Nonsynonymous T280M gene variant of CX3CR1 in South Indian population is associated with reduced risk for vascular disease in patients with diabetes mellitus

S Sumi PhD1*, Surya Ramachandran PhD1*, V Raman Kutty MD MPhil MPH2, Maulin M Patel MSc1, TN Anand MPH1, Ajit Mullassari MD DM3, CC Kartha MD FRCP4

BACKGROUND: Vascular inflammation leading to coronary artery disease (CAD) is a major complication of type 2 diabetes mellitus (T2DM). The chemokine receptor CX3CR1 is a key regulator in vascular injury-related inflammation.

OBJECTIVES: The authors examined the T280M and V249I gene variants of the CX3CR1 gene in patients with T2DM, CAD and associated with T2DM, to understand the effect of these polymorphisms on the disease phenotype.

METHODS: Whole blood samples were collected from 913 South Indian subjects comprising 160 patients with T2DM, 284 with T2DM and CAD, 198 with CAD and 271 healthy subjects. T280M and V249I variants of the CX3CR1 gene were genotyped in these study subjects using polymerase chain reaction (PCR)-restriction fragment length polymorphism analysis and PCR-DNA sequencing.

RESULTS: Multivariate logistic regression analysis revealed that the frequencies of 280M and 249I alleles were higher in controls than in cases in both the CAD and T2DM groups. Adjusted odds of T280M according to logistic regression were significant in all disease groups compared with healthy subjects. Both MM and II genotypes of T280M and V249I polymorphisms were found to have an atheroprotective role in CAD and T2DM. The association of these polymorphisms were then examined in patients with CAD who were also diagnosed with diabetes. The M allele of T280M polymorphism was associated with attenuated risk for CAD and T2DM.

CONCLUSIONS: The present study demonstrated a protective role of the T280M variant of CX3CR1 gene for vascular complication in patients with T2DM. This polymorphism may reduce the binding strength of the chemokine receptor, thus attenuating inflammation both in vascular wall and adipose tissue.

Key Words: Chemokine; Coronary artery disease; Polymorphism; Type 2 diabetes mellitus

METHODS

Study subjects and groups

Study subjects were recruited from three multispeciality centres of South India. The study was approved by institutional ethics committees of Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram and participating hospitals (Madras Medical Mission, Chennai, IRS hospital and Indian Institute of Diabetes, Thiruvananthapuram) and all study participants provided informed written consent. Subjects with hypertension, congenital heart disease, renal disease, cardiac disease other than CAD, microvascular disease and pregnancy were excluded. Whole blood samples (3 mL) after an overnight fast were collected from 913 subjects (age range 24 to 93 years). The subjects were grouped into four categories: T2DM (n=160); patients with T2DM and CAD (n=284); patients with CAD but no T2DM

*Authors who contributed equally

1Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram; 2Achutha Menon Centre for Health Science Studies, Sree Chitra Tirunal Institute for Medical Sciences and Technology, Thiruvananthapuram, Kerala; 3Madras Medical Mission, Chennai, India

Correspondence: Dr CC Kartha, Cardiovascular Diseases and Diabetes Biology, Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram, India 695014. Telephone 91-471-2529448, fax 91-471-2529505; e-mail ckartha@rgcb.res.in

This open-access article is distributed under the terms of the Creative Commons Attribution-Non-Commercial License (CC BY-NC) (http://creativecommons.org/licenses/by-nc/4.0/), which permits reuse, distribution and reproduction of the article, provided that the original work is properly cited and the reuse is restricted to noncommercial purposes. For commercial reuse, contact reprints@pulsus.com
The genotyping of length polymorphism


drop-1000 spectrophotometer (Thermo Scientific, USA) at 260 nm. Instructions. Quantity and quality of DNA was measured using nano-DNA blood mini kit (Qiagen, USA) according to the manufacturer’s instructions. Genomic DNA from peripheral blood was extracted using QIAamp genomic DNA extraction

Genomic DNA from peripheral blood was extracted using QIAamp DNA blood mini kit (Qiagen, USA) according to the manufacturer’s instructions. Quality and quantity of DNA was measured using nanodrop-1000 spectrophotometer (Thermo Scientific, USA) at 260 nm. Genotyping using polymerase chain-reaction-restriction fragment length polymorphism

The genotyping of CX3CR1 V249I (rs3732379) and T280M (rs3732378) polymorphisms were performed using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) techniques. DNA fragments containing polymorphisms were amplified using specific primers, CX3CR1: 5′-CCGAGGTCTCTCAGGAATTCT-3′ and 5′-TACGACATCAGGTTCCAGGAC-3′ (7). PCR conditions reactions were performed in a thermal cycler with an initial denaturation for 5 min at 94°C, 35 cycles of denaturing at 94°C for 30 s, annealing at 62°C for 30 s and extension at 72°C for 1 min. A final extension at 72°C for 7 min was also performed. The PCR products were digested with BsmI and PspI406I enzymes (New England Biolabs, United Kingdom). RFLP was performed by incubating the PCR product with 1 µL of restriction enzyme at 37°C for 2 h, followed by inactivation of enzyme at 65°C for 10 min.

PCRamplification

was performed, as mentioned earlier, and products were purified using gel band elution kit (GE Healthcare, USA). DNA sequencing based on Sanger dideoxy chain termination sequencing was performed on a DNA analyzer utilizing Bigdy terminator chemistry (ABI 3100, Applied Biosystems, USA). All sequences were analyzed using Sequencing analysis version 1.0 (Applied Biosystems, USA).

Statistical analysis

Statistical tests were performed using R software on the GNU platform (R Core Team, 2013, R: A language and environment for statistical computing, R Foundation for Statistical Computing, Austria). Data regarding continuous variables were expressed as mean ± SD and data of noncontinuous variables as frequency (n [%]); P<0.05 was considered to be statistically significant. Hardy-Weinberg equilibrium was tested for a goodness-of-fit using a χ2 test. The genetic variants and their risk for disease were computed according to ORs and 95% CIs by logistic regression analysis.

RESULTS

Clinical and baseline profile of study subjects

The study population consisted of 913 subjects, which included 198 with CAD (162 men and 36 women), 160 with T2DM and no associated vascular diseases (72 men and 88 women) and 284 diagnosed with
PCR sequencing; the results of both genotyping techniques corroborated well. A representative gel image of these polymorphisms is presented in Figure 1. Representative electrophograms of CX3CR1 T280M and V249I polymorphisms are shown in Figure 2. Logistic regression analysis of T280M and V249I polymorphisms revealed a reduced risk for CAD (Table 3). An OR of 0.07 (95% CI 0.04 to 0.12) was observed in patients carrying TM heterozygous and MM homozygous mutant genotypes when compared with patients carrying the TT wild genotype. The genotype frequency for V249I polymorphism was also found to be significantly different between patients and controls, with a protective OR 0.61 (95% CI 0.40 to 0.94) for the V1 heterozygous and II homozygous mutant genotypes compared with VV wild genotype. The calculated OR was <1, indicating that the II and MM genotype have a protective effect for CAD. These results were stable after adjustment for traditional risk factors (age, sex, lipid profile, HbA1c and lifestyle risk factors).

Similar results were obtained for the T2DM group. Genotype frequencies and risk estimates for T2DM are shown in Table 4. T280M polymorphism of CX3CR1 gene was associated with a reduced risk for developing diabetes in the South Indian population (OR 0.29 [95% CI 0.07 to 1.14]). VI heterozygous and II homozygous mutant genotypes versus VV wild genotype of V249I polymorphism had an OR of 0.49 (95% CI 0.13 to 1.54).

Effect of CX3CR1 gene variants on diabetic vascular disease
The CX3CR1 gene polymorphisms have been associated with a protective role for atherogenesis in patients with CAD. The analysis also
demonstrated similar results in patients with diabetes mellitus. The authors, therefore, investigated the role of these two gene variants in patients with both diabetes and CAD.

The risk estimates of patients in whom T2DM and CAD coexist are tabulated in Table 5. Regression analysis of T280M polymorphism revealed an OR of 0.71 (95% CI 0.25 to 2.23) for the T allele and 0.53 (95% CI 0.33 to 0.83) for the M allele in CAD compared with normal healthy subjects. For V249I polymorphism, an OR of 0.97 (95% CI 0.53 to 2.17) for the I allele was less prevalent compared with the V allele in all disease groups compared with normal healthy subjects. Logistic regression analysis shows that P<0.05 in patients with coronary artery disease (CAD), P<0.01 in type 2 diabetes mellitus (T2DM) patients and P<0.07 in patients with T2DM with CAD. No Number

![Figure 3](image)

**Figure 3** Bar diagram showing allelic distribution of CX3CR1 gene polymorphisms in study groups. A: Allelic distribution of T280M polymorphism: M allele is less prevalent compared with the T allele in the disease groups compared with normal healthy subjects (P<0.01). B: Allelic distribution of V249I polymorphism: I allele is less prevalent compared with V allele in all disease groups compared with normal healthy subjects. t test analysis shows that P<0.05 in patients with coronary artery disease (CAD), P<0.33 in type 2 diabetes mellitus (T2DM) and P<0.07 in patients with T2DM with CAD. No Number

Table 5

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Control</th>
<th>T2DM+CAD</th>
<th>Adjusted OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n=271)</td>
<td>(n=284)</td>
<td></td>
</tr>
<tr>
<td>CX3CR1 T280M</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T/T</td>
<td>39 (14.4)</td>
<td>88 (31)</td>
<td>1 (reference)</td>
</tr>
<tr>
<td>T/M</td>
<td>127 (46.9)</td>
<td>141 (49.6)</td>
<td>0.49 (0.31–0.76)</td>
</tr>
<tr>
<td>M/M</td>
<td>105 (38.7)</td>
<td>55 (19.4)</td>
<td>0.23 (0.14–0.38)</td>
</tr>
<tr>
<td>T/M + M/M</td>
<td>232 (85.6)</td>
<td>196 (69)</td>
<td>0.37 (0.24–0.57)</td>
</tr>
<tr>
<td>CX3CR1 V249I</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>V/V</td>
<td>124 (45.8)</td>
<td>151 (53.2)</td>
<td>1 (reference)</td>
</tr>
<tr>
<td>V/I</td>
<td>124 (45.8)</td>
<td>126 (44.4)</td>
<td>0.83 (0.59–1.17)</td>
</tr>
<tr>
<td>I/I</td>
<td>23 (8.5)</td>
<td>7 (2.5)</td>
<td>0.24 (0.10–0.60)</td>
</tr>
<tr>
<td>V/I + I/I</td>
<td>147 (54.2)</td>
<td>133 (46.8)</td>
<td>0.74 (0.53–1.03)</td>
</tr>
</tbody>
</table>

Data presented as n (%) unless otherwise indicated. *Logistic regression analysis adjusted for age, sex, cholesterol, glycated hemoglobin, smoking and alcohol. †P<0.05

**DISCUSSION**

We report the atheroprotective effect of CX3CR1 V249I and T280M polymorphisms studied in a South Indian population comprising patients with T2DM, CAD, and both T2DM and CAD. The purpose of the present study was to understand the role of these gene variants in diabetic vascular disease.

The gene variants of CX3CR1 are known to have a protective role in CAD and atherosclerosis by reducing chemokine-receptor interaction. The CX3CR1 receptor is involved in monocyte and leukocyte chemotaxis to vascular endothelium and promotes inflammation. Gene variants in this receptor are believed to attenuate this effect. The soluble form of FKN ligand-CX3CR1 signalling regulates crucial physiological functions necessary for immune regulation by attracting mononuclear cells from blood into the tissue. Membrane-bound FKN interacts with CX3CR1 on leukocytes and mediates rapid and firm adhesion of leukocytes (11,12).

Leukocyte infiltration of endothelial cells is mediated by FKN-CX3CR1 interaction results in various vascular impairments. We found that the V249I and T280M gene variants were associated with a reduced risk for CAD. Our results are similar to the findings of McDermott et al (6) and Moati et al (7). MM homozygous genotype of T280M polymorphism in CX3CR1 gene in some cohorts is reported to reduce the risk of acute coronary events (7) and coronary artery endothelial cell dysfunction (6). In contrast to the protective role of these variants in CAD and atherosclerosis, they are associated with a higher risk for developing systemic sclerosis-associated pulmonary arterial hypertension (13) and ischemic stroke (14,15).

The exact functional effects of the CX3CR1 280M and 249I alleles are unclear. The binding affinity of FKN chemokine (125Iodine labelled) to cells from CX3CR1-249I-280M homozygotes diminishes in comparison with its binding to wild-type CX3CR1-249I-280M cells (16). Both polymorphisms also result in ‘loss-of-function’ because the binding sites in cells was found to be significantly reduced in CX3CR1-249I-280M homozygotes compared with wild-type controls. In contrast, peripheral blood monocytes from individuals carrying the CX3CR1-249I-280M haplotype attach more to membrane-bound FKN than peripheral blood mononuclear cells from wild CX3CR1 V249I-280M donors, indicating a ‘gain-of-function’ (17).

Recently, there has been renewed interest in the CX3CR1 gene with the discovery of its role in the pancreatic β-cell insulin secretory pathway (8). With their comprehensive studies using CX3CR1 KO mice islets, Lee et al (8) observed that attenuation of FKN/CX3CR1 system underlie beta cell dysfunction and insulin secretion. The 280M and 249I
variants alter ligand-receptor binding affinity and are believed to influence an increased susceptibility to T2DM in the carrier. This assumption agrees, in part, with earlier studies by Shah et al. (5), involving patients with T2DM, and Sirios-Gagnon et al. (18) in obese individuals. They reported a positive association of CX3CR1 polymorphisms in their study subjects. This is, however, in sharp contrast to our findings. In our study, these two variants reduced the risk for T2DM. Homozygosity of mutant CX3CR1 alleles was more frequent in controls than cases. The 249I and 280M alleles were notably associated with the decreased risk for T2DM. Our results cannot be compared with studies investigating obesity (18) because we focused on T2DM and its associated macrovascular complications.

There is a significant paucity of data involving humans to further illustrate these contrasting evidences. The study by Mohan et al. (19) involving 2095 subjects with T2DM (the only study involving humans) had an OR of 1.20 with a modest test of significance of 0.05 after adjusting for variables such as age, sex and smoking lifestyle status. The Indian population is more prone to diabetes and its complications. Various studies have reported this trend. It is probable that the disparity in allele frequency is due to the interethnic differences between the South Indian Dravidian population analyzed in the present study and Caucasian-European populations in earlier studies.

These independent findings in CAD and T2DM prompted us to investigate the significance of CX3CR1 gene variants in patients with vascular diseases associated with diabetes mellitus. We analyzed 284 patients with T2DM diagnosed with CAD for the prevalence of these two genetic variants. The heterozygous TM and homozygous mutant TM genotypes of T280M variants were less prevalent in all patient groups. We report here, for the first time, that 280M genetic variants of CX3CR1 are also significantly associated with decreased risk for CAD in patients with T2DM. Diabetes mellitus and vascular inflammation leading to CAD is a multifactorial process involving various chemokines (20), scavenger receptors (20), monocytes, endothelial cells, vascular smooth muscle cells (22,23) and growth factors (24,25), which function in disarray, leading to the diabetic vascular disease phenotype. Polymorphisms in CX3CR1 result in reduced chemokine binding. The reduced FKN-CX3CR1 signalling would possibly be a compensatory mechanism to prevent the onset of vascular complications in diabetes.

CONCLUSIONS

The present study demonstrated a positive association for 280M allele of CX3CR1 gene conferring a protective role for diabetic vascular disease phenotype. The decreased binding of CX3CR1 with its ligand FKN leads to the attenuated interaction between monocytes and vascular endothelium. At the same time, the reduced binding due to these variants did not have a detrimental effect that led to a diabetic phenotype. This is plausible because altered CX3CR1 signalling may reduce adipose inflammation due to its altered chemokine activity.

DISCLOSURES: The authors have no financial disclosures or conflicts of interest to declare.

AUTHOR CONTRIBUTIONS: SS, SR and CCK conceived and designed the experiments, MMP, SS and SR performed the experiments, VRK and ATN performed statistical analysis of data, VRK, AM, CCK provided reagents, materials, analysis tools, SS and SR Prepared manuscript, AM and CCK critical review of manuscript. All authors read and approved the final manuscript.

ACKNOWLEDGEMENTS: The authors thank Professor M Radhakrishna Pillai, Director, Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram for providing funding and facilities for conducting this study. They also thank Dr NS Pratapchandran and Dr VRK Santosh for providing patient samples.