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RESEARCH ARTICLE

Gene expression profiling of coronary artery disease and its relation with different severities

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Abstract. Global gene expression profiling is a powerful tool enabling the understanding of pathophysiology and subsequent management of diseases. This study aims to explore functionally annotated differentially expressed genes (DEGs); their biological processes for coronary artery disease (CAD) and its different severities of atherosclerotic lesions. This study also aims to identify the change in expression patterns of DEGs in atherosclerotic lesions of single-vessel disease (SVD) and triple-vessel disease (TVD). The weight of different severities of lesion was estimated using a modified Gensini score. The gene expression profiling was performed using the Affymetrix microarray platform. The functional annotation for CAD was performed using DAVID v6.8. The biological network gene ontology tool (BiNGO) and ClueGO were used to explore the biological processes of functionally annotated genes of CAD. The changes in gene expression from SVD to TVD were determined by evaluating the fold change. Functionally annotated genes were found in an unique set and could be distinguishing two distinct severities of CAD. The biological processes such as cellular migration, locomotion, cell adhesion, cytokine production, positive regulation of cell death etc. enriched the functionally annotated genes in SVD, whereas, wound healing, negative regulation of cell death, blood coagulation, angiogenesis and fibrinolysis were enriched significantly in TVD patients. The genes *THBS1* and *CAPN10* were functionally annotated for CAD in both SVD and TVD. The 61 DEGs were identified, those have changes their expression with different severities of atherosclerotic lesions, in which 13 genes had more than two-fold change in expression between SVD and TVD. The consistent findings were obtained on validation of microarray gene expression of selected 10 genes in a separate cohort using real-time PCR. This study identified putative candidate genes and their biological processes predisposing toward and affecting the severity of CAD.

Keywords. coronary artery disease; atherosclerosis; severity of lesion; gene expression; candidate genes; biological processes; north Indian population.

Introduction

Coronary artery disease (CAD) is a major cause of morbidity and mortality worldwide (Lopez *et al.* 1998; Watkins 2004). Initiation of CAD involves the translocation and modification of low-density lipoprotein (LDL) into intima of coronary artery that facilitate the extravasation of monocytes from the blood through endothelial cells. The severity of lesion progresses due to inflammation and deposition of lipid-laden contents within the intima of the coronary artery (Libby and Theroux 2005; Vitruk 2013; Gregersen *et al.* 2016). The cellular and molecular mechanisms of underlying atherosclerotic plaque are distinct for different types of lesions (Stary *et al.* 1995). Local as well as systemic inflammation along with lipid-, lipoprotein- and cholesterol-mediated biological processes in the coronary arteries play a prominent pathophysiological role in initiation to progression of atherosclerosis (Hansson *et al.* 2002; Libby *et al.* 2002).

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The genetic susceptibility of populations under specific environmental conditions causes disparity in the incidence of CAD, subsequently the expression of the gene. The Indian population is more susceptible to early onset of CAD even in the absence of traditional risk factors (Enas and Yusuf 1999; Mohan *et al.* 2001; Ramachandran *et al.* 2001; Ghaffar *et al.* 2004). This may suggest that the high incidence of CAD may be related to the expression of a different set of genes in this population. It has been reported that gene expression patterns correlated with the extent of atherosclerotic severity right from the onset and progression of CAD (Wingrove *et al.* 2008; Sinnaeve *et al.* 2009).

However, the chronic, inflammatory (Libby and Theroux 2005; Gregersen *et al.* 2016) and multifactorial (Poulter 1999) nature of atherosclerotic CAD presents challenges to identify precise candidate genes for the disease. The dynamic behaviour of the genome is directed by the composite interaction of genetic susceptibility with environmental factors. Also, the severity of CAD supports the presence of various intermediate phenotypes in peripheral blood and that may support the differential gene expression with different severities of atherosclerotic lesions (Wingrove *et al.* 2008; Sinnaeve *et al.* 2009). Gene expression from whole blood is believed to mimic the expression of genes from atherosclerotic coronary artery (Sinnaeve *et al.* 2009). The evaluation of gene expression for the CAD has resulted in promising clinical application such as diagnosis and scoring in the severity of CAD (Elashoff *et al.* 2011; Joshi *et al.* 2013).

Disease risk prediction is based on traditional risk factors that allow solely evaluating the future CAD risk but not early diagnosis (Assmann *et al.* 2002; Conroy *et al.* 2003; Neuhauser *et al.* 2005; Berger *et al.* 2010). Early and accurate noninvasive diagnosis of obstructive CAD has been the major clinical hurdle in the last decade. Global gene expression analysis has identified 23 genes that are used as constituents in gene expression score (GES) test algorithms (Lansky *et al.* 2012; Fletcher *et al.* 2013; Thomas *et al.* 2013). This gene expression-based CAD severity scoring test has proven its potential for evaluation of CAD risk, diagnosis and future cardiac events. To implicate the clinical application of gene expression, sufficient data from various populations with different sets of condition are still required. We believe that, exploring functionally annotated differentially expressed genes (DEGs) for CAD and different severities of atherosclerotic lesions may add significant information toward implementation of clinical application of gene expression. This study aims to explore functionally annotated DEGs and their biological processes for CAD and for different severities of atherosclerotic lesions. We also aim to identify pattern changes in expression of DEGs in atherosclerotic lesions of single-vessel disease (SVD) and triple-vessel disease (TVD).

Materials and methods

Patients and controls

Global gene expression profiling was performed in 12 CAD nondiabetic male patients and six age-matched controls. Validation of microarray data was performed by quantitative estimation of 10 gene expressions using real-time-polymerase chain reaction (RT-PCR) after recruiting 40 CAD patients and 15 age-matched controls from north Indian population. Information for traditional risk factors, prior cardiac events, cardiac interventions and other relevant disease history was collected from previous medical records or while directly interviewing the patients. Patients diagnosed with chronic stable angina (CSA), admitted for coronary angiography and who consented to be part of the study were recruited.

The well-described methods, modified Gensini score (GS) (Montorsi *et al.* 2006) and numbers of disease vessels involved were employed for estimation of severity of CAD. Patients of the SVD group were having stenosis more than 95% left anterior descending artery only with a GS score of 20–30, whereas patients of the TVD group were having stenosis more than 95% of all three major epicardial vessels with a GS score of 50–60. The GS provides insight into the atherosclerotic lesion on the basis of luminal diameter and importance of particularly involved coronary segments.

All clinical assessments were performed in fasting venous blood samples including lipid profile, glucose, sodium, potassium, creatinine, haemoglobin, total leukocyte and platelet counts. The underlying liver disease and inflammation were ruled out in patients and controls by investigating the erythrocyte sedimentation rate (ESR), liver function tests (LFTs) and C-reactive protein (CRP). The patients with a history of cardiac events (unstable angina and myocardial infarction (MI)), acute illness and history of malignancy were excluded from the study.

Sample preparation and gene expression profiling

Venous blood sample (5 ml) was collected in ethylenediaminetetraacetic acid (EDTA)-coated vials after overnight fasting using the radial approach with a 5F catheter before coronary angiogram. Written informed consent was obtained from all recruited participants. Total RNA was isolated using the TriZol reagent standard protocol. The isolated total RNA was quantified on NanoDrop 2000 spectrophotometers; absorbance at $A_{260\,\mathrm{nm}}$ and the purity was estimated by the ratio of absorbance $A_{260\,\mathrm{nm}}/A_{280\,\mathrm{nm}}$. RNA integrity was confirmed by nondenaturing agarose gel electrophoresis. RNA was stored at −80°C in nuclease-free water for gene expression experiments. Genomewide transcript profiling was assessed using Gene Chip Human Genome U133-A2

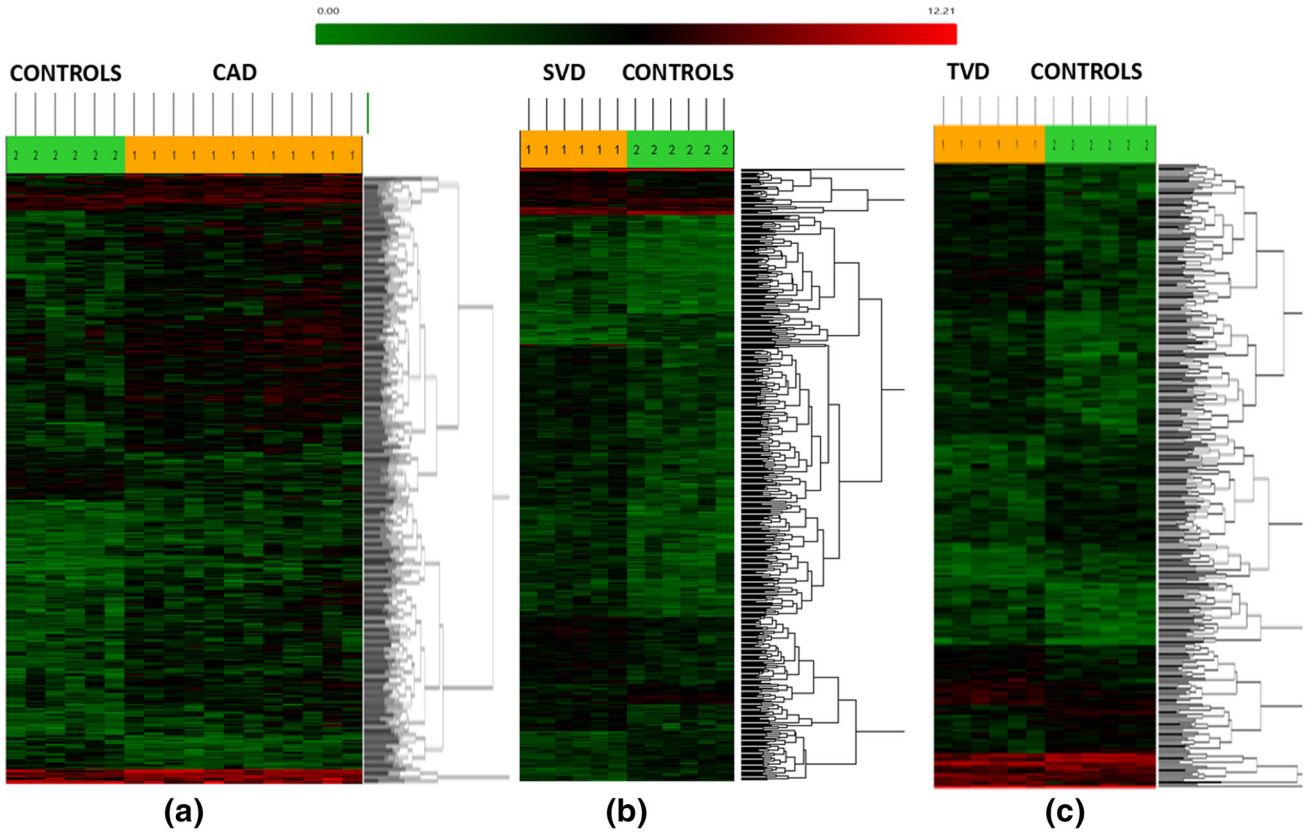

Figure 1. Hierarchical clustering analysis of DEGs for (a) CAD, (b) SVD, (c) TVD. The colour from green followed by dark green to red represents the lower to higher bi-weight (log 2) expression value of genes. The vertical axis clusters the DEGs and horizontal axis represents the samples.

oligonucleotide expression probe arrays (Affymetrix, Santa Clara, USA), comprising 22,000 probe sets. The experiments were performed according to the recommendations of the manufacturer (Lockhart *et al.* 1996). Data were normalized using a microarray suite 5 (MAS5) and quality control was evaluated using Affymetrix Expression Console software. The data have been reviewed and are available for public access at the Gene Expression Omnibus (GEO) repository under accession number GSE98583 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE98583). The analysis of differential expression of genes, data visualization and hierarchical clustering analysis (figure 1) were performed using Affymetrix Transcriptome Analysis Console (TAC) software (Torres *et al.* 2015). Functional annotation of DEGs for CAD was performed using Database for Annotation, Visualization and Integrated discovery v6.8 (DAVID v6.8) provided by the National Institutes of Health (NIH) (Huang *et al.* 2007). The genes were identified for persistent upregulation and downregulation by observing the fold change for SVD and TVD directly on output DEG list. The functionally annotated genes were subjected to assessment of biological processes in cytoscape v3.4.0 using the plug-ins BiNGO (Maere *et al.* 2005) and ClueGO.

Validation of microarray cohort gene expression with real-time PCR

A validation cohort of 40 CAD patients included 20 patients each in SVD and TVD and 15 coronary angiogram defined control subjects was recruited to validate the microarray data with the same inclusion criteria. The genes selected for real-time validation were *APOA1*, *CFTR*, *SRC*, *ICAM1*, *ESR1*, *THBD*, *SHANK2*, *ITGA1*, *IFT22* and *ZGAT*. The genes *APOA1* and *CFTR* expression represented CAD in all patients, *SRC* and *ICAM1* represented CAD in SVD and ESR1 and THBD showed modulated expression in TVD patients. Persistent upregulation was validated by estimating the expression of *SHANK2* and *ITGA1*. *IFT22* gene showed 13.3-fold in SVD and 4.0-fold in TVD, and were selected for real-time validation of higher expression in SVD patients. *ZGPAT* expression showed downregulation in SVD and upregulation in TVD, and its expression was validated using real-time PCR.

Table 1. Clinical characteristics of participants recruited to microarray cohort.

Clinical parameter	All patients CAD ($n = 12$)	Controls ($n = 6$)	P value for all patients versus controls	CAD (SVD) ($n = 6$)	P value for SVD versus controls	CAD (TVD) ($n = 6$)	P value for TVD versus controls
Age (year) male	55.5±8.31	52.0±7.4	0.387	56.1±8.5	0.391	55.0±8.8	0.540
Systolic BP (mm of Hg)	125.9±7.2	125.1±10.0	0.857	123.5±7.9	0.756	128.3±6.1	0.523
Diastolic BP (mm of Hg)	80.9±6.5	80.8±5.8	0.979	80.0±8.2	0.844	81.8±4.9	0.757
Pulse (per min)	76.4±3.1	79.6±1.9	0.036	77.8±2.7	0.210	75.0±3.0	**0.011**
Body mass index (kg/m^2)	25.7±1.7	27.4±2.9	0.141	26.5±1.7	0.515	24.9±1.6	0.109
Risk factors							
Hypertension (%)	50.0	33.3	0.494	66.7	0.558	0.0	–
Smoking							
Current (%)	0.0	0.0	–	0.0	–	0.0	–
Former	8.3	0.0	–	16.7	–	0.0	–
Tobacco chewing							
Current	16.7	0.0	–	33.3	–	0.0	–
Former	0.0	0.0	–	0.0	–	0.0	–
Laboratory investigations							
Serum creatinine (mg/dL)	0.9±0.3	0.9±0.3	0.874	0.8±0.2	0.483	1.1±0.2	0.392
Sodium (mg/dL)	138.3±3.1	139.8±1.8	0.304	137.8±3.8	0.279	138.8±2.5	0.455
Potassium (mg/dL)	4.1±0.2	4.3±0.3	0.306	4.2±0.2	0.502	4.1±0.3	0.309
Total leukocytes count (TLC) (×1000/μL)	7.9±1.5	7.9±1.5	–	7.4±1.7	0.668	8.3±1.3	0.629
Platelet count (PLT) (×1000/μL)	153.9±42.8	154.5±60.7	0.981	151.5±30.4	0.916	156.3±55.6	0.958
Hemoglobin (g/dL)	13.3±1.5	13.5±2.6	0.858	13.1±1.9	0.781	13.5±0.9	0.911
Glucose (mg/dL)	92.9±9.4	89.3±6.3	0.416	91.1±13.5	0.770	94.6±1.9	0.079
Total cholesterol (TC) (mg/dL)	138.7±20.8	134.5±7.4	0.538	145.6±17.9	0.189	131.8±22.8	0.795
Triglyceride (TG) (mg/dL)	117.5±31.2	105.6±35.0	0.437	119.3±27.6	0.471	115.8±37.0	0.636
High-density lipoprotein (HDL) (mg/dL)	35.3±10.6	31.8±9.4	0.507	36.6±14.1	0.503	34.0±6.7	0.658
Low-density lipoprotein (LDL) (mg/dL)	77.7±21.5	79.0±19.5	0.906	81.0±26.7	0.885	74.5±16.7	0.677
Very low-density protein (VLDL) (mg/dL)	25.5±10.8	22.6±6.4	0.568	28.0±13.7	0.409	23.0±7.5	0.936
Medications used before hospitalization							
Aspirin (%)	25.0	16.6		100		50.0	
Clopidogrel (%)	16.7	16.6		33.3		33.3	
Ramipril (%)	16.7	33.6		16.1		16.7	
Metoprolol (%)	25.0	0.0		100		50.0	
Statin (%)	25.0	33.3		16.1		33.3	
Medication advised at discharge							
Clopidogrel (%)	100	0.0		100		100	
Aspirin (%)	100	0.0		100		100	
Ramipril (%)	100	0.0		100		100	
Metoprolol (%)	100	0.0		100		100	
Statin (%)	100	0.0		100		100	
Nitrates (%)	100	0.0		100		100	

Primers for real-time PCR amplification were designed using the PrimerQuest program (IDT, Coralville, USA; table 1 in electronic supplementary material at http://www.ias.ac.in/jgenet/). The first stand cDNA synthesis protocol was used for cDNA synthesis, using MLV reverse transcriptase (SuperScript VILOTM cDNA, Invitrogen, Carlsbad, USA) and priming by random primers (Invitrogen) under PCR conditions 10 min at 25°C, 60 min at 42°C and the reaction was terminated at 85°C for 5 min. A total volume of 20 μL reaction mixture was prepared in duplicate with a concentration of 1 ppm of each forward and reverse gene-specific primers, 2 μL of synthesized cDNA and 5 μL SYBR green Master Mix. Real-time quantitative PCR was performed with the lightcycler LC480 (Roche, USA) using SYBR green PCR technology with the SYBR premix (Applied Biosystem, Foster City, USA). The PCR cycling was as follows: 50°C for 2 min for 1 cycle, 95°C for 10 min for 1 cycle, 95°C for 15 s and 60°C for 1 min for 40 cycles. The *GAPDH* gene was used for each sample as an internal control. The end point used in the real-time PCR quantification, cycle threshold (Ct), was defined as the PCR cycle number that crosses an arbitrarily placed signal threshold. Gene expression was presented using a modification of the 2^{-Ct} method (Livak and Schmittgen 2001). The expression of each housekeeping gene was presented as 2^{-Ct} where Ct (Ct time $\times$ −Ct time 0) and time 0 represents the 1$\times$ expression of each gene. Data were analysed using LightCycler 480 software, ver. 1.5 (Roche, USA). Additionally, the specificity was confirmed by performing dissociation curves and specific PCR products were further verified using agarose electrophoresis.

Statistical analysis

The demographic profiles of patients and controls were analysed for their difference in CAD, severity of CAD groups using the SPSS 20.0 statistical package (SPSS, Chicago, USA). The values of continuous data were presented as mean±standard deviation, analysed by Student's *t*-test and one-way ANOVA test. Categorical variable data were analysed using the chi-square test and presented in numbers and percentage. A P value <0.05 was considered as statistically significant. The differential expression for CAD and severity of CAD analysed using Affymetrix TAC software with $P<0.05$ and linear fold changes ≥ 2.0 using the un-paired ANOVA statistical test. The DEGs of this study were compared with the genes list from the global gene expression study by searching for gene symbols on the find tool in Microsoft Excel 2016.

The study was approved by institutional ethics committee (IEC code: 2013-67-SRF-70). All procedures performed in this study involving recruitment of human participants were in accordance with the ethical standards of the institutional ethics committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Results

Demographics

The gene expression data from 12 CAD patients (mean age: 55.5±8.31 years) and controls (mean age: 52.0±7.4 years) were analysed to find DEGs for CAD, SVD and TVD. Patients and controls had no significant difference in height, weight, body mass index (BMI), systolic and diastolic blood pressure, also no significant difference was noticed in risk factors like hypertension, smoking status and tobacco chewing. The clinical variables like ESR, LFTs, CRP, hemoglobin, total leukocytes count, platelet count and lipid profile were not significantly different in patients compared with controls. Ages of patients in the SVD and TVD groups were comparable (56.1±8.5 versus 55.0±8.8, P=0.82). Patients with different severities of CAD had no significant difference in risk factors, systolic and diastolic blood pressure, pulse, height, weight, BMI and laboratory investigations. The demographic profiles of the patients and controls are summarized in table 1. Clinical characteristics of a validation cohort were comparable with no significant difference in patients compared with the controls (table 2 in electronic supplementary material). Patients and controls were selected to evaluate gene expression profiling in the microarray as well as in the validation cohort, both of which were comparable for all clinical parameters. Since patients and controls were comparable for all clinical parameters other than the presence and severity of CAD, the differential gene expression is expected to be due to CAD and its severity alone.

Gene expression profile for CAD and severity of CAD

Gene expression data analysis for 12 CAD patients and six controls listed 394 DEGs of which 331 were found to be upregulated and 63 were found to be downregulated ($\geq$2.0-fold change, $P<0.05$). The expression analysis in comparison with severity of CAD identified 424 genes upregulated and 97 genes downregulated DEGs, in SVD compared with the controls and 239 genes upregulated and 125 genes downregulated in TVD compared with the controls (tables 3–5 in electronic supplementary material). A distinct differential gene expression pattern was obtained in the TVD patients with 170 upregulated genes and 394 downregulated genes in comparison with the SVD patients. The gene *CYP3A5* had >10-fold upregulation and *DCC*, *THRAP3* and *SH3GL2* had >10-fold downregulation in patients with TVD when compared with SVD.

Table 2. Functionally annotated genes for CAD and different severities of lesion (SVD and TVD).

	Functionally annotated genes for SVD		Functionally annotated genes for TVD		Functionally annotated genes for CAD	
	Symbol	Gene name	Symbol	Gene name	Symbol	Gene name
1	*ALX4*	ALX homeobox 4	*ADAMTS6*	ADAM metallopeptidase with thrombospondin type-1 motif 6	APOA1	Apolipoprotein A1
2	*APOA1*	Apolipoprotein A1	*CAPN10**	Calpain 10	APOA4	Apolipoprotein A4
3	*APOC3*	Apolipoprotein C3	*CD300A*	CD300a molecule	CAPN10*	Calpain 10
4	*ARHGAP35*	Rho GTPase activating protein 35	*CEL*	Carboxyl ester lipase	CYP2C19	Cytochrome P450 family 2 subfamily C member 19
5	*CAPN10**	Calpain 10	*ESR1*	Estrogen receptor 1	HFE	Hemochromatosis
6	*CAPN5*	Calpain 5	*EXT1*	Exostosin glycosyltransferase 1	IL6ST	Interleukin 6 signal transducer
7	*CCL11*	C–C motif chemokine ligand 11	*GRK4*	G protein-coupled receptor kinase 4	MTHFR	Methylenetetrahydrofolate reductase
8	*CYP2C19*	Cytochrome P450 family 2 subfamily C member 19	*HLA-DRB1*	Major histocompatibility complex, class II, DR beta 1	NES	Nestin
9	*CYP2C9*	Cytochrome P450 family 2 subfamily C member 9	*HNF4A*	Hepatocyte nuclear factor 4 alpha	NRXN3	Neurexin 3
10	*CYP3A4*	Cytochrome P450 family 3 subfamily A member 4	*HTR1D*	5-Hydroxytryptamine receptor 1D	PTGIS	Prostaglandin I2 synthase
11	*DNASE1*	Deoxyribonuclease 1	*IL1RN*	Interleukin 1 receptor antagonist	PTPRD	Protein tyrosine phosphatase, receptor type D
12	*DTNB*	Dystrobrevin beta	*LAMA4*	Laminin subunit alpha 4	SORCS3	Sortilin-related VPS10 domain containing receptor 3
13	*EBF2*	Early B-cell factor 2	*LARGE1*	LARGE xylosyl- and glucuronyltransferase 1		
14	*G6PC2*	Glucose-6-phosphatase catalytic subunit 2	*MCTP1*	Multiple C2 and transmembrane domain containing 1		
15	*HFE*	Hemochromatosis	*MSR1*	Macrophage scavenger receptor 1		
16	*ICAM1*	Intercellular adhesion molecule 1	*MSX2*	Msh homeobox 2		
17	*ID3*	Inhibitor of DNA binding 3, HLH protein	*PALLD*	Palladin, cytoskeletal associated protein		
18	*IL6ST*	Interleukin 6 signal transducer	*RSRP1*	Arginine- and serine-rich protein 1		

Table 2 (*contd*)

	Functionally annotated genes for SVD		Functionally annotated genes for TVD		Functionally annotated genes for CAD
19	*LAMA4*	Laminin subunit alpha 4	*THBD*	Thrombospondin 1	
20	*LBP*	Lipopolysaccharide binding protein	*THBS1**	Thrombomodulin	
21	*MTHFR*	Methylenetetrahydrofolate reductase	*VANGL1*	VANGL planar cell polarity protein 1	
22	*NES*	Nestin	*VEGFB*	Vascular endothelial growth factor B	
23	*NRG1*	Neuregulin 1	*ZNF492*	Zinc-finger protein 492	
24	*SMAD3*	SMAD family member 3			
25	*TGFB2*	Transforming growth factor beta 2			
26	*THBS1**	Thrombospondin 1			

The functionally annotated genes for CAD represented in gene symbols and genes names. Total of 26 and 23 genes were functionally annotated with CAD in SVD and TVD. The genes with * were functionally annotated in SVD as well as TVD.

The genes *GULP1*, *PMCHL1*, *CAPN10*, *IFT22* and *HLA-DRB4* were upregulated and *ARSJ* was downregulated by more than 10-fold in CAD as a whole.

In comparison of different severities of CAD, 23 DEGs identified to be progressively upregulated from the control group to SVD to TVD (table 6 in electronic supplementary material). Four genes had consistent downregulation with the severity of CAD (SVD to TVD). SVD had significantly higher expression in 32 genes compared with TVD and controls, and two genes were noticed to be downregulated in SVD and upregulated in TVD (table 7 in electronic supplementary material). In total, 13 genes were found to have more than two-fold difference in expression change between SVD and TVD.

Functional annotation for DEGs

The DEGs identified for CAD and severity of CAD were projected for functional annotation in the online tool DAVID ver. 6.8. Functional annotation identified 12 genes involved with CAD from all DEGs identified for CAD patients compared with the controls. Whereas, functional annotation analysis for different severities of CAD identified 26 DEGs for SVD showed functional enrichment with atherosclerosis, CAD, MI and acute coronary syndrome (ACS). Similarly, the TVD group had 23 DEGs, and those were found to have functional annotation for atherosclerosis and MI (table 2). Another set of six DEGs *CBL*, *FKBP5*, *IL1RN*, *IL2RA*, *HLA-DRB1* and *HLA-DRB4* of the TVD group belongs to the processes of inflammation. The functional annotation identified that these genes were unique to SVD and TVD, except for *THBS1* and *CAPN10* endorsed CAD in patients with SVD as well as TVD. The genes *APOA4*, *NRXN3*, *PTGIS*, *PTPRD* and *SORCS3* were unique to all CAD patients, whereas the genes *APOA1*, *CYP2C19*, *HFE*, *IL6ST*, *MTHFR* and *NES* were found representing CAD in all patients and SVD. The functionally annotated DEGs in TVD patients largely reported enriched to MI. However, in patients with SVD most of the functionally annotated DEGs were enriched to CAD and ACS.

Biological network gene ontology (GO) analysis of functionally annotated genes

The analysis of overrepresented GO terms of biological processes (adjusted $P<0.05$) enriched to functionally annotated genes for CAD and its different severities of atherosclerotic lesions (SVD and TVD) (table 3) revealed that *APOA1*/4 plays a major role for CAD in this dataset. The GO terms GO30300; regulation of intestinal cholesterol absorption, GO17873; positive regulation of cholesterol esterification, GO17872; regulation of cholesterol esterification, GO33700; phospholipid efflux, GO65005; protein–lipid complex assembly, GO34377 and plasma

Table 3. Biological processes enriched to functionally annotated genes of CAD, SVD and TVD.

GO-ID	*P* value	Adjusted *P* value	Biological processes	Functionally annotated genes
			Biological processes for CAD	
30300	9.66E–06	3.54E–03	Regulation of intestinal cholesterol absorption	*APOA1*, *APOA4*
10873	1.35E–05	3.54E–03	Positive regulation of cholesterol esterification	*APOA1*, *APOA4*
10872	1.80E–05	3.54E–03	Regulation of cholesterol esterification	*APOA1*, *APOA4*
33700	2.89E–05	3.83E–03	Phospholipid efflux	*APOA1*, *APOA4*
65005	4.24E–05	3.83E–03	Protein–lipid complex assembly	*APOA1*, *APOA4*
34377	4.24E–05	3.83E–03	Plasma lipoprotein particle assembly	*APOA1*, *APOA4*
44255	5.22E–05	3.83E–03	Cellular lipid metabolic process	*PTGIS*, *APOA1*, *APOA4*, *CYP2C19*, *IL6ST*
70103	8.39E–04	1.27E–02	Regulation of interleukin-6-mediated signaling pathway	*IL6ST*
70104	8.39E–04	1.27E–02	Negative regulation of interleukin-6-mediated signaling pathway	*IL6ST*
70106	8.39E–04	1.27E–02	Interleukin-27-mediated signalling pathway	*IL6ST*
10106	8.39E–04	1.27E–02	Cellular response to iron ion starvation	*HFE*
42221	4.78E–03	3.29E–02	Response to chemical stimulus	*CAPN10*, *APOA4*, *CYP2C19*, *IL6ST*, *NES*
			Biological processes for SVD	
32879	1.26E–08	1.64E–05	Regulation of localization	*TGFB2*, *CCL11*, *SMAD3*, *CAPN10*, *LAMA4*, *APOA1*, *APOC3*, *NRG1*, *IL6ST*, *THBS1*, *ICAM1*
30334	6.20E–08	2.14E–05	Regulation of cell migration	*TGFB2*, *CCL11*, *SMAD3*, *LAMA4*, *IL6ST*, *THBS1*, *ICAM1*
30335	7.03E–08	2.14E–05	Positive regulation of cell migration	*TGFB2*, *CCL11*, *SMAD3*, *IL6ST*, *THBS1*, *ICAM1*
51272	1.05E–07	2.14E–05	Positive regulation of cellular component movement	*TGFB2*, *CCL11*, *SMAD3*, *IL6ST*, *THBS1*, *ICAM1*
40017	1.05E–07	2.14E–05	Positive regulation of locomotion	*TGFB2*, *CCL11*, *SMAD3*, *IL6ST*, *THBS1*, *ICAM1*
30155	1.67E–07	2.43E–05	Regulation of cell adhesion	*TGFB2*, *SMAD3*, *LAMA4*, *NRG1*, *THBS1*, *ICAM1*
1817	1.04E–06	1.05E–04	Regulation of cytokine production	*TGFB2*, *SMAD3*, *APOA1*, *LBP*, *IL6ST*, *THBS1*
50776	2.52E–06	2.19E–04	Regulation of immune response	*TGFB2*, *SMAD3*, *APOA1*, *LBP*, *IL6ST*, *ICAM1*
42221	1.86E–06	1.73E–04	Response to chemical stimulus	*CYP2C9*, *TGFB2*, *CCL11*, *SMAD3*, *CAPN10*, *ID3*, *LBP*, *CYP2C19*, *CYP3A4*, *IL6ST*, *THBS1*, *NES*
48522	7.70E–06	4.79E–04	Positive regulation of cellular process	*TGFB2*, *CCL11*, *SMAD3*, *APOA1*, *EBF2*, *NRG1*, *THBS1*, *ICAM1*, *CAPN10*, *ALX4*, *ID3*, *LBP*, *IL6ST*
48518	2.27E–05	9.27E–04	Positive regulation of biological process	*TGFB2*, *CCL11*, *SMAD3*, *APOA1*, *EBF2*, *NRG1*, *THBS1*, *ICAM1*, *CAPN10*, *ALX4*, *ID3*, *LBP*, *IL6ST*
1819	2.86E–05	1.01E–03	Positive regulation of cytokine production	*SMAD3*, *LBP*, *IL6ST*, *THBS1*
			Biological processes for TVD	
61041	3.24E–05	2.30E–02	Regulation of wound healing	*THBD*, *VEGFB*, *THBS1*
51918	9.17E–05	3.26E–02	Negative regulation of fibrinolysis	*THBD*, *THBS1*
51917	1.59E–04	3.27E–02	Regulation of fibrinolysis	*THBD*, *THBS1*
30194	1.85E–04	3.27E–02	Positive regulation of blood coagulation	*THBD*, *THBS1*
50820	2.75E–04	3.27E–02	Positive regulation of coagulation	*THBD*, *THBS1*
2690	5.10E–04	3.27E–02	Positive regulation of leukocyte chemotaxis	*VEGFB*, *THBS1*
51897	5.10E–04	3.27E–02	Positive regulation of protein kinase B signalling cascade	*VEGFB*, *THBS1*

Table 3 (*contd*)

GO-ID	*P* value	Adjusted *P* value	Biological processes	Functionally annotated genes
43066	1.97E–03	3.73E–02	Negative regulation of apoptosis	*MSX2*, *VEGFB*, *ESR1*, *THBS1*
43069	2.07E–03	3.73E–02	Negative regulation of programmed cell death	*MSX2*, *VEGFB*, *ESR1*, *THBS1*
2687	9.30E–04	3.27E–02	Positive regulation of leukocyte migration	*VEGFB*, *THBS1*
42221	7.11E–04	3.27E–02	Response to chemical stimulus	*THBD*, *IL1RN*, *MSX2*, *CAPN10*, *HNF4A*, *HTR1D*, *ESR1*, *THBS1*
10033	1.22E–03	3.27E–02	Response to organic substance	*THBD*, *IL1RN*, *MSX2*, *CAPN10*, *ESR1*, *THBS1*
32879	3.45E–03	4.30E–02	Regulation of localization	*MSR1*, *CAPN10*, *LAMA4*, *VEGFB*, *THBS1*

GO, gene ontology; *P* value of significance was adjusted by the Benjamini and Hochberg false discovery rate correction.

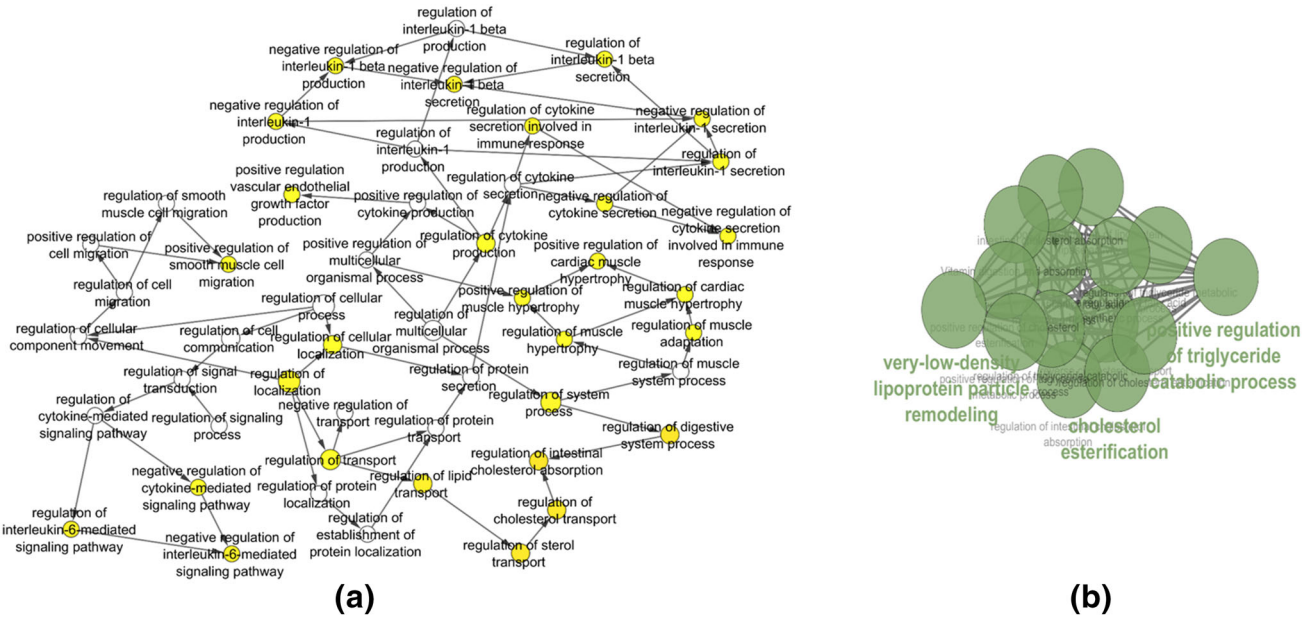

Figure 2. (a) GO term networks and (b) biological processes of functionally annotated genes for CAD as whole clustered in similar functions. Biological processes enriched to functionally annotated genes for CAD represented in GO terms a BiNGO analysis and clustered to similar functions (ClueGO analysis). The significance of enrichment is represented by yellow color ($P<0.05$).

lipoprotein particle assembly were enriched most significantly to *APOA1*/4 as BiNGO analysis. The genes *PTGIS*, *APOA1*/4, *CYP2C19* and *IL6ST* enriched to GO term GO44255; cellular lipid metabolic processes. *IL6ST* is enriched to regulation of the interleukin-6-mediated signalling pathway; GO70103. The GO10106 enriched to HFE-mediated cellular response to iron ion starvation. The term response to chemical stimulus GO42221 is enriched to CAPN10, APOA4, CYP2C19, IL6ST and NES (figure 2, a&b).

The GO term analysis of biological processes for functionally annotated genes related to severity of CAD (SVD and TVD) revealed that different severities may be differentiated based on the underlying biological processes involved. The biological processes related to the CAD in SVD (lower severity) were most significantly enriched to GO terms such as regulation of localization; GO32879 (*TGFB2*, *CCL11*, *SMAD3*, *CAPN10*, *LAMA4*, *APOA1*, *APOC3*, *NRG1*, *IL6ST*, *THBS1* and *ICAM1*), regulation of cell migration; GO30334 (*TGFB2*, *CCL11*, *SMAD3*, *LAMA4*, *IL6ST*, *THBS1* and *ICAM1*) and positive regulation of cell migration; GO30335. The genes *TGFB2*, *CCL11*, *SMAD3*, *IL6ST*, *THBS1* and *ICAM1* enriched to the GO terms; GO51272 and GO40017, those were described for positive regulation of cellular component movement and positive regulation of locomotion, respectively. The GO term GO30155 was described related to the regulation of cell adhesion enriched to functionally annotated genes *TGFB2*, *SMAD3*, *LAMA4*, *NRG1*, *THBS1* and *ICAM1*. Also, the functionally annotated

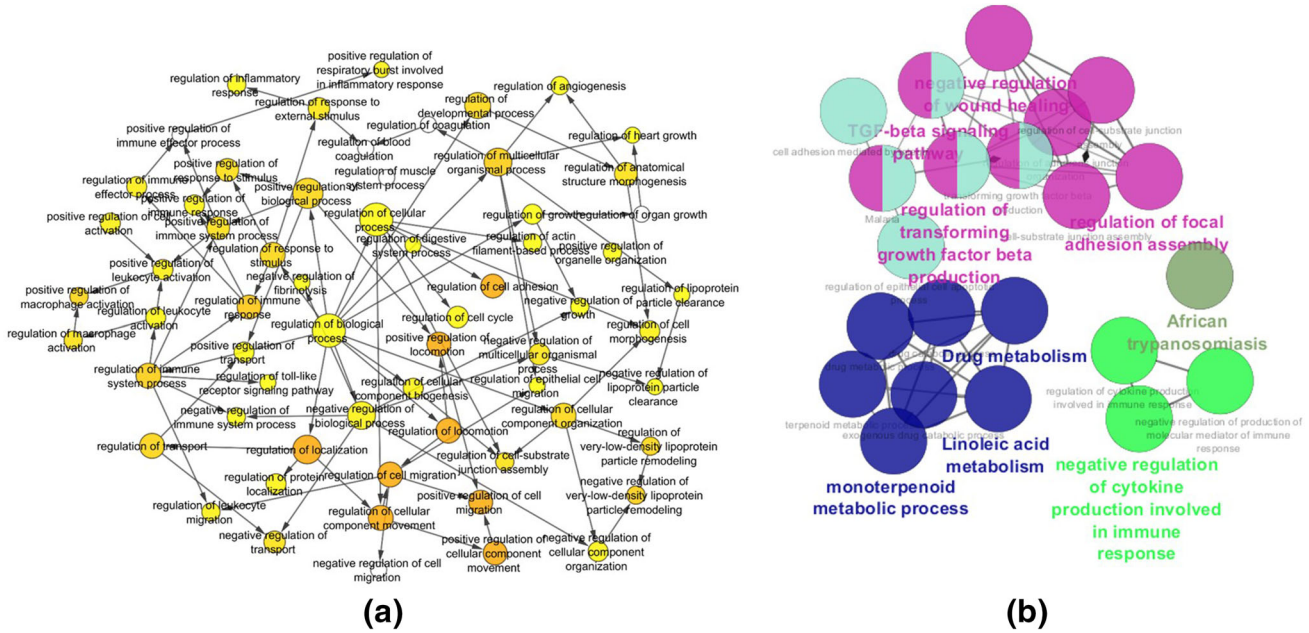

Figure 3. (a) GO term networks and (b) clustered biological processes in similar function for 26 functionally annotated DEGs identified for CAD in SVD. The colour gradient of node from yellow to reddish yellow represents the increasing significance ($P<0.05$) of association for biological processes with SVD in BiNGO network and different colours represent the clustering of biological processes in similar functions with significance of association $P<0.05$ in ClueGO network. The GO network shows the biological processes of regulation of immune response, regulation of biological processes, regulation of locomotion, positive regulation of apoptosis noticed significant in SVD and nonsignificant in TVD. The biological processes cell adhesion and immune response were clustered for SVD.

genes for SVD enriched to GO1817; regulation of cytokine production, GO42221; response to chemical stimulus and regulation of immune response; GO50776 and positive regulation of cell death (GO; 43068, 1817) were significantly associated with lower severity of CAD (SVD) (figure 3, a&b).

The BiNGO and ClueGO analyses for functionally annotated genes of CAD in TVD reveal the GO terms, GO61041; regulation of wound healing (*THBD*, *VGEFB* and *THBS1*), regulation of fibrinolysis; GO51918 and GO51917, regulation of blood coagulation; GO30194 and GO50820 (*THBD* and *THBS1*). The genes such as *THBD*, *IL1RN*, *MSX2*, *CAPN10*, *HNF4A*, *HTR1D*, *ESR1* and *THBS1* were enriched to response to chemical stimulus. The genes *MSX2*, *VEGFB*, *ESR1* and *THBS1* were found to be related to GO terms positive regulation of angiogenesis and negative regulation of apoptosis; GO43066 and GO43069, respectively (figure 4, a&b).

The biological processes enriched to different severities reveal changing underlying pathophysiology of CAD with different severities. For lesser severity of disease (SVD), the operating processes are those involved in cell migration, locomotion, adhesion, cytokine production, immune response and positive regulation of cell death. Whereas, for higher severity of CAD, i.e. TVD, processes significantly altered are those involved in wound healing, blood coagulation, angiogenesis, negative regulation of cell death etc.

Gene identified differentially expressed for CAD from global expression profiling

While comparing DEGs of our study that are intercepted with the other global gene expression studies for CAD, we were able to identify the genes *ACO2*, *TP53I11*, *GAMT*, *PDK2*, *PDE9A*, *DDX11*, *IGHA1*, *GTF2I* and *ID3* as shown in PREDICT and CATHGEN study, *TP53I11*, *HLCS*, *ZMYND10* and *CA12* reported by Sinnaeve *et al.* (2009), genes *WIF1*, *DCX*, *COL4A3*, *SERPINB13*, *MYH10*, *GGA2* and *CES1* intercepted to DEGs listed by Wingrove *et al.* (2008), *MAP7*, *TP53I11*, *TNFRSF21*, *FSTL3*, *ARHGEF10*, *VEGFB*, *DGKA*, *ID3* and *HLA-DOA* reported in a study by Taurino *et al.* (2010) and Abdullah *et al.* (2012) reported DEGs *NSUN7*, *ERAP2* and *MAPRE3* intercepted in our study. Two genes *MPRIP* and *MAP7* were identified to be reported differentially expressed for CAD from south Indian population (Arvind *et al.* 2015) and this study (table 8 in electronic supplementary material).

The evaluation of DEGs' interception in different global gene expression studies involving different populations to identify common genes for CAD, yielded very few genes. Therefore, identification of a unique signature for CAD globally as treatment target and early diagnostic biomarker needs to be further explored. The expression patterns of DEGs may be very different among studies; however, the underlying biological processes related

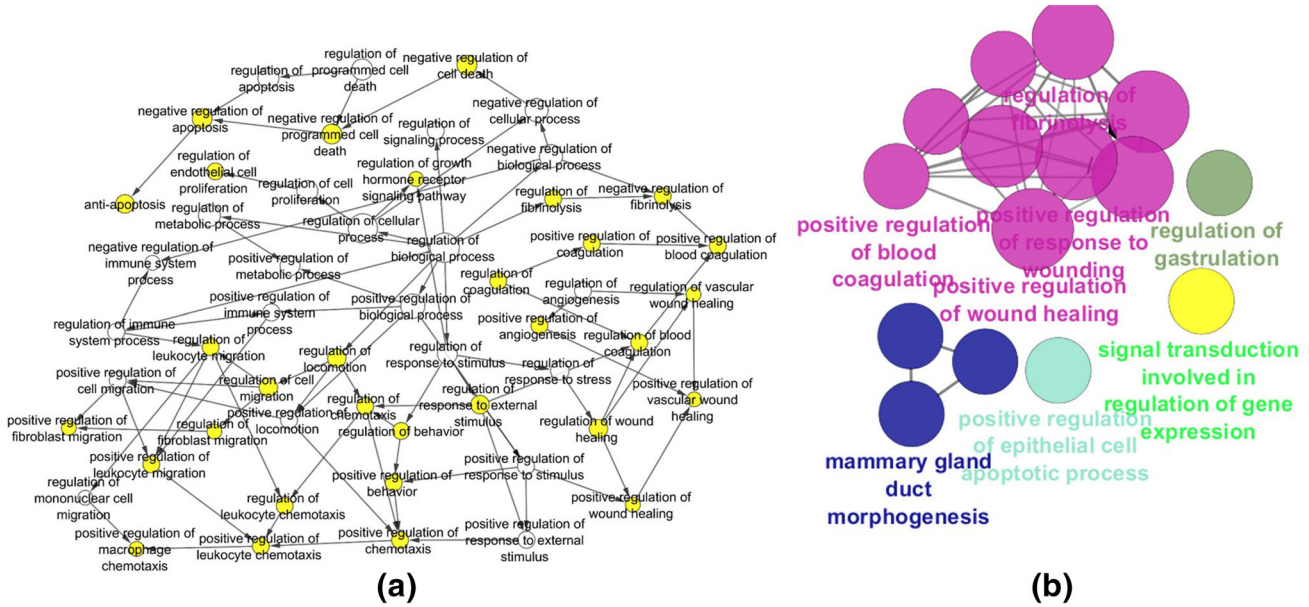

Figure 4. (a) GO term networks and (b) clustered biological processes in similar function for 23 functionally annotated DEGs for CAD in TVD. The colour gradient of node from yellow to reddish yellow represents the increasing significance ($P<0.05$) of association for biological processes with CAD in BiNGO network and different colours represent the clustering of biological processes in similar functions with significance of association $P<0.05$ in ClueGO network. The biological processes such as coagulation, wound healing, positive regulation of apoptosis, regulation of fibrinolysis are significant in TVD and nonsignificant in SVD. Similarly, regulation of wound healing, coagulation fibrinolysis were found significantly clustered for TVD.

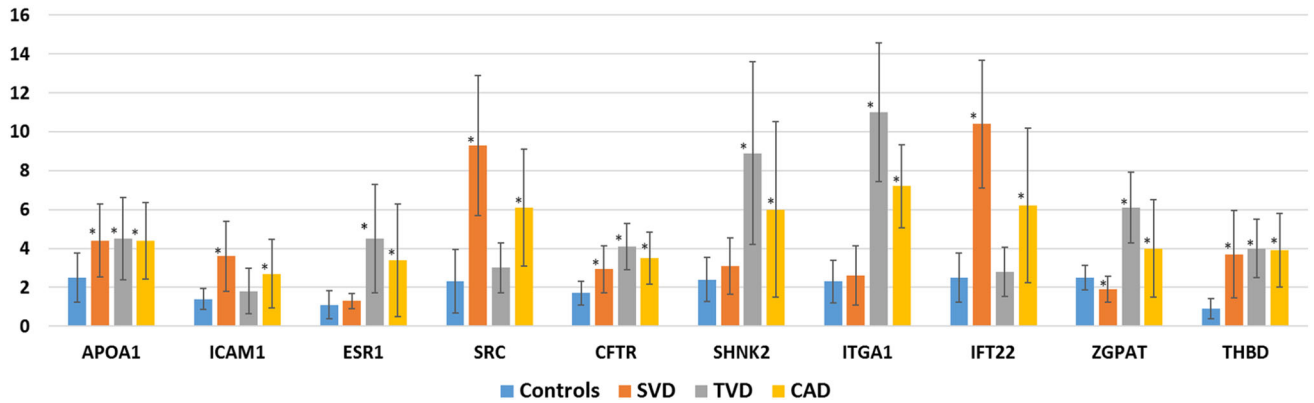

Figure 5. The mean fold expression of 10 genes validating the expression patterns of microarray data using real-time PCR for CAD, SVD and TVD compared with the controls. The horizontal axis shows the genes name and vertical axis represent expression of the genes. The colours of bar, blue, brown, grey and yellow are assigned for the controls, CAD, SVD and TVD groups, respectively. *$P<0.05$ compared with the controls.

to those genes have shown significant degree of similarity.

Validation of microarray data

The mean expression fold change was calculated for genes *APOA1*, *CFTR*, *SRC*, *ICAM1*, *ESR1*, *SHANK2*, *ITGA1*, *IFT22*, *ZGPAT* and *THBD* (figure 5). The analysis results replicated the results of expression as represented by microarray cohort. The mean mRNA expression fold changes of genes *SHAKK2* and *ITGA1* was highest in TVD followed by SVD patients followed by the controls. *IFT22* showed higher expression (10.48±4.29, 2.81±1.26 and 2.35±1.09) in SVD followed by TVD and controls. The gene *ZGPAT* was downregulated in SVD patients (2.56±0.62 versus 1.96±0.67) and upregulated in TVD patients (6.17±1.82 versus 2.56±0.62). *THBD* showed higher expression value in TVD patients. The gene *APOA1* had higher mean expression in CAD patients (4.49±1.97 versus 2.50±1.25, $P=0.001$); SRC (9.33±3.59

versus 2.38±1.63, $P<0.001$) and ICAM1 (3.61±1.79 versus 1.48±0.54, $P<0.001$) for SVD patients and *ESR1* (4.53±2.80 versus 1.11±0.72, $P<0.001$) for TVD patients compared with the controls.

Discussion

This is the first study looking at the differential expression of genes reflecting different severities of atherosclerotic CAD. Interestingly, functionally annotated genes for CAD were different for SVD and TVD, and reflecting distinct involved biological processes. In addition, a total of 61 genes were found to have differing expression in lesions of different severities. These include genes having persistent upregulation and downregulation, genes having significantly higher expression in SVD and the genes having downregulation in SVD and upregulation in TVD. This indicates that these genes may have peculiar expression patterns reflecting two distinct severities of lesions. Since attempts are being made to formulate a gene expression scoring system of 23 genes with different severities of atherosclerotic lesions (as measured by invasive techniques such as quantitative coronary angiogram (McPherson *et al.* 2013), computerized tomography angiogram (Voros *et al.* 2014) and intravascular ultrasound with radiofrequency backscatter analysis (IVUS/VH) (Joshi *et al.* 2013)), therefore, our study has potential to further contribute towards development of a gene expression profile score in Asian Indians, who per se have earlier onset and greater severity of CAD when compared with the West. We, henceforth, discuss the relevance of separate DEGs for CAD (as a whole), SVD and TVD separately.

A focussed functional annotation analysis of DEGs enriched to CAD identified *APOA1*/4, *CYPC19*, *IL6ST*, *PTGIS* and *MTHFR* genes which are well-known for CAD as reported by various studies. *APOA1* and *APOA4* are genes involved in cholesterol absorption from the intestine, cholesterol esterification and reverse cholesterol transport (Steinmetz and Utermann 1985). Polymorphisms in *CYP2C19* involved in drug metabolism and synthesis of cholesterol, steroids and other lipids, increase risk of atherosclerosis (Ercan *et al.* 2008). *IL6ST* is known to have a role in plaque development and increases the risk of developing MI and atherosclerosis (Luchtefeld *et al.* 2007). *PTGIS* encodes prostacyclin and its physiological antagonist thromboxane A2 known to promote atherosclerosis (Lemaitre *et al.* 2009; Di Taranto *et al.* 2012). The chromosomal region 1p36.3 mapped methylenetetrahydrofolate reductase (*MTHFR*) gene encodes an enzyme regulating the metabolism of homocysteine (Hcy) and is accepted as a potential candidate gene for CAD (Frosst *et al.* 1995). The gene *NES* has been associated with vulnerable plaque (Meng *et al.* 2008; Fittipaldi *et al.* 2014). The new genes identified from this study which modulate CAD are *NRXN3*, *PTPRD* and *SORCS3*. *NRXN3* has been reported to modulate waist circumference (Heard-Costa *et al.* 2009). *PTPRD* regulates plasma Hcy levels. *SORCS3* belongs to a family of vacuolar protein sorting receptors, a group of molecules involved in intracellular trafficking, other constituents of this family (*SORT1*) have been associated with atherosclerosis (Lane *et al.* 2012). The mechanistic contribution of these genes towards driving atherosclerosis merits further investigation.

Functional annotation of DEGs for severity of CAD identified a unique pattern of genes for SVD and TVD. After functional annotation, genes *THBS1* and *CAPN10* were found to be representing CAD in SVD and TVD. *THBS1* play a role in promoting platelet aggregation (Isenberg *et al.* 2007) as well as inhibition of angiogenesis (Ren *et al.* 2006) and *CAPN10* gene was reported associated with type-2 diabetes mellitus (Horikawa *et al.* 2000) and atherosclerosis (Goodarzi *et al.* 2005).

Genes *CYP2C9*/19, *ICAM1*, *SMAD3* and *TGFB2* functionally annotated for SVD have been reported in association with CAD. *ICAM1* plays an important role in movement of leukocytes into vascular intima through cell adhesion and endothelium activation (Hayflick *et al.* 1998; Blankenberg *et al.* 2003) and development of atherosclerosis (Poston *et al.* 1993). The gene *CYP2C9* involved in metabolism of arachidonic acid to epoxyeicosatrienoic acids, which plays a key role in antiapoptotic, inflammatory and thrombotic activity and endothelial homeostasis (Zeldin 2001; Spiecker and Liao 2005; Zordoky and El-Kadi 2010). Similarly, the roles of *SMAD3* and *TGFB2* have been reported in association with CAD through the TGF-β/SMAD3 signalling pathway (Peng *et al.* 2016).

The genes functionally annotated with CAD in TVD play a role in atherosclerosis. The *ESR1* gene identified for TVD, encoded oestrogen receptors which have atheroprotective behaviour, expressed in arterial wall and reported as a potential candidate for CAD (Lehtimäki *et al.* 2002). The genes *CEL38* (Vahtola *et al.* 2011) and *PALLD39* (Shiffman *et al.* 2005) are known to be associated with MI. Modified LDL cholesterol uptake is mediated by the macrophage scavenger receptor 1 (*MSR1*) which has a mechanistic role in driving atherogenesis (Durst *et al.* 2009). TVD patients had higher expression of the *THBD* gene which encodes for thrombomodulin, an endothelial-specific type-I membrane receptor that binds thrombin and associates with higher risk of MI (Salomaa *et al.* 1999; Nakagawa *et al.* 2001). Thus, the genes functionally annotated with CAD in our dataset are emerging as candidate genes for CAD and our study reporting the candidate genes are annotated to different severities.

The quantum of severity of the atherosclerotic lesion could be distinguished on the basis of related biological processes revealed on biological process analysis of functionally annotated genes for SVD and TVD. Cellular localization, migration, cell adhesion, positive regulation of cell death and cytokine production processes mark the atherosclerotic lesion for SVD, whereas, wound healing,

negative regulation of cell death, blood coagulation, angiogenesis and fibrinolytic biological processes are involved in atherosclerotic lesion of TVD patients. Processes involved in SVD may reflect those described in earlier stages of atherosclerosis, while those in TVD may reflect later processes in an established atherosclerotic plaque (Falk 2006). Our study is unique in describing different processes based on the severity of atherosclerotic coronary artery involvement.

In two previously reported studies, 14 (Wingrove *et al.* 2008) and 19 (Sinnaeve *et al.* 2009) DEGs have been identified in relation to severity of CAD, compared with our study where 13 DEGs had significant two-fold difference in expression between SVD and TVD. In these previous studies, CAD scoring systems such as percentage of stenosis (Wingrove *et al.* 2008) and CAD index (Sinnaeve *et al.* 2009) had employed to define different ranges of severity of CAD that includes only major epicardial arteries without consideration of diseased sub-branches. There might have existed an overlap between differing severities when percentage of stenosis and CAD index scores were employed to quantify the extent of CAD. Also, the approach of using a correlation analysis may not be able to explore the differences in candidate genes and their biological processes in two distinct severities of CAD. Hence, we have used a different approach, where we have utilized the number of vessels involved and a modified Gensini scoring system to distinguish distinct two severities of CAD, thereby overcoming the skewed effect transition between control subjects to less severe CAD and to higher CAD. Also, in the study reported by Sinnaeve *et al.* (2009), most of the patients were with early lower severity (mild and moderate). Wingrove *et al.* (2008) selected samples from two different cohorts for expression profiling including patients of ACS and might have influenced gene expressions. Candidate genes *MMP9*, *IL1B* and *SOD2* were identified commonly in peripheral blood gene expression representing CAD in three ethnicities: Indian, Chinese and Malays (Abdullah *et al.* 2012). The genes associated with improvement in vascular function, i.e. oxidative phosphorylation and mitochondrial function in cardiac rehabilitation have been explored by whole blood expression analysis (Chiara *et al*. 2010). The putative candidate genes explored for CAD, involved in inflammation, immune response, cell regulation, proliferation and apoptotic processes (Wingrove *et al.* 2008; Elashoff *et al.* 2011; Arvind *et al.* 2015) may distinguish and affect severity of CAD as reported from our study, such as immune response, positive regulation of apoptosis and cell regulation indicates lesser severity of CAD (SVD), whereas, negative regulation of apoptotic processes and inflammation represents the CAD largely in higher severity (TVD).

Very few genes were common among different global gene expression profiling studies for CAD, which may be due to differences in study design, sample processing, array platform used and study populations. However, the reported biological processes and pathways involved have significant degree of similarities.

Limitation of our study was that we have studied the gene expression in peripheral blood rather than using diseased tissue. Previous studies have reported that whole blood mimics the expression patterns of genes in diseased coronary arteries (Sinnaeve *et al.* 2009) and blood cells can be used as surrogate tissue to explore the perturbations occurring in remote tissues (Pool-Zobel *et al.* 2004; Rockett *et al.* 2004). The peripheral whole blood cell-mediated inflammation and immune response play a major contributing role in initiation as well as progression of atherosclerotic CAD (Hansson *et al.* 2002; Libby *et al.* 2002). Also, whole blood is readily available compared with tissues for collection and use in clinical practice. This made the peripheral whole blood suitable to explore underlying pathophysiology of disease to evaluate changes in the expression of genes. This study has been performed in nondiabetic, male patients from North India. Small sample size can be explained in terms of high cost for sample processing. Epigenetic mechanisms may also play a role in CAD, which have not been evaluated in our study.

Similar to various global gene expression studies for CAD, we too observed that only few DEGs from our study are common to other studies. Our study supports the fact that different severities of atherosclerotic lesions may be supported by distinct different sets of candidate genes as well as related biological processes. This may help in understanding the underlying mechanisms of progressing atherosclerotic lesion and thus identification of treatment targets for different severities of CAD. Validation further in different populations as well as different sets of conditions may strengthen the clinical utility.

In conclusion, this study has explored several potential putative candidate genes for CAD, particularly functionally annotated genes and their biological processes distinguishing and affecting different severities of CAD.

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