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Variant at 9p21 rs1333049 is associated with age of onset of coronary artery disease in a Western Indian population: a case control association study

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Summary

The 9p21 chromosomal region has been associated with coronary artery disease (CAD) in many genome wide association studies (GWAS). To date no information exists regarding the rs1333039 SNP which showed the strongest association in the WTCCC GWAS with CAD risk in the Indian population. The present study attempts to replicate the findings in the Indian population.

Genotyping for rs1333049 was done in 229 cases and 151 controls by allele-specific real-time assay.

A higher frequency of the risk allele rs1333049C was seen in cases (0·60) as compared with controls (0·49), which associated with CAD risk both in univariate (OR = 1·564, 95%CI = 1·154–2·119, $P = 0·003$) and multivariate analysis (OR = 2·460, 95%CI = 1·139–5·314, $P = 0·022$). Increased frequency of the risk allele was seen in younger individuals with CAD where 40% individuals in the age group 30–55 years had the CC genotype as compared with 29 and 24·5% in the age group 56–65 years and >65 years, respectively (CC versus GG, $P = 0·045$). Higher incidence of the CC genotype was seen in MI patients, but missed significance when compared with controls (OR = 1·361, 95%CI = 0·954–1·942, $P = 0·084$).

In conclusion, the rs1333049 variant is significantly associated with CAD risk and also with age of onset in the Western Indian population. However there are differences in the haplotype structure of this SNP with the neighbouring rs10757278 SNP, these differences emphasize the importance of genotyping all risk variants at this locus which could underlie the differences in risk susceptibility to CAD across populations.

1. Introduction

The 9p21 chromosomal region is a well-characterized locus showing association with coronary artery disease (CAD). Several genome wide association studies (GWAS; Helgadottir *et al.*, 2007; McPherson *et al.*, 2007; Samani *et al.*, 2007) and meta-analysis (Palomaki *et al.*, 2010; Preuss *et al.*, 2010) have implicated this region with increased risk of CAD, MI as well as progression of disease. Replication studies with different SNPs in this region in diverse populations including Irish (Meng *et al.*, 2008), Japanese (Hinohara *et al.*, 2008; Hiura *et al.*, 2008), Koreans (Hinohara *et al.*, 2008), Indians (AshokKumar *et al.*, 2011; Bhanushali *et al.*, 2011), Chinese (Ding *et al.*, 2009), etc. have corroborated these findings. In addition, SNPs in this locus have also been associated with increased risk of abdominal aortic aneurysm (AAA) (Bown *et al.*, 2008) and ischaemic stroke (Smith *et al.*, 2009).

However, the association of 9p21 SNPs with the extent or severity of CAD has been disputable with some studies stating that 9p21 SNP under study predicts severity of the disease (Ye *et al.*, 2008; Dandona *et al.*, 2010) and another the contrary (Chen *et al.*, 2009). The study by Anderson *et al.* (2008) also concludes that variants at the 9p21 locus robustly predict angiographic CAD prevalence, but not CAD extent or myocardial infarction. These findings have led to suggestions that the 9p21 locus acts at an early stage (as an 'initiator') of coronary atherosclerosis (Horne *et al.*, 2008).

In the Indian context, there have been a few studies that have evaluated genetic variants at this locus. In a

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study from South India (AshokKumar *et al.*, 2011), GWAS identified SNPs rs2383207 and rs10757278 at the 9p21 locus and nine additional flanking SNPs were evaluated for increased risk of CAD. In the North Indian population study (Kumar *et al.*, 2010), six SNPs were genotyped in a case-control association study in which three SNPs (rs10116277, rs1333040 and rs2383206) present at the locus 9p21 were significantly associated with CAD. Two other studies have also found increased risk of CAD with the rs10757278 variant (Maitra *et al.*, 2009; Bhanushali *et al.*, 2011).

However to date, there have been no data on the rs1333049G > C variant regarding its frequency as well as association with CAD risk in the Indian population. The rs1333049 G > C on the 9p21 locus had the strongest association in the Wellcome Trust Case Control consortium (WTCCC, 2007) study and German myocardial infarction (MI) family study ($P = 1{\cdot}80 \times 10^{-14}$ and $3{\cdot}40 \times 10^{-6}$, respectively). This SNP has also been associated with recurrent MI and cardiac death (Buysschaert *et al.*, 2010).

The high propensity of Indians to develop CAD necessitates that we conduct studies on the genetic markers associated with increased risk in this population. Hence, the current study was conducted with the aim of determining the frequency of this SNP as well as association with CAD risk in a select western Indian region (Indo Europeans).

2. Materials and methods

(i) *Subjects*

The study was performed on a total of 365 unrelated individuals, which consisted of 229 patients with CAD confirmed by coronary angiography: > 50% stenosis in one or more arteries and stable or unstable angina and 151 controls: examined clinically and investigated by electrocardiography and treadmill test to exclude CAD. Informed consent was obtained from all the subjects. The study is in accordance with the Helsinki declaration and was approved by the local ethical committee. A detailed case record form pertaining to information on demographics, medical history and coronary risk factors such as presence of diabetes, hypertension, smoking, lifestyle and current medication was completed for each participant through personal interviews and through perusal of their medical records.

Blood specimens were collected after an overnight fast of 12 h by venipuncture using the vacutainer system from Becton Dickinson (Franklin Lakes, NJ, USA) in the anticoagulant EDTA as well as plain bulb for serum. Serum, EDTA plasma samples were separated by centrifugation and aliquots were preserved at −20 °C until analysis.

(ii) *Biochemical parameters*

The laboratory parameters, namely serum total cholesterol (TC), triglyceride (TG) and high-density lipoprotein cholesterol (HDL-C) levels were determined by routine enzymatic endpoint methods (X Imola; Randox Laboratories Ltd., UK). Low-density lipoprotein-cholesterol (LDL-C) and VLDL cholesterol were calculated according to Friedwald′s formula.

(iii) *SNP genotyping*

The genomic DNA was isolated from peripheral blood using QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany). DNA yield and purity was determined by measuring absorbance at 260/280 nm. The rs1333049 genotypes were determined for all study participants using an allele-specific real-time PCR Taqman genotyping assay. The DNA was standardized to 10 ng/ml.

(iv) *Statistical analysis*

Allele frequency was calculated as the number of occurrences of the test allele in the population divided by the total number of alleles. Any deviation of the genotype frequencies from the Hardy–Weinberg equilibrium (HWE) was assessed by Fischer's exact test. Chi-square (χ) tests were used for comparison of binary variables across groups. The non-parametric Kolmogorov–Smirnov test was used as test of normality for the quantitative variables, those parameters that did not show a normal distribution were log transformed during regression analysis. To determine risk for CAD unadjusted odds ratios were determined by Cochran–Mantel–Haenszel statistics and adjusted odds ratio by multivariate logistic regression. SNPStat online software tool was applied to determine the association of the 9p21 variant rs1333049 with CAD and also to determine the model of association, if any (Sole *et al.*, 2006). Routine statistical analysis were carried out with the SPSS v 15 software (SPSS Inc., Chicago, IL) and GraphPad StatMate 2.0 (GraphPad software Inc) was used to determine the power of the study. Under the significance level of $P = 0{\cdot}05$, minor allele frequency between 0·25 and 0·40, assuming population disease prevalence between 5 and 10% and main genetic effect between 1·5 and 1·2, our study design can reach > 85% power when the relative risk (RR) is 1·5 and 50% when it is 1·2.

3. Results

Table 1 displays means and standard deviations for the study subjects for relevant biochemical

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Table 1. *Clinical and demographic details of the study population*

Characteristic	Cases[a] ($n=229$)	Controls ($n=151$)
Age (years)	54 ± 10	49 ± 11
Gender (male %)	89**	70
[b]Smoker (%)	44**	27
Diabetic (%)	38**	13
Hypertensive (%)	55**	21
Family history (%)	51*	33
[c]Alcohol consumption (%)	20	19
TC (mg/dl)	162 ± 50	192 ± 38
TG (mg/dl)	143 ± 84	154 ± 97
LDL-C (mg/dl)	97 ± 45	125 ± 31
HDL-C (mg/dl)	38 ± 13	42 ± 12
VLDL-C (mg/dl)	29 ± 17	29 ± 20
SVD (%)	39	NA
DVD (%)	8	NA
TVD (%)	53	NA
MI (%)	55	NA

Mean ± SD for continuous variables. SD, standard deviation; NA, not applicable; TC, total cholesterol; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; VLDL-C, very-low-density lipoprotein cholesterol; APOA1, apolipoprotein A1; APOB, apolipoprotein B; TG, triglycerides; SVD, single-vessel disease; DVD, double-vessel disease; TVD, triple-vessel disease; MVD, multiple-vessel disease; MI, myocardial infarction.
*$P<0{\cdot}001$; **$P<0{\cdot}0001$.
[a] Includes individuals on lipid lowering medications.
[b] Smokers includes ex-smokers for more than 5 years.
[c] Alcohol consumption indicates individuals with >5–7 pegs/week.

characteristics as well as risk factors. Statistically significant differences were seen in the smoking status ($P<0{\cdot}01$), gender ($P<0{\cdot}0001$), presence of family history ($P<0{\cdot}0001$), diabetes ($P<0{\cdot}0001$) as well as hypertension ($P<0{\cdot}0001$) in cases versus controls. Genotypic and allelic frequencies of the rs1333049 SNP are shown in Table 2. No significant departure from the HWE was seen. A higher frequency of the rs1333049 C allele was seen in cases as compared with the controls (0·60 versus 0·49), which was significant (OR = 1·564, 95%CI = 1·154–2·119, $P=0{\cdot}003$) in the univariate analysis. The univariate analysis also indicated a much higher OR on comparison of CC versus GG genotype (OR = 2·814, 95%CI = 1·426–5·578, P value = 0·001). In the logistic regression analysis, female gender was associated with decreased CAD risk (OR = 0·241, 95%CI = 0·092–0·062, $P=0{\cdot}004$) even after accounting for the differences in their ratio in cases and controls, whereas rs1333049 with increased CAD risk (OR = 2·460, 95%CI = 1·139–5·314, $P=0{\cdot}022$) as seen in Table 3. To determine if this variant is associated with age of onset of the disease chi-square analysis was done (Table 4). A higher frequency of the risk allele was seen in the younger age group with 40% of individuals in age group 30–55 years having the CC genotype as compared with 29 and 24·5% in age group 56–65 years and >65 years, respectively. These findings were significant but only when the CC genotype was compared with the GG genotype. This may be indicative of a recessive model of inheritance, where lack of the G allele may be responsible for the disease risk *per se*. The association of rs1333049 (C allele) with the end-point of non-fatal MI was also determined (Table 5). Although a higher frequency of the CC genotype was seen in MI patients, it was not statistically significant (allelic OR = 1·361, 95%CI = 0·954–1·942, $P=0{\cdot}084$). However in agreement with the previous table (Table 2), the combined OR of MI+CAD versus controls was highly significant (OR = 1·540, 95%CI = 1·333–2·094, $P=0{\cdot}005$). The rs1333049 SNP was also in strong linkage disequilibrium with the rs10757578 SNP (previously studied) (Bhanushali *et al.*, 2011) with D' statistic, 0·794; r statistic, 0·726 and $P<0{\cdot}01$. The haplotype frequencies are indicated in Table 6. In LD block (rs1333049, rs10757278), the two most frequent haplotypes accounted for >85% of the chromosomes (Hap 1, CG 43 and 56% in controls and cases respectively and Hap 2, GA 42 and 31% in controls and cases, respectively).

4. Discussion

The magnitude of CAD represents a major socio-economic impact especially in the developing nations of South-Asia (Yusuf *et al.*, 2001; Gaziano *et al.*, 2010). It is well known that CAD arises from a combination of environmental and genetic factors (Yusuf *et al.*, 2004; Damani & Topol, 2007). To date several GWA studies have highlighted the importance of the 9p21 chromosomal region with CAD/MI risk (Helgadottir *et al.*, 2007; McPherson *et al.*, 2007; Samani *et al.*, 2007; O'Donnell *et al.*, 2011). However, it is crucial to replicate these findings in independent studies as well as to extend and verify its validity in different ethnic populations. The rs1333049 was identified in WTCCC GWA study as the strongest SNP associated with CAD risk (WTCCC, 2007). Although other variants at the 9p21 locus have been studied in Indians (Kumar *et al.*, 2010; AshokKumar *et al.*, 2011; Bhanushali *et al.*, 2011; Maitra *et al.*, 2009), no information is available to date on this variant. The results of our study determine for the first time the frequency of this SNP in the select population from Western India as well as confirm the association with CAD risk. The study also sheds light on the LD of rs1333049 with rs10757278 which was found to be

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Table 2. *Genotypic and allelic frequency of 9p21 (rs1333049) in cases and controls*

rs1333049	Genotype frequencies *n* (%)			Major	Minor	OR[a] C		OR[a] CC		OR[a] CG	
	GG	CG	CC	G	C	versus G	95% CI *P* value	versus GG	95% CI *P* value	versus GG	95% CI *P* value
Cases ($n=229$)	29 (12·5)	125 (54·5)	75 (33)	0·40	0·60	1·564	1·154–2·119 0·003**	2·814	1·426–5·578 0·001**	1·994	1·096–3·633 0·022*
Controls ($n=151$)	37 (24·5)	80 (53)	34 (22·5)	0·51	0·49						

SNP, single-nucleotide polymorphism OR [a]Unadjusted Odds ratio derived using Cochran–Mantel–Haenszel statistics. 95% CI, 95% Confidence Interval. Statistically significant at $*P<0·05$, $**P<0·01$.

associated in another GWA study (Helgadottir *et al.*, 2007).

In the present study, the C allele of the rs1333049 SNP has a frequency of 0·49 in controls which is similar to that seen in Europeans (0·49), Han Chinese (0·48) and Japanese (0·51) but significantly different to the Yoruban population (0·17) and Kenyan population (0·24) (HapMap data).

Although the rs1333049 was seen to be in strong LD with the rs10757278 (previously studied), $D'=0·796$ however the haplotype structure was not in near complete LD as seen in another study where the D' was 0·99 (Chen *et al.*, 2009). The haplotype structure in our population is also at variance with the HapMap CEU population where the rs10757278 and this SNP are practically equivalent with linkage $r^2=1$ (HapMap data), unlike in our population where the r statistic is 0·726.

The results of our study confirm that the rs1333049 is significantly associated with CAD risk in the Indian population, which is in agreement with findings in other ethnic populations. In the present study, the OR was 1·564, 95% CI = 1·154–2·119, $P=0·003$ in the allele count model, which is higher than that seen in the Japanese (OR = 1·30, 95%CI = 1·13–1·49, $P=0·00027$) or Koreans (OR = 1·09, 95%CI = 1·02–1·38, $P=0·025$) in the same model (Hinohara *et al.*, 2008). Furthermore, comparison of the CC genotype versus the GG genotype indicated a much higher OR = 2·814, 95%CI = 1·426–5·578, $P=0·001$. In the discovery GWA study the OR for the WTCCC and the German data were 1·37 and 1·33 for the risk allele (95% CI = 1·26–1·48, $P=1·80\times10^{-14}$ and 95% CI = 1·18–1·51, $P=6·80\times10^{-6}$, respectively).

When the cases were further differentiated based on age a much higher frequency of the CC genotype was seen in the premature CAD group (30–55 years). Similar findings were seen in the Irish population (Meng *et al.*, 2008) where three SNPs at the 9p21 locus, which included the rs1333039 ($P=3·08\times10^{-7}$) were strongly associated with early onset CAD. The rs1333049 polymorphism was also associated with an earlier age of disease onset in two coronary disease cohorts (Ellis *et al.*, 2010). The coronary disease cohort study (CDCS) patients homozygous for the high-risk C allele had an age of onset 2–5 years earlier for coronary disease ($P=0·005$), angina ($P=0·025$), MI ($P=0·022$) and percutaneous transluminal coronary angioplasty ($P=0·009$). The post myocardial infarction (PMI) participants with the CC genotype were 3 years younger on admission ($P=0·009$). The homozygous CC genotype of rs1333049 was also seen to confer a magnified risk of early-onset and severe CAD in diabetic patients (Wang *et al.*, 2011). Statistically significant association between 9p21 SNPs and heart disease that varied by age at disease onset was also seen in a meta-analysis (Palomaki

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Table 3. *Multivariate logistic regression analysis of association of rs1333049 with CAD risk*

Variable	β-coefficient	OR	95% CI	*P* value
Constant	−2·552	0·0077	0·012–0·597	0·014
Sex	−1·423	0·241	0·0923–0·629	0·004**
Age	0·0410	1·042	1·005–1·080	0·025*
Smoking	1·433	4·190	1·515–11·589	0·006*
Alcohol	−1·021	0·360	0·124–1·045	0·060
Diabetes	0·935	2·546	0·948–6·839	0·064
Family history	0·401	1·493	0·697–3·202	0·303
rs1333049 CC versus GG	0·900	2·460	1·139–5·314	0·022*
rs1333049 CC versus CG+GG	0·488	1·629	0·925–2·866	0·091

*$P<0·05$, **$P<0·005$.

Table 4. *Association of rs1333049 with age of onset*

	Genotype frequencies *n* (%)			Chi-square; df; *P* value		
Age of onset	GG (%)	CG (%)	CC (%)	Genotypes CC, CG, GG	Genotypes CC : CG+GG	Genotypes CC:GG
30–55 years ($n=103$)	8 (8%)	54 (52%)	41 (40%)	$\chi^2=7·41$ df=4	$\chi^2=4·46$ df=2	$\chi^2=6·18$ df=2
56–65 years ($n=77$)	14 (18%)	41 (53%)	22 (29%)	$P=0·116$	$P=0·107$	$P=0·045$*
>65 years ($n=49$)	7 (14·5%)	30 (61%)	12 (24·5%)			

χ^2, Chi-square; df, degree of freedom; *Statistically significant at $P<0·05$.

Table 5. *Genotype frequency and association of rs1333049G>C with MI as endpoint*

	Genotype frequencies					
[a]Phenotype	GG (%)	CG (%)	CC (%)	Allelic OR	95% CI	*P* value
MI ($n=120$)	22 (18)	60 (50)	38 (32)	1·361[b]	0·954–1·942[b]	0·084[b]
CAD ($n=97$)	7 (7)	57 (59)	33 (34)	0·755[c]	0·502–1·134[c]	0·169[c]
Controls ($n=151$)	37 (24·5)	80 (53)	34 (22·5)	1·540[d]	1·333–2·094[d]	0·005[d]*

* Statistically significant at $P<0·01$.
[a] Data on 217 individuals (cases), for 12 patients with ischaemic heart disease it was not clear if the endpoint was MI.
[b] OR, 95% CI, *P* value indicate OR, 95% CI and *P* value for MI versus controls.
[c] OR, 95% CI, *P* value indicate OR, 95% CI and *P* value for MI versus CAD.
[d] OR, 95% CI, *P* value indicate OR, 95% CI and *P* value for MI+CAD versus controls.

et al., 2010) although the magnitude of association was small.

Although the association of the 9p21 region with CAD risk is well established, the association with clinical outcomes still remains unclear. The Global Registry of Acute Coronary Events Genetics (GRACE) study found the rs1333049 at-risk C allele to be significantly and independently associated with recurrent MI. Moreover, inclusion of the rs1333049 into the GRACE risk score was also seen to improve classification for recurrent MI or cardiac death (Buysschaert *et al.*, 2010). Similarly strong association of 6 SNPs at the 9p21 locus (rs1333049 included) with MI was also seen particularly in patients with a positive family history (Scheffold *et al.*, 2011). These findings were in agreement with a GWA study, where the rs1333049 was strongly associated ($P=7·58\times10^{-19}$) with coronary artery calcification

Table 6. *Haplotype frequencies of rs1333049G>C and rs10727578A>G*

	rs1333049	rs10757278	Total	Controls	Cases
1	C	G	0·51	0·43	0·56
2	G	A	0·35	0·42	0·31
3	G	G	0·09	0·09	0·09
4	C	A	0·05	0·06	0·04

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(CAC) and MI (O'Donnell, 2011). The meta-analysis by Preuss *et al.* (2010) indicated that the rs1333049 conferred a 29% increase in risk for MI per copy ($P = 2 \times 10^{-20}$) (Preuss *et al.*, 2010).

In contrast the association of 9p21 SNPs (rs2383206, rs2383207, rs10757274 and rs10757278) with occurrence of nonfatal MI or death could not be established by Horne and colleagues (Horne *et al.*, 2008). Similarly in another study though the rs1333049 (C) was associated with greater atherosclerotic plaque, it did not associate with MI when disease severity was accounted for (Dandona *et al.*, 2010). Variable results have also been seen with respect to disease progression with one study indicating the sequence variation in this locus to influence atherosclerosis development and progression (Ye *et al.*, 2008) and another indicating no effect either on disease severity or progression (Chen *et al.*, 2009). In the present study, also no significant association of the risk allele with MI was seen. The conflicting results seen to date in phenotypic studies indicate that still more needs to be done to understand the direct as well as indirect mechanisms underlying the effects of this locus.

In conclusion, the rs1333049 SNP at the 9p21 locus is not only significantly associated with CAD risk, which is in concordance with results across several other ethnic groups, but also with age of onset in the Western Indian population. However, there are differences in the haplotype structure of this SNP with the neighbouring rs10757278 SNP. These differences emphasize the importance of genotyping all risk variants at this locus, as it could underlie the differences in risk susceptibility to CAD, which varies across populations. More importantly the high propensity of Indians to develop CAD emphasizes the need for genetic studies in this population. This can also have implications for prevention as adding the 9p21 allele to traditional risk factors was seen to modestly improve CAD risk prediction in the intermediate categories in the population studied (Brautbar *et al.*, 2009).

Thus although the study has limitations, that of sample size and consequently the statistical power to examine multiple interactions, these findings provide for the first time data on frequency and association of the rs1333049 variant with CAD and larger studies are warranted in this high-risk population.

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Statement of Interest

None.

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