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ICAM-1 K469E polymorphism is a genetic determinant for the clinical risk factors of T2D subjects with retinopathy in Indians: a population-based case–control study

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ABSTRACT

Objective: Elevated levels of intercellular adhesion molecule-1 (ICAM-1) are demonstrated in diabetes complications. The current study aims to understand association of K469E (rs5498) in *ICAM-1* gene, in type 2 diabetic (T2D) subjects with retinopathy.

Design: Case–control study.

Setting: Sankara Nethralaya Diabetic Retinopathy Epidemiology and Molecular Genetic Study, an epidemiology study (on prevalence of diabetic retinopathy in T2D subjects (T2DR) from south India) and outpatient department of Sankara Nethralaya, a tertiary care hospital, in Chennai, India.

Participants: A total of 356 T2D subjects of >15 years of diabetes duration, with (n=199) and without (n=157) retinopathy.

Methods: The rs5498 polymorphism was genotyped by direct sequencing. Multivariate analysis for various clinical covariates was done using SPSS V.14. Comparative assessment of structure stability, folding rate of the variants were assessed using bioinformatics tools like STRIDE, MuPro, ModellerV97, fold rate server, etc.

Results: The AA genotype of rs5498 was seen at a higher frequency in the retinopathy group (p=0.012). The risk for diabetic retinopathy (DR) increased in the presence of AA genotype (OR=1.89–4.82) after the sequential addition of various clinical covariates. Multivariate logistic regression analysis showed 8.26 times high risk for developing DR in the AG genotype (p=0.003). Structural superimposition of ICAM-1 wild type (K469) and variant (E469) showed 0.943 Å of backbone root mean square deviation as calculated by PYMOL software. A difference in the fold rate time was also observed between the wild type (5.4/s) and variant (3.3/s).

Conclusions: This study shows that allele A of rs5498 in *ICAM-1* is a putative risk predisposing allele for T2D retinopathy and its clinical covariates in Indian population. The folding rate of the protein decreases for the A allele implicating a potential effect on the structure and function of ICAM-1.

ARTICLE SUMMARY

Article focus

- Intercellular adhesion molecule-1 (ICAM-1) is a biomarker for inflammation and endothelial cell dysfunction and increased levels have been implicated in diabetic retinopathy (DR) pathology both in human and animal models. Strong heritability and genetic control on ICAM-1 expression have been observed by genome-wide association studies.
- The present study investigates the association of K469E (rs5498) polymorphism with DR in type 2 diabetic patients with ≥15 years duration of diabetes from south Indian population.

Key messages

- The AA genotype of rs5498 is a putative risk predisposing genotype for DR (OR=1.89–4.82) when adjusted for clinical covariates.
- The single-nucleotide polymorphism (SNP) imposes a change in the folding rate of the ICAM-1 protein that has potential functional implications.
- These results indicate low antigenicity of incobotulinumtoxinA.

Strengths and limitations of this study

- The detailed clinical evaluation and homogeneity of the study subjects, representing the southern part of India, is the main strength of the current study.
- The statistical approach that has accounted for the clinical confounding factors for DR has proved the strong correlation of the rs5498 with DR.
- With the limited available literature on the possible effect of rs5498 on ICAM-1 structure and function, the bioinformatics results provided an additional knowledge on the putative effect on the protein folding by the SNP through structure superimposition analysis that has potential futuristic research implications.
- Functional characterisation is the potential limitation of the study that could have further helped in proving the positive association observed for the AA genotype. The lack of correlation of serum ICAM-1 levels and sample size are other limitations.

INTRODUCTION

Diabetic retinopathy (DR) is reaching an alarming proportion in developing countries. In India DR has been reported as the sixth cause of blindness with an age-adjusted prevalence of 18% in diabetes subjects from rural and urban populations.[1 2]

DR is characterised by retinal vessels basement membrane thickening, loss of pericytes and endothelial cells, blood–retinal barrier breakdown, capillary non-perfusion, microaneurysms, haemorrhages and neovascularisation.[3] Several molecules and biochemical pathways like polyol pathway, activation of protein kinase C, formation of advanced glycation end products (AGEs), oxidative stress, upregulation of growth factors, adhesion molecules, etc have been implicated in the pathogenesis of DR. Recent research insights describe DR as a retinal disease associated with vascular neuroinflammation.[3] Molecular and functional characterisation of the diabetic retina from animals, humans and cell culture studies have shown an increase in leukostasis, cytokines and growth factors resulting in breakdown of the blood–retinal barrier, thus implying the role of inflammation in DR.[3–5] In lieu of such inferences, many anti-inflammatory molecules are being tested in recent years as a target for a possible remedy in DR.

Intercellular adhesion molecule-1 (ICAM-1) is a biomarker for endothelial cell dysfunction and inflammation that mediates leucocyte influx and persistent retinal leukostasis, retinal vascular leakage, capillary non-perfusion and endothelial cell injury and death subsequently resulting from Fas/FasL-mediated apoptosis.[6] Its levels are upregulated along with the integrin ligands in patients with DR and retina of animal models.[6] A decrease in the adherent retinal leucocytes have also been observed in ICAM-1 knock out animal models[5] demonstrating the role of ICAM-1-mediated inflammation in DR pathogenesis.

Genetic variants in *ICAM-1* gene have been shown to regulate the expression level and have been widely studied for possible genetic association with a range of degenerative and inflammatory diseases including diabetic retinopathy.[7 8–15] The K469E (rs5498) polymorphism in exon 6 of *ICAM-1* gene has been shown to influence the binding of ICAM-1 on endothelial cells and LFA-1 and Mac-1 on leucocytes, mediating leukostasis and its migration in an inflammatory environment.[16] This domain is essential for the structure and function of ICAM-1.[17] Recent genome-wide association studies have demonstrated a strong correlation between rs5498 and sICAM-1 levels.[18] In the present study, we have investigated the association of the K469E polymorphism with retinopathy in type 2 diabetes (T2D) subjects from south India.

MATERIALS AND METHODS

Sample collection

The patients were recruited prospectively from SNDREAMS (Sankara Nethralaya Diabetic Retinopathy Epidemiology and Molecular Biology Study), an epidemiology study to understand the prevalence of DR in south Indian population[1] and from the outpatient departments of Sankara Nethralaya, a tertiary eye care hospital in Chennai.

The study subjects were enrolled between the years 2003 and 2010. The study protocol adhered to the Declaration of Helsinki and approved by institutional review board. After an informed consent all the subjects underwent detailed history, physical examination and pedigree analysis. Ocular examination included 45° fundus photograph using four-field stereoscopic digital photography that were graded by two independent observers in a masked fashion and the grading agreement showed a high κ value of 0.83.[1] The diagnosis of DR was based on the modified Klein classification of the Early Treatment Diabetic Retinopathy Study scale.[1] The methodology of sample selection were as described earlier.[19 20] The inclusion criteria for the study participants were T2D, south Indian origin and ≥40 years of age. The duration of diabetes varied between cases (≥10 years) and controls (≥15 years). Subjects with sight threatening diabetic retinopathy (STDR, inclusive of severe non-proliferative diabetic retinopathy (NPDR), proliferative diabetic retinopathy (PDR) or clinically significant macular oedema (CSMO)) constitute the case group (DR+, n=199) and those without any signs of DR were included as the controls (DR−, n=157). Age-related macular degeneration (AMD), other hereditary retinal disease and non-south Indian origin were the exclusion criteria.

Genotyping

DNA was extracted from the peripheral blood samples by conventional phenol chloroform method[21] and NucleoSpin Blood XL maxi kit method (Macherey-Nagel, Duren, Germany) according to the manufacturer's instructions. The genomic region flanking the K469E (rs5498; accession no. NT_011295) polymorphism in exon 6 of *ICAM-1* was amplified with forward (5′-CTTGAGGGCACCTACCTCTG-3′) and reverse (5′-CATTATGACTGCGGCTGCTA-3′) using the following protocol: initial denaturation at 94°C for 5 min followed by 35 cycles of denaturation (94°C/30 s) primer annealing (60°C/1 min) and extension (72°C/1 min) followed by final extension at 72°C for 7 min. Genotype scoring was performed by direct sequencing in ABI PRISM 3100 Avant genetic analyser (Applied Biosystems, Foster City, California, USA).

Statistical analysis

Deviation from Hardy-Weinberg equilibrium(HWE) for genotypes was analysed. Statistical analyses were performed using SPSS software (for Windows V.14.0; SPSS Science, Chicago, Illinois, USA). The results were expressed as mean SD if the variables were continuous and as percentage if the variables were categorical. The Student's t test and χ^2 test were performed to compare continuous variables and proportions among groups, respectively. Distribution of genotypes and alleles

between the case and the control groups were compared using χ^2 test. To assess the specific effect of the genotypes on the various clinical factors, multivariate analysis was performed in the DR+ group. Multivariate analysis with stepwise sequential addition of various clinical variables was performed in the DR+ group with the three genotypes GG, AG and AA as the dependent variable. OR and 95% CIs were calculated and p<0.05 was considered significant.

Bioinformatic analysis

Sequence alignment analysis and the effect of polymorphism on structure and function of the protein are performed with BLAST (basic local alignment search tool), Polyphen-2 (polymorphism phenotyping v2) and SIFT (sorting intolerant from tolerant). Conservatonal analysis using ConSurf 9 was done with Swiss-Prot Accession ID: P05362 as the reference sequence.[22] Three-dimensional structural co-ordinates of the ICAM-1 having the natural variant of rs5498, elucidated using x-ray crystallography at resolution of 2.70 Å was retrieved from Protein Data bank (PDBID: 2OZ4). The structure for K469 variant elucidated using Modeller9V7[23] and validated for quality by checking the stereo chemical and energetic aspects. The structural stability of the wild and variant was analysed through potential energy of the molecule. The effect of these variants at the cell adhesion was also studied through protein dimerisation and interaction with integrin α-M and β-2 using MUpro.[24]

RESULTS

In the current study we have analysed the frequency distribution of the K469E (A>G, rs5498) polymorphism in T2D subjects from south Indian population and analysed for the putative association of the same with DR. The demographic details of the study participants are given in table 1.

The genotypes were in HWE (p>0.05) The observed and expected frequencies for the homozygous, heterozgous variant did not deviate and were found to be consistent with HWE (p=0.313 and 0.316 for cases and controls, respectively. The AA genotype showed a higher frequency of distribution in the DR+ group when compared with the DR− (p=0.012); OR=1.94 (95% CI 1.06 to 3.55; table 2).

Results of multivariate analysis adjusted for the various clinical risk factors for DR are given in table 3. A negative association was observed for age/body mass index and age/high-density lipoprotein (HDL) in genotypes GG and AG, respectively. A positive association was observed for insulin usage and HbA1c in all the genotypes in addition to microalbuminuria which showed 8.26 times high risk for developing DR in the AG genotype.

Table 4 shows the multivariate logistic analysis performed with the genotypes (GG vs AG, GG vs AA and AG vs AA) as the independent variables and DR status as the dependent variables. Unadjusted analysis was performed initially, followed by sequential adjustment of the various clinical factors (covariates) mentioned in table 4. Significant value of p<0.05 and OR>1.0 was observed for the AA genotype when compared with the other genotypes {unadjusted p=0.923, OR=1.02, 95% CI 0.62 to 1.70 for GG vs AG; p=0.032, OR=1.94, 95% of CI 1.06 to 3.55 for GG vs AA; and p=0.019, OR=1.89, 95% of CI 1.11 to 3.22 for AG vs AA}. After adjusted for all the covariates, maximum OR with statistical significance was observed

Table 1 Baseline characteristics of the study participants

Variables	DR− control (n=157)	DR+ case (n=199)	p
Age (years)*	64.32±9.01	58.81±8.63	<0.0001
Male gender (n, %)	98 (62.4)	128 (64.3)	0.711
Duration of T2DM (years)*	18.44±6.18	17.74±5.45	0.261
User of insulin	34 (21.7)	83 (41.7)	<0.0001
Age at diabetes onset (years)*	44.88±9.19	41.06±9.25	<0.0001
HbA1c (%)*	7.55±1.97	9.23±2.69	<0.0001
HbA1c (mmol/mol)*	58.97±21.51	77.39±29.37	
Systolic BP (mm Hg)*	133.06±18.24	135.82±20.07	0.180
Diastolic BP (mm Hg)*	77.04±9.38	81.52±10.66	<0.0001
BMI* (kg/m^2)	25.33±7.78	23.79±5.36	0.031
History of hypertension	66 (42.0)	78 (39.2)	0.587
Smokers	23 (14.6)	37 (18.6)	0.321
Total cholesterol (mmol/l)*	4.29±1.10	4.05±1.03	0.069
HDL cholesterol (mmol/l)*	1.06±0.31	1.09±1.06	0.763
Triglycerides (mmol/l)*	1.17±0.61	1.12±0.54	0.541
Microalbuminuria	29 (19.9)	86 (58.5)	<0.0001
Macroalbuminuria	3 (2.1)	14 (9.5)	0.010

*Data are M±SD. p<0.05: statistically significant.
BMI, basal metabolic index; BP, blood pressure; DR+, T2D subjects with retinopathy; DR−, T2D subjects without retinopathy; HbA1C, glycosylated haemoglobin; HDL, high-density lipoprotein; n, total subjects; T2DM, type 2 diabetes mellitus.

Table 2 Distribution of ICAM-1 rs5498 genotype and allele frequencies in DR+ and DR− groups

Genotype	Groups		p Values		
	DR− controls (n=157)	DR+ case (n=199)		Dominant AA+AG=GG	Recessive AA=AG+GG
GG	44 (28.0)	47 (23.6)	0.344	0.4	0.01*
AG	84 (53.5)	92 (46.2)	0.173		
AA	29 (18.5)	60 (30.2)	0.012*		
Alleles					
G	172 (54.8)	186 (46.7)			
A	142 (45.2)	212 (53.3)	0.033*		

*p<0.05—significant p value.
DR+, T2D subjects with retinopathy; DR−, T2D subjects without retinopathy; ICAM-1, intercellular adhesion molecule-1.

for AA (p=0.004; OR=4.82; 95% CI 1.64 to 14.14) when compared against AG genotype.

Bioinformatics analysis

Conservational analysis using ConSurf 9[22] predicted the amino acid at position 469 to be of highly variable nature. Further, multiple sequence alignment of *ICAM-1* in different species showed the E469 genotype as a conservative variant. Possible pathogenic effects inferred using Polyphen and SIFT, predicted the variant to be benign and tolerable. Moreover, sequence-based stability analysis using MUpro[24] showed an increase in stability for the natural variant (E469) when compared with the wild (K469) with a confidence score of 0.10666991. Folding rate (using Fold rate server) was predicted to be 5.54/s and 3.3/s for the wild and variant proteins, respectively.

Three-dimensional structure of K469 ICAM-1 variant was modelled using the natural variant (PDBID: 2OZ4) as a template. The generated structure was loop refined using loop.py module of Modeller9v7. The steric clashes and bad contacts were removed using What-If server. Structural quality of the protein was assessed by checking the Ramachandran plot and ProQ server and tabulated (table 5). Moreover, the structure of wild and variant were energy optimised using optimised potential for liquid simulations force field for 1000 runs of steepest descent. Backbone superimposition of wild and variant forms of ICAM-1 showed RMSD (root mean square deviation) deviation of 0.943 Å. Since structural superimposition studies showed mild deviation (figure 1), secondary structural analysis were performed using STRIDE[25] a software tool for secondary structure assignment from atomic resolution protein structures,

Table 3 Multivariate analysis between DR+ and DR− group for ICAM-1 rs5498 genotypes and the clinical covariates with the DR status as the dependable variable

Characteristics	GG		AG		AA	
	DR+		DR+		DR+	
Number	OR (95% CI)	p	OR (95% CI)	p	OR (95% CI)	p
Age (years)	0.91 (0.84 to 0.98)	0.019*	0.95 (0.91 to 0.99)	0.016*	0.92 (0.84 to 1.01)	0.077
Male sex	3.28 (0.86 to 12.44)	0.081	0.94 (0.43 to 2.05)	0.880	1.82 (0.46 to 7.10)	0.391
Duration of DM (years)	0.93 (0.82 to 1.06)	0.277	0.99 (0.94 to 1.06)	0.880	0.95 (0.82 to 1.06)	0.404
User of insulin	4.35 (1.09 to 17.32)	0.037*	2.66 (1.24 to 5.69)	0.012*	5.44 (1.03 to 28.90)	0.047*
Age at diabetes onset (years)	0.95 (0.89 to 1.01)	0.091	0.96 (0.93 to 1.00)	0.054	0.97 (0.90 to 1.04)	0.356
HbA1c (DCCT) (%)	1.47 (1.14 to 1.89)	0.003*	1.32 (1.11 to 1.57)	0.002*	1.48 (1.07 to 2.04)	0.017*
HbA1c (IFCC) mmol/mol)	1.04 (1.01 to 1.06)		1.03 (1.01 to 1.04)		1.04 (1.01 to 1.07)	33
Systolic BP (mm Hg)	0.99 (0.96 to 1.03)	0.693	1.01 (0.98 to 1.02)	0.664	1.03 (0.98 to 1.07)	0.234
Diastolic BP (mm Hg)	1.08 (0.99 to 1.18)	0.065	1.04 (1.00 to 1.08)	0.048*	1.08 (0.99 to 1.17)	0.069
BMI	0.83 (0.70 to 0.97)	0.020*	0.93 (0.85 to 1.01)	0.102	0.84 (0.71 to 1.01)	0.059
History of hypertension	1.70 (0.48 to 5.97)	0.407	0.69 (0.32 to 1.46)	0.332	1.26 (0.34 to 4.74)	0.731
Smokers	1.17 (0.22 to 6.29)	0.857	1.10 (0.42 to 2.86)	0.846	3.72 (0.45 to 31.02)	0.225
Total cholesterol (mmol/l)	0.29 (0.08 to 1.05)	0.060	0.99 (0.48 to 2.04)	0.972	0.13 (0.01 to 1.39)	0.092
HDL cholesterol (mmol/l)	0.55 (0.01 to 55.94)	0.798	0.03 (0.00 to 0.80)	0.037*	6.83 (0.04 to 127.59)	0.471
Triglycerides (mmol/l)	0.40 (0.03 to 5.75)	0.504	0.41 (0.07 to 2.49)	0.337	3.34 (0.07 to 15.72)	0.540
Microalbuminuria	2.06 (0.21 to 20.26)	0.537	8.26 (2.06 to 33.11)	0.003*	3.59 (0.29 to 43.63)	0.316

*p<0.05—significant p value.
BMI, basal metabolic index; BP, blood pressure; DCCT, Diabetes Control and Complications Trial; DM, diabetes mellitus; DR+, T2D subjects with retinopathy; DR−, T2D subjects without retinopathy; HbA1C, glycosylated haemoglobin; HDL, high-density lipoprotein; ICAM-1, intercellular adhesion molecule-1; IFCC, International Federation of Clinical Chemistry and Laboratory Medicine.

Table 4 Multivariate logistic analysis in DR+ group with sequential addition of clinical covariates with ICAM-1 rs5498 genotypes as the dependable variables

Characteristics Number	DR+ GG vs AG OR (95% CI)	P	GG vs AA OR (95% CI)	p	AG vs AA OR (95% CI)	p
Unadjusted	1.02 (0.62 to 1.70)	0.923	1.94 (1.06 to 3.55)	0.032*	1.89 (1.11 to 3.22)	0.019*
Age	1.02 (0.60 to 1.74)	0.931	1.97 (1.05 to 3.69)	0.035*	1.92 (1.10 to 3.34)	0.022*
Age+gender	1.02 (0.60 to 1.74)	0.932	2.00 (1.06 to 3.77)	0.031*	1.93 (1.10 to 3.36)	0.021*
Age+gender+DD	1.01 (0.59 to 1.73)	0.958	2.00 (1.06 to 3.77)	0.031*	1.94 (1.11 to 3.39)	0.020*
Age+gender+DD+insulin	1.04 (0.61 to 1.80)	0.873	2.28 (1.19 to 4.36)	0.012*	2.12 (1.20 to 3.75)	0.010*
Age+gender+DD+insulin+HbA1c	1.31 (0.73 to 2.34)	0.368	1.83 (1.25 to 2.69)	0.002*	2.12 (1.16 to 3.85)	0.014*
Age+gender+DD+insulin+HbA1c+systolic BP	1.26 (0.70 to 2.28)	0.439	1.79 (1.22 to 2.65)	0.003*	2.15 (1.17 to 3.93)	0.013*
Age+gender+DD+insulin+HbA1c+systolic BP+diastolic BP	1.28 (0.70 to 2.32)	0.425	1.81 (1.21 to 2.70)	0.004*	2.12 (1.15 to 3.91)	0.016*
Age+gender+DD+insulin+HbA1c+systolic BP+diastolic BP+BMI	1.31 (0.71 to 2.42)	0.377	1.97 (1.29 to 3.01)	0.002	2.21 (1.18 to 4.13)	0.013*
Age+gender+DD+insulin+HbA1c+systolic BP+diastolic BP+BMI+SMK	1.31 (0.71 to 2.41)	0.392	1.99 (1.29 to 3.06)	0.002*	2.22 (1.18 to 4.16)	0.013*
Age+gender+DD+insulin+HbA1c+systolic BP+diastolic BP+BMI+SMK+microalbumin	1.25 (0.60 to 2.59)	0.552	2.04 (1.26 to 3.32)	0.004*	3.17 (1.41 to 7.08)	0.005*
Age+gender+DD+insulin+HbA1c+systolic BP+diastolic BP+BMI+SMK+microalbumin+triglyceride	0.68 (0.27 to 1.75)	0.430	1.77 (0.98 to 3.21)	0.059	3.75 (1.28 to 10.98)	0.016*
Age+gender+DD+insulin+HbA1c+systolic BP+diastolic BP+BMI+SMK+microalbumin+triglyceride+HDL	0.74 (0.27 to 1.97)	0.543	1.75 (0.97 to 3.18)	0.064	4.17 (1.39 to 12.53)	0.011*
Age+gender+DD+insulin+HbA1c+systolic BP+diastolic BP+BMI+SMK+microalbumin+triglyceride+HDL+cholesterol	0.67 (0.25 to 1.84)	0.439	1.80 (0.98 to 3.34)	0.060	4.07 (1.34 to 12.35)	0.013*
Age+gender+DD+insulin+HbA1c+BMI+cholesterol+HDL+SMK+triglyceride+microalbumin+HOHT	0.63 (0.23 to 1.72)	0.365	1.72 (0.98 to 3.02)	0.061	3.56 (1.23 to 10.03)	0.020*
Gender+onset of diabetes+insulin+HbA1c+systolic BP+diastolic BP+BMI+SMK+cholesterol+HDL+triglyceride+microalbumin	0.67 (0.26 to 1.76)	0.417	1.89 (1.03 to 3.49)	0.041*	4.82 (1.64 to 14.14)	0.004*

*Significant p value.
BMI, basal metabolic index; BP, blood pressure; DD, duration of diabetes; DM, diabetes mellitus; DR+, T2D subjects with retinopathy; DR–, T2D subjects without retinopathy; HbA1C, glycosylated haemoglobin; HDL, high-density lipoprotein; HOHT, history of hypertension; ICAM-1, intercellular adhesion molecule-1; SMK, smoking.

Table 5 Comparison of the structural properties of the wild (KK) and variant (EE) proteins for SNP rs5498 of ICAM-1 gene

Experimental type	Wild Homology modelling	Variant PDBID: 2OZ4
		Resolution: 2.70 Å
Residues in most favoured regions	95.5%	88.8%
Residues in additional allowed region	4.0%	10.8%
Residues in generously allowed regions	0.4%	0.4%
Residues in disallowed regions	0.0%	0.0%
G-factor	−0.04	0.13
Bond lengths		
Main chain	99.8%	100%
Within limits	0.2%	0.0%
Bond angles		
Main chain	:93.9%	100%
Within limits	6.1%	0.0%
Planarity		
Planar groups	100%	100%
Within limits	0.0%	0.0%
Energy minimisation using Gromacs for 1000 runs of steepest descent	−2.9554834e +04	−3.7942434e +04

ICAM-1, intercellular adhesion molecule-1; SNP, single nucleotide polymorphism.

that, however, did not show any significant change between the wild and the variant.

DISCUSSION

In the present study, the frequency distribution of K469E (rs5498) polymorphism in the *ICAM-1* gene was analysed for its possible association with T2D retinopathy from south India. A higher frequency of AA genotype is being observed in the DR+ when compared with the DR− group (OR=1.94 (95% CI 1.06 to 3.55; p, 0.012)). The frequency of rs5498 polymorphism observed in the current study

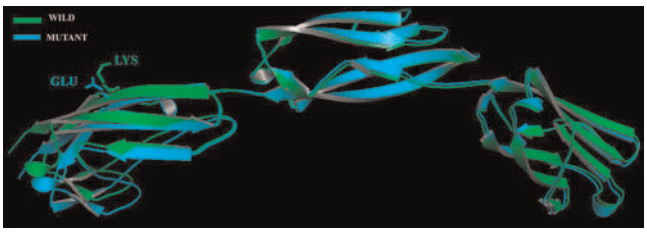

Figure 1 Structural superimposition of intercellular adhesion molecule-1 wild type K469 (green) and variant E469 (blue) with a 0.943 Å of backbone root mean square deviation, as calculated by PYMOL. The variant residues are shown in stick representation.

simulates the other reports from India on ICAM-1 gene single nucleotide polymorphisms (SNPs).[26 27]

The association of rs5498 AA genotype with DR identified in the current study simulates the Japanese and Chinese study on PDR populations.[13 14] The A allele of rs5498 was also observed to confer disease susceptibility in type 1 diabetes (T1D) patients with nephropathy of Swedish Caucasian origin.[12] A high heterozygous index was observed in the current study is similar to that observed in GoKinD study.[28] However, another similar study from Caucasian cohort shows significant association with GG genotype suggesting the population difference per se.[15]

Our results also differ from another similar study in India, by Balasubbu *et al*.[29] The possible reasons for the observed differences between our study and the other reports from India could be attributed to the area of sampling and the types of DR included in the study. The present study represents a more homogenous population from the same geographical area of southern India against that of Balasubbu *et al* which represents a hospital-based population. We have included STDR patients with PDR, NPDR or CSMO while the ARAVIND study includes only PDR similar to the Caucasian study.

Strong heritability factor has been observed for circulating levels of sICAM-1 by bivariate quantitative genetic analyses[30] and upregulated expressions are being reported in animal models and PDR patients.[6] Such elevated levels are reported to be influenced by glycaemic control, disturbances in lipid metabolism, obesity and insulin resistance, etc which are also important clinical determinants of DR. Hence we performed a multivariate logistic regression analysis for the different genotypes between DR+ and DR− groups after adjusting these parameters.

In the current study, we observed an OR of 8.0 (95% CI 2.06 to 33.11) for the heterozygous genotype (AG) in the DR group after adjusting for microalbuminuria (table 3) and a genotype-dependent risk was observed after the sequential addition of the gender (table 4). Plasma levels of ICAM-1, vascular cell adhesion molecule 1 were increased in T2D patients with microalbuminuria thus suggesting a significant correlation between the same.[31] In GoKinD population allele G has been detected to confer a decreased risk susceptibility to the development of diabetic nephropathy in female T1D patients.[28]

ICAM-1 expression has been reported to share a common genetic modulation with traits related to obesity, insulin resistance and HDL3 cholesterol.[32] We observed significant p value with OR>1.0 for the AA genotype after the sequential addition of lipid biomarkers (table 4). An abnormal endothelial activation after an oral lipid meal, coupled with an increased oxidative stress is being observed in patients with familial history of T2D.[33] High-fat meals are shown to increase ICAM-1 and other adhesion molecules in normal and diabetic subjects.[33] Similarly, a recent study on the coeffects of inflammation and endothelial dysfunction and insulin resistance on hypertension in a large Asian population reports elevated levels of biomarkers for inflammation and endothelial cell dysfunction including

sICAM-1.[34] The ICAM-1 GG (E469E) genotype was reported as an independent contribution factor of plasma fibrinogen level as well as HDL cholesterol and urinary albumin excretion in atherosclerosis.[11] However, we did not identify any statistically significant association for the GG genotype with these two parameters in the current study.

Collectively, multivariate analysis after sequential addition of various DR risk factors reveal a high susceptibility for DR (OR>1, table 4) in the AA genotype of rs5498 for all the DR covariates. Interestingly, these parameters are reported to have a significant effect on ICAM-1 expression.[32–34] Estimation of ICAM-1 levels in individuals with E469K polymorphism has been reported to be associated with many inflammatory and infectious diseases. However, population differences are seen.[35–37] These differences could be due to the dietary influence/epigenetic silencing of ICAM-1 expression by hypermethylation as reported in animal and human models.[38 39]

Any disease-associated polymorphism can possibly mediate the effect either through altered expression or function of the protein. The rs5498 is located at three-base position upstream of the splice donor site that produces an alternatively spliced short isoform (ICAM-1-S) that has no transmembrane or intracellular domain and speculated to influence the ICAM-1 signal transduction and cell–cell contact including Fas–FasL interaction.[40] Thus, correlated decrease in Fas-associated death domain-like interleukin-1-β-converting enzyme-inhibitory protein long form (FLIPL) mRNA expression and apoptosis suggests a putative role of the polymorphism in regulating apoptosis by modifying the inflammatory immune responses.[40] Comparison of the RNA splicing patterns in cells expressing G/G (469E) and A/A (469K) genotypes showed a comparatively higher expression of ICAM-1-S mRNA in A/A cells.[40]

The polymorphism rs5498 results in a non-conservative change from lysine to glutamic acid in the fifth immunoglobulin-like domain of ICAM-1[14] that is essential for dimerisation, surface presentation and solubilisation of the protein.[17] We therefore performed a bioinformatic analysis to study the putative effect of this SNP on the ICAM-1 structure and thus its influence on the expression. As per the sequence analysis, the variant K469E was shown as benign without any significant secondary structural change. The x-ray structure of ICAM-1 consists of Ig-like C2-type domain 3, 4 and 5 that consist of four intradisulphide bridges as per Swiss-Prot annotation and it includes 237–290, 332–371, 403–419 and 431–457 (http://www.uniprot.org/uniprot/P05362). It could therefore be inferred that the variant K469E does not affect the disulphide bridges. Interestingly, structure superimposition of the two variants (figure 1), revealed a 0.943 Å deviation of backbone RMSD as calculated by the software PYMOL thus suggesting a structural effect of the SNP. The difference in the fold rate time observed between the KK (5.4/s) when compared with the EE (3.3/s) variant highlights the need of further dimerisation studies.

Our results indicate that the AA genotype of *ICAM-1* (rs5498) gene increases the risk predisposition for retinopathy in T2D patients in south Indian population. Clinical covariates such as microalbuminuria, lipid biomarkers, etc show a genotype-dependent (AG/AA) increase in the risk for DR among T2D patients. Bioinformatics analysis of rs5498 showed a deviation in the structure and folding rate of the ICAM-1 protein. These observations emphasise the need for further studies to identify the molecular mechanism connecting the SNP, expression, protein structure and function.

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