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Pilot Genome wide Linkage Analysis in Asian Indian Families with Coronary Artery Disease

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Abstract

Background and Objectives: Asian Indians show an inherent predisposition to premature Coronary Artery Disease (CAD) with a strong family history and therefore serve as a suitable population for identifying novel genes linked to CAD. We performed pilot linkage analysis on a subset of Asian Indian families selected from the *Indian Atherosclerosis Research Study* (IARS) to identify putative loci linked to CAD.

Methods & Findings: We performed linkage study on six multiplex families consisting of 31 affected sibling pairs (ASPs). Families were ascertained through the proband who had angiographically confirmed CAD, with age at onset < 60 years for males and <65 years for females. *Linkage mapping set v 2.5-MD10*, comprising of 400 fluorescent labeled microsatellite markers were genotyped in 31 ASPs. Quantitative trait loci (QTL) analysis was carried out for sixteen atherothrombotic biomarkers and non parametric linkage analysis was performed by affected sib-pair method. There was suggestive evidence of linkage at 4q21.21, 6q22.33, 6q23, 6q24.2 and 8q24.1 to CAD (Logarithm of Odds – LOD score ≥ 1; $p<0.05$). Bioinformatics analysis of significant linkage peaks identified key genes associated with inflammation and immune response. QTL analysis revealed suggestive evidence of linkage to Xp22.3 locus for total cholesterol (LOD =1.7) and at various loci on chromosomes 1,2,4,11 and X for Fibrinogen, Interleukin 2, Apolipoprotein A1, High density lipoprotein cholesterol and Apolipoprotein B (LOD >1; $p<0.05$), respectively.

Conclusion: Novel loci on chromosome 4,6 and 8 has shown suggestive evidence of linkage to CAD in this initial study; their role in the etio-pathogenesis of CAD remains to be established

Key words: affected sibling pair, Asian Indians, coronary artery disease, linkage, microsatellite markers.

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Introduction

Coronary Artery Disease (CAD) in a common cause of death and disability in the world. Presence of strong family history and premature disease onset in Asian Indians indicate a significant role for genetic factors in the etiopathogenesis of CAD (1). In this regard, the Indian Atherosclerosis Research Study (IARS), a genetic epidemiological study, comprising of CAD patients and their relatives with strong family history of cardiovascular disease (CVD), offers a suitable platform to investigate the contribution of genetic factors (2).

Non-parametric linkage analysis based on affected sibling pairs (ASPs) serves as a useful method to test for linkage between microsatellite markers and CAD (3). A number of linkage studies have been reported on ASPs that have helped to identify specific CAD loci on chromosome 1, 2, 3, 7, 16, 20, X etc (4-11). The key issues with linkage analysis have been their limited success in identifying putative candidate genes for CAD and the lack of reproducibility of the study findings that has been attributed to factors such as different cohort sizes, variable clinical phenotype, gene-environment interactions etc. Nevertheless, studies undertaking fine mapping of loci with significant linkage have been moderately successful in identifying novel CAD genes such as KALRN, NPY, FAM5C, MEF2A etc (8, 10-12). Furthermore, studies on quantitative trait loci (QTL) have identified several interesting novel loci linked to various candidate atherothrombotic biomarkers namely lipids (13-15), inflammation (16), coagulation (17), obesity markers(18), vascular markers of sub clinical atherosclerosis (19) and so on.

Despite the enormous burden of heart disease in India, there is very limited information on the genetic architecture of Indians (17, 20-33); what little is known suggests that the Indian population is a genetic potpourri of unique social and religious divides that makes it a very interesting topic for study. Availability of large multiplex families in the IARS provides opportunities to unravel the genetic risk factors for CAD. This paper discusses the findings of a pilot linkage study performed on six multiplex families selected from the IARS cohort, comprising of 31 ASPs, with an objective to identify novel loci for CAD as well as QTL loci for candidate biomarkers in a predisposed cohort of Asian Indians.

Methods

Study Cohort

A total of six families comprising of 21 CAD affected siblings and 10 unaffected siblings were selected from the ongoing Indian Atherosclerosis Research Study (IARS) cohort. These subjects were enrolled during May 2004 to May 2005 in Phase I of the study and comprised of CAD patients, their affected and unaffected family members including siblings, spouse, offspring above 18 years of age and parents. There was a total of 31 ASPs - 1 family each with 15 ASPs (family # 87), 6 ASPs (family # 76) and 1 ASP (family # 232) and 3 families with 3 ASPs (family # 13, #131, #232), respectively (**Fig. 1**).

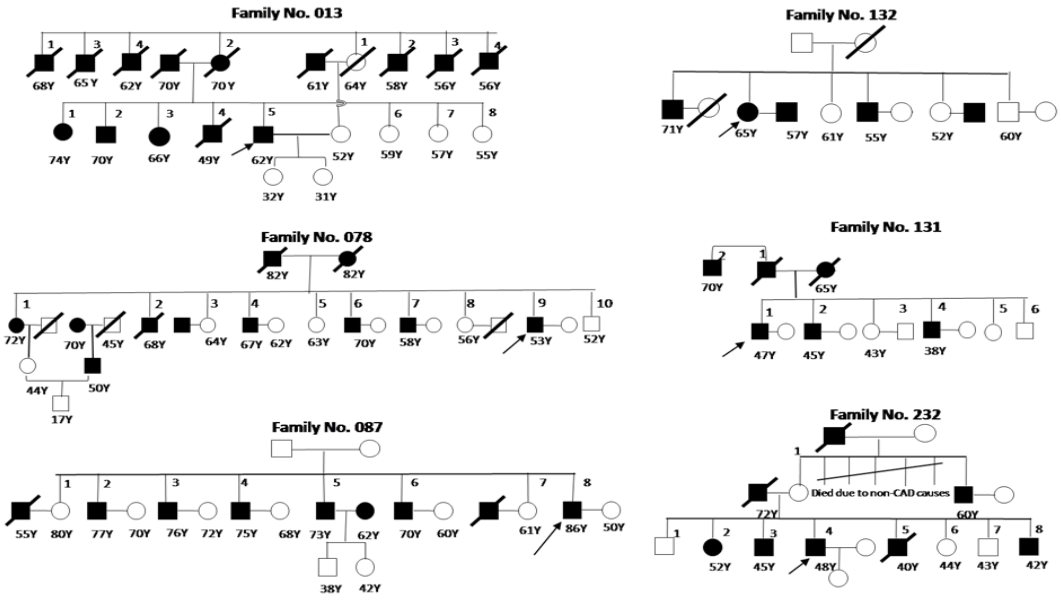

Figure 1. Pedigree of six families selected for linkage study.

A detailed design of the IARS has been previously described (2). To describe briefly, the IARS is an ongoing epidemiological study, with an objective to investigate the genetic factors associated with CAD, as also their interaction with traditional risk factors among Asian Indians living in India. Family members were recruited through the proband with history of premature CAD from Narayana Hrudayalaya multispecialty hospital and other hospital/clinics in Bangalore city and from the Asian Heart Institute in Mumbai, India. Representative participants were recruited from North, South, East and West of India. Patients showed clinical evidence of stable / unstable angina or myocardial infarction event, diagnosed by coronary angiogram and echocardiogram (ECG) and treated with standard medication or coronary angiography followed by percutaneous transluminal coronary angioplasty (PTCA) or coronary artery bypass graft (CABG). Probands were selected based on predefined inclusion-exclusion criteria which included those having age of disease onset 60 years or less for men and 65 years or less for women. Unaffected subjects were asymptomatic at recruitment and showed normal ECG readings. Participants were not suffering from any other major illness at the time of enrolment and were free of concomitant infection. Participation in the study was by informed, signed, voluntary consent. The IARS protocol has been approved by the institutional ethics committee and designed as per the Indian Council of Medical Research (ICMR) guidelines on bioethics (34).

All study participants provided fasting blood and urine samples. Detailed demographics, anthropometrics, vital parameters, medical history, medication and pedigree information were recorded for each participant through personal interviews. Prevalence of type 2 diabetes, hypertension and CVD was ascertained based on self-report of physician's diagnosis and/or use of prescription medications along with perusal of medical records.

DNA extraction and Genotyping

Genomic DNA was isolated from whole blood using salting out procedure (35) and quantified using Nanodrop ND-1000 (Thermo Scientific, Washington, USA) and real time polymerase chain reaction (RT-PCR, Applied Biosystems, USA). The ABI Prism *Linkage* Mapping Set v 2.5-MD10 that comprises of 400 fluorescent labeled microsatellite markers, spaced approximately 10cM apart and covers the entire whole genome, was used for linkage analysis. Thermal cycling conditions for PCR were based on manufacturer's instructions. Following amplification, the PCR products were pooled along with Gene Scan^{TM}- 500 LIZ^{TM} size standard and analyzed on ABI Prism 3130XL genetic analyzer. Commercially available CEPH DNA sample as well as two in-house samples was used for quality control. Following standardization, PCR amplification and analysis was carried out in two batches of 14 and 22 samples. CEPH DNA was used as positive control with each batch of PCR set up, 3130XL run and during analysis.

Genotyping and assignment of Allele calls

Genotyping based on allele calls were performed using *GeneMapper version 4.0* software (ABI, USA) and a macros based algorithm that was developed in-house. All genotypes, including those that passed the internal quality control of GeneMapper were manually read and independently verified by at least two individuals. Genotypes were rejected and samples were re-analyzed in case there was no consensus on the allele calls. The *PedCheck* program (36) was used to detect genotype inconsistencies, not in agreement with the Mendelian inheritance pattern. Such genotypes were reanalyzed and either corrected, or else deleted from the study.

Linkage analysis

Non Parametric linkage analysis was performed based on the affected sibling pair method to test for linkage between microsatellite markers and CAD (3). *MERLIN* program was used for linkage analysis (37). Appropriate input files were created using *Mega2* (38). Both single point and multipoint linkage analysis was performed using the $MERLIN_{all}$ and $MERLIN_{pairs}$ options in *MERLIN*. Significant linkage was calculated based on LOD score value. LOD stands for logarithm of the odds (to the base 10). Linkage was assigned based on predefined significance criteria (39).

Analysis of Quantitative trait loci (QTL)

QTL analysis was performed for various candidate biomarkers. For the purpose of measuring biomarker levels, venous blood was collected in evacuated tubes after an overnight fast of 12 to 14 hours (Vacuette®, Greiner Bio-One GmbH, Vienna, Austria). Serum cholesterol and triglycerides were estimated by standard enzymatic analysis following manufacturer's guidelines (Randox Laboratories, UK); High Density Lipoprotein–Cholesterol (HDL-c) was estimated after precipitation of non-HDL fractions with a mixture of 2.4mmol/l phosphotungstic acid and 39mmol/l magnesium chloride and Low Density lipoprotein-cholesterol (LDL-c) was calculated using Friedwald formula (40). Immunoturbidimetry was employed to measure Lipoprotein(a) levels using reagents from Randox Laboratories, UK; Apolipoproteins A1 and B100 were measured with reagents from Orion Diagnostica, Finland in a Cobas-Fara II Clinical Chemistry Autoanalyser (Roche, Switzerland). Normal human serum pool (NHP) was prepared in-house and run with each batch of tests. The inter assay coefficients of variation (CV) for commercial controls and NHP range was 4.9-7.0% for total cholesterol, 6.1-7.7% for triglyceride, 7.1-

12.2% for HDL-cholesterol, 3.3-5.2% for Lp(a), 9.9-14.2% and 10.7-13.9% for apolipoprotein A1 and B100 respectively. Plasma Interleukin 6 was measured by ELISA (R&D systems, USA); interassay CV for NHP was 4.3%. Plasma hsCRP levels were measured using the Roche latex Tina quant kit (Roche Diagnostics, Switzerland); interassay CV of NHP was 7.85%. secretory phospholipase A2 levels were determined using a sandwich immunoassay specific for type IIa (Cayman Corporation, USA) with a sensitivity limit of 15pg/ml; interassay CV of NHP was 5.37%.

Height, weight, waist and hip circumference and blood pressure (BP) was measured for each participant. BMI was calculated as a ratio of weight in kg to height in meter2. The 'QTL' option in MERLIN program was used for linkage analysis for these quantitative traits.

Results

Cohort

There were 17 males and 4 females in the CAD affected group (N=21) and 6 males and 9 females in the unaffected sibling group (N=15). Frequencies of diabetes and smokers were higher among the affected subjects. Mean age at onset was 50.47 years for males and 59.50 years for females. **Table 1** provides a summary of clinical profile of the study participants.

Linkage analysis to CAD

We utilized the data obtained from the affected sibling group only for performing linkage analysis,. Based on analysis on 31 ASPs from 6 multiplex families, we observed suggestive

Table 1. Clinical profile of study participants.

Description	CAD affected (N=21)		Unaffected siblings (N=15)	
	Male (N=17)	Female (N=4)	Male (N=6)	Female (N=9)
Mean age	61 y	64.75 y	43.83 y	53.22 y
Diabetes	8 (47%)	2 (50%)	1 (16.7%)	2 (22.2%)
Hypertension	2 (11.8%)	4 (100%)	3 (50%)	5 (55.6%)
Ever smoked	14 (82.4%)	-	2 (33.3%)	-
Mean age at CAD onset	50.47 y	59.5 y	-	-

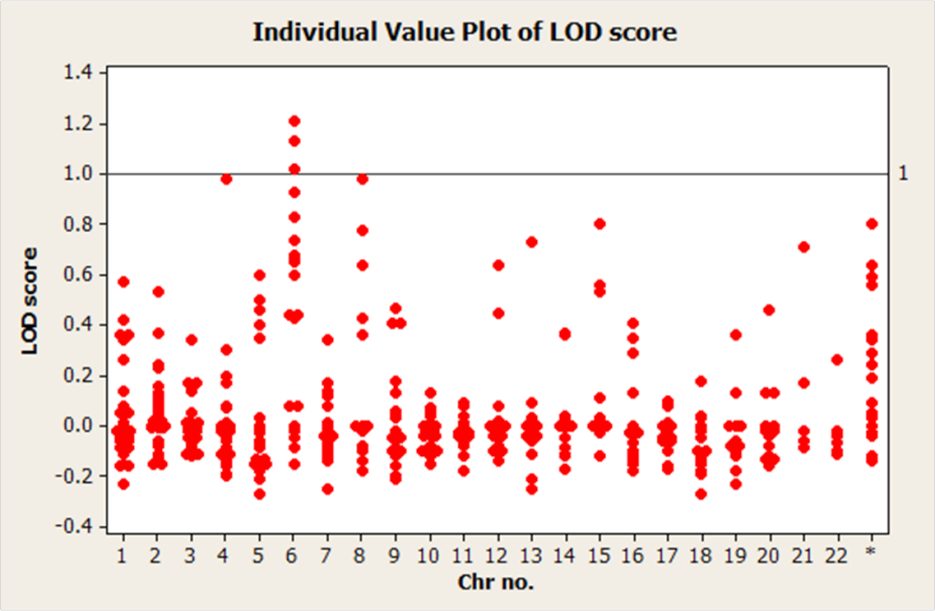

Figure 2. Individual value plot of LOD score plotted against chromosome number for the 400 microsatellite markers.

evidence of linkage for CAD at 4q21.21, 6q22.33, 6q23 and 6q24.2 and 8q24.1 with a LOD score value of ~ 1 at a nominal significance of $p < 0.05$. **Table 2** summarizes the list of all microsatellite markers that show a LOD score >0.40 while **Fig. 2** depicts loci showing suggestive evidence of linkage to CAD. Age and gender were used as covariates.

Analysis of Quantitative Trait Loci (QTL)

QTL analysis showed suggestive evidence of linkage at chromosome Xp22.3 for TC (LOD=1.7), and various loci on chromosomes 1, 2, 4, 11 and X for fibrinogen, IL2, ApoA1, HDL-c and ApoB (LOD score above 1), respectively (**Fig. 3**). Interestingly, D13S159 marker showed significant suggestive linkage to 13q32.2 region (LOD=0.9), a validated locus for the F7 gene. The complete list of suggestive quantitative trait loci with LOD score >0.88 for the various biomarkers are shown in **Table 3.**

Bioinformatics analysis

We performed bioinformatics analysis on putative candidate genes underlying loci that showed suggestive evidence of linkage to CAD (P<0.05). For this, we used the NCBI database to identify all those genes lying at approximately 1 LOD unit (1Mb) upstream and downstream from those microsatellite markers showing suggestive linkage to CAD (**Figure 2**). We identified several interesting genes or gene products. The APO A1 gene located on 4q21 locus that encodes for Apolipoprotein A1 and PLA2G7 (Phospholipase A2, group VII) gene located at *6q21* region that encodes for Lp-PLA(2), an inflammatory protein; inflammatory genes, namely IL20RA, IL22RA2, IFNGR1, TNFAIP3 in the 6q23 chromosomal region, the CRP gene located at 8q24 and the *MEF2A* (myocyte enhancer factor 2A) and *HSP90B2P* (heat shock protein90B2P) genes on the *15q26* chromosomal region are considered to be some of the important atherothrombotic genes.

Table 2. List of potential Chromosomal loci showing suggestive evidence of linkage to CAD (p value ≤ 0.05).

Marker	Map position (cM)	LOD score	P value	Chromosomal loci
D1S2836	285.75	0.57	0.05	1p36.3
D4S392*	78.97	0.98	0.02	4q21.21
D5S419	39.99	0.60	0.05	5p13.1
D6S462	99.01	0.83	0.03	6q16.1-q16.3
D6S292*	136.97	1.21	0.009	6q22.33
D6S262*	130.0	1.02	0.02	6q23
D6S308	144.46	1.13	0.011	6q24.2
D6S1574	9.18	0.67	0.04	6p25.1
D6S309	14.07	0.60	0.05	6p25.3
D8S1784	118.15	0.78	0.03	8q23
D8S270	103.69	0.64	0.04	8q21.3
D8S514*	130.0	0.98	0.02	8q24.1
D12S1723	164.63	0.64	0.04	12q24.3
D13S1265	98.82	0.73	0.03	13q32.2
D15S120	112.58	0.80	0.03	15q26
D15S205	78.92	0.56	0.05	15q24
D21S263	27.40	0.71	0.04	21q21.3
DXS991	52.50	0.80	0.03	Xp11.22
DXS1060	15.12	0.64	0.04	Xp22.3
DXS987	22.18	0.59	0.05	Xp22.2
DXS1126	27.59	0.56	0.05	Xp22

*potential loci with LOD score ~ 1

Table 3. List of QTL loci showing suggestive evidence of linkage to various atherothrombotic biomarkers (p value ≤ 0.05).

Biomarker	Marker	Chromosomal Loci	LOD Score
Total Cholesterol	DXS1060	Xp22.3	1.07
	DXS8051	Xp22.3-p22.2	1.69
	DXS1214	Xp21.2	0.88
	DXS990	Xp11.22 / Xq13.1-q21	0.88
	D5S433	5q11.2-q13.3	1.04
	D7S484	7p15-p14	1.03
	D7S510	7p14-p13	1.05
	D19S420	19q13.1-q13.2	0.91
Triglycerides	DXS986	Xq13.1-q21	1.1
	DXS990	Xp11.22 / Xq13.1-q21	0.88
	D4S426	4q35	0.9
Low-Density Lipoprotein-cholesterol	D7S513	7p21.3	1.11
	D7S493	7p21	1.08
	D7S560	7p11	1
	D9S161	9p21.2	0.98
	DXS986	Xq13.1-q21	1.06
	DXS990	Xp11.22 / Xq13.1-q21	1.02
High Density Lipoprotein-cholesterol	D2S112	2q21-q22	0.93
	D11S904	11p14.2	1.26
	D19S418	19q13.4	0.94
Apolipoprotein A1	D3S1267	3q13.2-q21	1.06
	D4S391	4p14	0.95
	D4S392	4q	1.19
	D4S2964	4q21.1	1.05
	D6S470	6p24.3	1.02
	D13S156	13q22, 13q	0.93
	D15S130	15q25-q26, 15q23-q26.3 / 7p15.3	0.93
	D19S221	19p13.2	1.018
Apolipoprotein B100	DXS986	Xq13.1-q21	1.21
	DXS990	Xp11.22 / Xq13.1-q21	1.05
	D13S175	13p12	0.92
Factor VII.c	D5S406	5q11.2-q13.3	0.9
	D6S434	6q16.3-q21	0.98
	D13S159	13q32.2	0.89
Fibrinogen	D1S2785	1q41-q42	1.03
	D1S2842	1q42.2-q43 / 11p15.1	1.48
	D4S405	4p14 / 4p15.1 - q12	1.02
	D12S86	12q24	1.08
	D15S131	15q23	0.9
Secretory Phospholipase A II	D12S83	12p13.2-q24.1	0.88
	D15S1007	15q12-q15	0.94

Interleukin 6	D2S112	2q21-q22	0.9
	D2S2330	2q23-q24	1.3
	D6S1581	6q24	0.9
	D9S1826	9q, 9q34, 9q34.3	1.01
Lipoprotein(a)	D7S513	7p21.3	1.11
	D7S493	7p15, 7p21	1.08
	D7S516	7p15.2-p15.1	1
	D9S161	9p21.2 / 17q22-24	0.98
	DXS986	Xq13.1-q21	1.06
	DXS990	Xp11.22 / Xq13.1-q21	1.02
High sensitive C-reactive protein	D2S165	2p21-p22	0.95
	D8S284	8q24	0.88
Diastolic blood pressure	D2S391	2p16-p21	0.89
	DXS1001	Xq24-q25, Xq27	0.88
Systolic blood pressure	D4S1592	4q12	0.99
	D8S505	8q13	1.16
	D13S153	13q14.2	1.03
	D14S985	14q32-q32.2	0.93
	D14S292	14q22-21	0.92
Body Mass Index	D15S1012	15q13.1-q15.1	1
	D18S53	18p11.32-p11.23	1.16
Waist Hip Ratio	D1S468	1p36.3	0.98
	D6S470	6p24.3	0.89
	D12S86	12q15-14	0.88

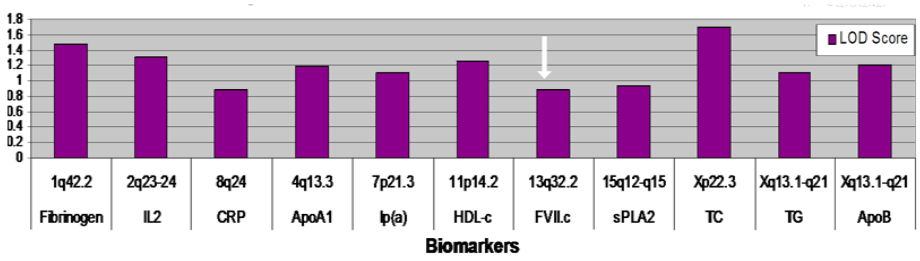

Figure 3. QTL analysis showing suggestive evidence of linkage of various chromosomal loci for the atherothrombotic biomarkers. Arrow denotes evidence of linkage of plasma FVII.c levels to 13q32.2 region.

Discussion

We carried out pilot linkage study using affected sibling pairs showing premature onset of CAD, selected from six Asian Indian families with a positive family history for CVD. To our knowledge, this is the first study of its kind, performed on the Indian population. We have identified several potential loci with suggestive evidence of linkage to CAD in the present study. Despite the low power of the study due to the small sample size and no single locus passing the threshold for confirmed linkage to CAD, we were able to identify several putative loci that have been reported to harbor putative candidate genes regulating inflammation and immune response process. This finding is of particular importance given our current understanding of atherosclerosis as a chronic inflammatory and auto-immune disease (41, 42).

There are several positive aspects to the study. Presence of family history has been traditionally considered as an independent risk factor for CAD (1). In the IARS, all subjects have

a strong family history of CVD, which can possibly enrich the CAD associated genes. Another important aspect is the selection of CAD affected subjects with premature onset of the disease; the average age at onset for CAD in the present study was around 50 years for males and less than 60 years for females.

Given the difficulty in recruiting large multi generation families, affected sibling pairs offer an attractive alternative method to conventional multi-generation pedigrees for undertaking linkage studies. The underlying basis of this method is the analysis of the pattern of sharing of risk alleles between the affected sibling pairs. Such an approach has been popular across various studies on CAD (4, 6-10, 43). An important consideration here is that since CAD is a late-onset disease, the parental generation may not be alive for participation in the study. In such an instance, siblings rather than parents-offspring-trios may be useful for linkage analysis. Furthermore, such studies also facilitate investigation on contributions of environmental factors, given that proband and their siblings belong to the same generation, may be of comparable age and might be exposed to similar environmental triggers. Furthermore, multi-point linkage analysis that simultaneously uses multiple markers to test for linkage at any given chromosomal locus serves as a powerful tool as it utilizes the haplotype information to infer IBD relation between affected sibling pairs (44).

Using the powerful tool of bioinformatics and the enormous genetic data available in public domain such as NCBI, we identified several interesting genes surrounding loci that showed suggestive evidence of linkage to CAD in the present study. For example, the PLA2G7 gene located on 6q21.1 chromosomal region encodes for the inflammatory protein, Lp-PLA(2). Over 25 prospective epidemiological studies have demonstrated the association of elevated Lp-PLA(2) levels with primary CVD events, recurrent events and stroke as reviewed by Corsan et al (45). The PLA2G7 was shown to be a potential functional candidate gene for CAD based on independent replication in two large cohorts, the CATHGEN and GENECARD (46). Further, strong multivariate-association was shown with Lp-PLA(2) activity for MEF2A (myocyte-specific enhancer factor-2), a DNA binding regulatory protein directed towards muscle-specific genes, in the Framingham Heart Study (47). Interestingly, the MEF2A gene is located within the 15q26 region that is yet another potential locus identified in the present study. In addition, inflammatory genes such as the IL20RA, IL22RA2, IFNGR1 and TNFAIP3 that play a key role in inflammation-induced atherosclerosis are also known to reside in the 6q23 region. Other salient findings include the 4q21 locus that harbors the APOA1 gene and the 8q24 locus that harbors the CRP gene, both of which are considered as important biomarkers of CAD progression (48-51).

We obtained suggestive evidence of linkage at the 13q32.1 locus for the FVII.c phenotype by QTL analysis. It is of interest to note that QTL analysis carried out previously in a subset of the IARS cohort showed suggestive linkage evidence of F7 SNP with FVII.c levels (LOD score – 1.82; $P = 0.002$) (17).

In conclusion, although the results of our pilot linkage study on genome wide scan using microsatellite markers in a selective cohort of Asian Indians is initial; they have helped to identify interesting loci showing suggestive evidence of linkage to CAD. These loci are known to harbor critical genes associated with inflammation and immune response which is in tune with our current understanding that atherosclerosis is an infection-mediated immuno-modulatory disease (52-55). Our early findings provide a basis for further investigations on a larger ASPs cohort already enrolled in the IARS. These findings will be integrated with the ongoing study on candidate genes, supported by functional and gene expression studies. Additionally, in depth bioinformatics analysis will be carried out on published linkage, QTL and genome wide association datasets to correlate and gauge the true potential of the interesting loci obtained in the present study. Such a convergent genomic approach will help to define a prioritized list of genetic markers for better risk stratification in the Asian Indian population.

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Competing Interests

The authors have no conflicts of interest to disclose.

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