this region is also implicated in type 2 diabetes, abdominal aortic aneurysm and ischemic stroke (Saxena *et al*. 2007; Helgadottir *et al*. 2008; Matarin *et al*. 2008).

Asian Indians have a high susceptibility to CAD and its associated risk factors like type 2 diabetes and hypertension (Arvind *et al*. 2002; Shanker *et al*. 2007). Further, Asian Indians have higher plasma levels of proinflammatory markers compared to their European counterparts (Chambers *et al*. 2001). Recent evidence suggests that differences exist between the patterns of CAD in men of Indian and European origin (Tillin *et al*. 2007). Although there have been some reports showing association of genetic polymorphisms with heart disease in Asian Indians, so far no study has been conducted on the 9p21 common variants in CAD patients belonging to this population (Kooner *et al*. 2008; Maitra *et al*. 2008; Rai *et al*. 2008; Shanker *et al*. 2008). In view of these facts, genotyping of common variants located in this novel candidate region in the Asian Indian population is of significance.

The present pilot study was conducted to investigate the association of a 9p21 variant, rs10757278 (*A*/*G*), in a limited number of samples from Asian Indians affected with CAD. This genomic variant has been previously implicated in heart disease in other populations (Helgadottir *et al*. 2007; Abdullah *et al*. 2008; Shen *et al*. 2008). Genotyping was performed by a novel method and confirmed in selected samples by sequencing. The risk allele (*G*) was found to be associated with CAD in men, even after correction for age, diabetes and hypertension. The association can be explained by a recessive model of inheritance of the protective allele (*A*). Based on these preliminary findings, high throughput investigations on the involvement of multiple variants are being initiated from this region.

Materials and methods

Study population and samples

All participants included in this pilot study were recruited from Bangalore and Mumbai, in the ongoing Indian Atherosclerosis Research Study (IARS) (Maitra *et al*. 2008). All study participants were residing in the Indian subcontinent for a minimum of three generations. In addition, the specific inclusion criteria for the patients were a history of CAD, which included stable and unstable angina and myocardial infarction diagnosed by echocardiogram (ECG) and treated based on the catheter lab availability with standard

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medication or coronary angiography followed by percutaneous transluminal coronary angioplasty or coronary artery bypass graft, age at onset of 50 years or less, and the presence of family history of CAD. Out of 154 unrelated CAD patients, 57 participants suffered from chronic ischemia with angina, 45 participants had MI and 52 participants suffered from recurrent coronary disease event. Disease severity was defined by the number of diseased vessels based on the coronary angiogram report. Similarly, 160 unrelated volunteers from the population, matched by the geographic region of habitatation, who had no apparent clinical symptom or history of CAD or family history of heart disease, and who were also free from any concomitant infection or major illness at the time of enrollment, were selected as controls. The prevalence of diabetes and hypertension was ascertained based on the diagnosis of a clinician and relevant medical records of medication and laboratory tests. All participants with past or present history of smoking were considered as smokers. The recruitment of participants and collection of samples and relevant information were performed by the clinical research team of Thrombosis Research Institute (TRI), India, under the supervision of a clinician. Blood samples were collected by informed voluntary consent, as per the IARS protocol approved by the institutional ethics committee of TRI and the Indian Council of Medical Research (ICMR) guidelines, as per the declaration of Helsinki. Plasma and genomic DNA were isolated from the whole blood samples using a procedure previously described (Miller *et al*. 1988). The present study had a power of over 80% to detect a 1.4-fold increase of allele frequencies, assuming a 40% prevalence of the rare allele in the control group and a type I error probability of 0.05.

Lipid assays

Plasma total cholesterol (TC) and triglyceride (TG) were assayed by reagents and standards from the Randox laboratories (Crumlin, UK) while high-density lipoprotein-cholesterol (HDL-c) was assayed by reagents from the Bayer diagnostics (Berkshire, UK), controls from the Randox (UK) and standards from the Dade Behring (Milton Keyneo, UK). The low-density lipoprotein-cholesterol (LDL-c) was assayed by applying the Friedwald's formula for samples with TG value less than 400 mg/dl (Friedewald *et al*. 1972). The interassay coefficients of variation (CV) for the commercial controls and normal serum pool ranged from 4.9% to 7.0% for TC, 6.1% to 7.7% for TG and 7.1% to 12.2% for HDL-c.

SNP genotyping

Since information on the genomic variation is not available on the Asian Indians, the linkage disequilibrium (LD) data of the 9p21 SNPs rs10757278, rs1333049, rs10757274 and rs2383206 for the Asian populations (CHB and JPT) were reviewed from the HapMap database (International HapMap Consortium 2005). The variant rs10757278 was selected for genotyping since it is in high LD with the other SNPs in this region (pair-wise $r^2 > 0.8$). A rapid and cost effective method was developed in-house to genotype rs10757278 based on PCR using modified allele specific primers using an ABI 9700 PCR instrument and subsequent SYBR Green I dye based dissociation curve analysis using an ABI 7500 Real Time PCR system (Applied Biosystems, Foster City, USA) (Papp *et al*. 2003). Briefly, about 20 ng of genomic DNA was used for PCR using the olignucleotide primers 281F-A (5′-CGC GGC CGG CCA GGG TGT GGT CAT TCC GGA AA), 281F-G (5′-AGG GTG TGG TCA TTC CGG CAG) and 335R (5′- GAG AAA CTA CTC TGT CTT GAT TCT GCA T) and SYBR Green I PCR Master Mix (Applied Biosystems, Foster City, USA). The PCR conditions were 95°C for 12 min and 35 cycles of 95°C for 10 s and 60°C for 30 s. The DNA dissociation data was imported in an analysis tool based on the Open Office.org Calc v2.0 software (OpenOffice.org), developed in-house to automatically plot the dissociation curves, detect the allele specific dissociation peaks and convert them into genotype calls. To confirm the genotypes obtained, a 319-bp fragment encompassing this SNP was amplified in 58 samples with the primers 168F (5′-AAG CTT CTA AAC TAA CAA ACA GCC AAT) and 486R (5′-TGA TAG CTC AAC TAG AAA AAC AAG AGA AA) and sequenced bidirectionally using the PCR primers and Big Dye Terminator v3.1 sequencing chemistry (Applied Biosystems, Foster City, USA) and analysed in a 3130 x l automated genetic analyzer with SeqScape v2.5 software (Applied Biosystems, Foster City, USA).

Data analysis

Pair-wise r^2 values (LD) of SNPs were estimated in the HapMap data with the Haploview v3.32 software (Barrett *et al*. 2005). Any deviation of the genotype frequencies from the Hardy–Weinberg proportions was assessed by the χ^2 test. The association between the genotypes and CAD was evaluated in the data obtained as well as in 10,000 Monte Carlo simulations, by the Cochran–Armitage trend test in R package v2.6.1 (R-package.org) and SNPStat online software tool (Sole *et al*. 2006). Routine statistical analyses were carried out with the SPSS v15 software (SPSS, Chicago, USA). The associations between the clinical covariates and CAD was analysed by logistic regression, the genotypes and the number of diseased vessels by the χ^2 test and that between the genotypes and the age at onset of CAD by the analysis of variance method. Normality of the plasma lipid levels was assessed by the Kolmogorov–Smirnov test and the Q–Q plots, and the data was subsequently log-transformed for normalization. The association between the genotypes and the plasma lipid levels was assessed by multivariate analysis of variance, with and without covariate adjustment for age and statins. Since this was a preliminary study, a P value of 0.05 or less was considered as statistically significant. All quantitative values are represented as mean ± standard error of the mean.

Results

Clinical characteristics

Detailed information on the study participants is provided in table 1. The mean age at onset of CAD was 48.14 ± 0.59 yr. CAD patients were found to have significantly elevated body mass index (BMI), waist-hip ratio and systolic blood pressure compared to the controls ($P < 0.05$). Diabetes and hypertension were present among 48.4% and 53.6% of the patients, respectively.

Genotype analysis

Resequencing of the rs10757278 in the selected samples confirmed the genotypes obtained by the real time PCR and did not reveal any novel polymorphisms in the neighbouring region of this variant. There was no significant departure from the Hardy–Weinberg equilibrium ($P > 0.05$). The risk allele (*G*) frequency was found to be 0.56 in the cohort, 0.59 in the cases and 0.52 in the controls. It was significantly associated with CAD in men with an odds ratio (OR) of 2.19 (95% CI: 1.04 – 4.64) ($P_{observed} = 0.0106$) (table 2). This association retained significance even after adjustment for the clinical covariates of age, diabetes and hypertension (OR = 2.15, 95% CI: 1.02 – 4.6) or after 10,000 Monte Carlo simulations ($P_{empirical} = 0.0098$). The corresponding unadjusted and adjusted population attributable risk (PAR) percentage was 47.1% (95% CI: 7.7% – 69.7%) and 46.6% (95% CI: 6.2% – 69.6%), respectively. The association can be explained by a recessive model of inheritance of the protective allele (*A*) (table 3). No significant evidence of association of the genotypes with diabetes, hypertension and smoking were observed in the present cohort (see table 1 in electronic supplementary material at http://www.ias.ac.in/jgenet/).

Genotype–phenotype analysis

The association between rs10757278 and the number of disease vessels was assessed in 134 cases for whom the information on the disease vessel status was available (see table 2 in electronic supplementary material). The frequency of the *GG* and the *AA* genotypes was found to be 35.4% and 11.4%, respectively among individuals with three or more diseased vessels and 17.6% and 23.5%, respectively among those with one diseased vessel. Similarly, the mean age at onset of CAD was 47.29 ± 1.11 yr in the individuals harbouring the *GG* genotype as opposed to 49 ± 1.57 yr for those harbouring the *AA* genotype. However, these differences in the genotype frequencies or the age at onset did not attain statistical significance. No significant association between the genotypes and the mean plasma lipid levels could be detected either in cases or in controls (see table 3 in electronic supplementary material).

Discussion

In this pilot study, rs10757278 was found to be associated with CAD in Asian Indians. Despite the inherent genetic susceptibility to heart disease, the Asian Indian

Table 1. Clinical characteristics of study participants. Continuous and categorical data were assessed using 2-tailed Student's *t*-test and chi-square test respectively in SPSS v15 software. Data are presented as mean standard error of mean or number (%).

	Case ($n = 154$)	Control ($n = 160$)	*P* value
Age (years)	53.18 ± 0.64	43.98 ± 0.37	< 0.001
BMI (kg/m^2)	26.42 ± 0.35	25.4 ± 0.33	0.033
Waist/hip ratio	0.95 ± 0.004	0.91 ± 0.01	< 0.001
Systolic blood pressure (mm Hg)	125.97 ± 1.3	119.7 ± 1.28	0.001
Diastolic blood pressure (mm Hg)	81.74 ± 0.64	81.42 ± 0.7	0.733
Gender (men/women)	130/24	97/63	< 0.001
Smoking (ever) (%)	65 (42.2)	34 (21.2)	< 0.001
Diabetes (%)	74 (48.4)	4 (2.6)	< 0.001
Hypertension (%)	82 (53.6)	15 (9.7)	< 0.001
Statin (%)	111 (72.1)	0	–

BMI, body mass index.

Table 2. Association of rs10757278 with coronary artery disease.

	Number of samples (%)								OR (95% CI)	
	Genotype *AA*		Genotype *AG*		Genotype *GG*					
Gender	Case	Control	Case	Control	Case	Control	[a]$P_{observed}$	[b]$P_{empirical}$	Unadjusted	[c]Adjusted
All	20 (13.0)	33 (20.6)	85 (55.2)	86 (53.8)	49 (31.8)	41 (25.6)	0.065	0.074	1.74 (0.91 – 3.38)	1.73 (0.90 – 3.37)
Men	17 (13.1)	24 (24.7)	70 (53.8)	52 (53.6)	43 (33.1)	21 (21.6)	0.0106	0.0098	2.19 (1.04 – 4.64)	2.15 (1.02 – 4.6)
Women	3 (12.5)	9 (14.3)	15 (62.5)	34 (54)	6 (25)	20 (31.7)	0.7484	0.8514	1.17 (0.26 – 7.33)	1.15 (0.25 – 7.28)

[a]P_{observed}, uncorrected *P* value calculated by Cochran–Armitage trend test. [b]$P_{\text{empirical}}$, permutation *P* value calculated using 10000 Monte Carlo simulations. OR, odds ratio; CI, confidence interval. [c]OR adjusted for age, diabetes and hypertension.

Table 3. Assessment of genetic models of rs10757278.

		Unadjusted			[a]Adjusted		
Model	Genotype	[b]OR (95% CI)	*P* value	AIC	OR (95% CI)	*P* value	[c]AIC
	GG	1			1		
Codominant	AG	0.81 (0.48 – 1.38)	0.078	415.3	0.76 (0.35 – 1.65)	0.043	237.1
	AA	0.45 (0.22 – 0.91)			0.27 (0.09 – 0.81)		
	GG	1			1		
Dominant	AG - AA	0.71 (0.42 – 1.18)	0.18	416.6	0.61 (0.29 – 1.29)	0.19	239.7
	GG - AG	1			1		
Recessive	AA	0.51 (0.27 – 0.96)	0.034	413.8	0.33 (0.13 – 0.84)	0.016	235.5
	GG - AA	1			1		
Overdominant	AG	1.09 (0.69 – 1.73)	0.7	418.2	1.26 (0.65 – 2.44)	0.5	240.9
Log-additive	–	0.69 (0.48 – 0.98)	0.035	413.9	0.55 (0.33 – 0.93)	0.023	236.2

[a]*P* values were obtained from logistic regression modeling after adjustment for age, gender, diabetes and hypertension.
[b]OR, odds ratio; CI, confidence interval; [c]AIC, Akaike information criterion.

population has not yet been substantially analysed from the perspective of cardiovascular genomics. Recent reports have indicated that the landscape of genetic diversity of the Asian Indians does not completely complement that observed in the HapMap populations (Indian Genome Variation Consortium 2008). The preliminary findings of this study implicate for the first time, a 9p21 common variant in CAD in Asian Indian patients and hence might provide important comparative information about this variant among the different populations. We are conducting an ongoing analysis based on multiple 9p21 variants and a substantially larger age and gender-matched cohort including patients with and without family history of heart disease.

Although of limited sample size, our study is based on patients belonging to well characterized families with verified pedigrees and with documented history of CAD. However, the small number of samples from affected women precluded any meaningful evaluation of the marker in this group. The average age of the controls were less than that of the CAD patients even though the comparison is probably justified as it has been previously observed by us that these clinical comorbidities such as diabetes, hypertension or metabolic syndrome appear to set in a decade or so earlier than the onset of clinical CAD i.e., around the middle of the fourth decade of life among the IARS cohort (Kanjilal *et al*. 2008). Additionally, the association remained significant even after adjustment for age, gender, diabetes and hypertension implying that the present observations might not be substantially influenced by these covariates.

The South Asian populations in general, and the Asian Indian population in particular, have a high prevalence of CAD. The estimated prevalence of cardiovascular disease in Asian Indians is about 10.5% (Sharma and Ganguly 2005; Goyal and Yusuf 2006). The risk of CAD in the Asian Indians is about three to four times higher than in the Caucasians, six times higher than in the Chinese and 20 times higher than in the Japanese populations. Our findings in the present study provide further evidence of the involvement of the 9p21 region in the etiopathology of CAD in Asian Indians. The odds ratio and the population attributable risk percentage for this CAD associated variant in men remain as the highest reported for rs10757278 to date (Helgadottir *et al*. 2007; McPherson *et al*. 2007; Samani *et al*. 2007; Wellcome Trust Case Control Consortium 2007; Hinohara *et al*. 2008; Shen *et al*. 2008). These findings might be of importance in light of the high prevalence of heart disease in the Asian Indians. Although the association between the genotypes and the number of diseased vessels did not achieve statistical significance, the observed trend, if validated in a large sample size, might elucidate the relationship between the genotypes and disease severity. The presence of a risk allele has been found to have a greater effect at an early age (Zdravkovic *et al*. 2002). A marginally lower age at onset was observed among the patients harbouring the *GG* genotype compared to those with the *AA* genotype which is in agreement with a previous finding (Helgadottir *et al*. 2007). Our findings also indicate that this implication of rs10757278 in CAD might be independent of lipid risk factors.

The variant rs10757278 is located in a region neighbouring the *CDKN2A* and *CDKN2B* genes and some exons of *ANRIL* (Helgadottir *et al*. 2007; Broadbent *et al*. 2008). *CDKN2A* and *CDKN2B* gene products are known to have important regulatory roles in cell proliferation, ageing and apoptosis, processes which are also important in the etiopathology of CAD and atherosclerosis. Multiple cell types involved in atherosclerosis have been found to express ANRIL, an antisense RNA hypothesized to be a part of the cellular transcriptional machinery. Given the strong implication of this genomic region in CAD, the exact molecular mechanism of its involvement remains to be confirmed.

Although our findings are in general agreement with the observed trend in other populations, the association was relatively stronger in the present cohort. Future studies based on a large cohort should be undertaken to elucidate the molecular mechanism underlying the observed association and to assign a functional role of this genomic region in the etiopathology of heart disease and its comorbidities in the Asian Indian population.

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