

Association of chemokine receptor *CX3CR1* V249I and T280M polymorphisms with chronic kidney disease

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ABSTRACT

The chemokine fractalkine (*CX3CL1*) and its receptor *CX3CR1* are involved in the activation of leukocytes. Two common single-nucleotide polymorphisms of the *CX3CR1* gene, V249I and T280M, have been associated with reduced fractalkine signaling, leading to decreased adhesive function and leukocyte chemotaxis. We hypothesized that variation in the *CX3CR1* gene could be associated with chronic kidney disease (CKD), a disease of inflammatory activation. We studied the association between *CX3CR1* V249I and T280M polymorphisms, and fractalkine and highly sensitive C-reactive protein (hs-CRP) levels in 123 patients with CKD and 100 healthy controls (HCs). Genotype analysis was done by polymerase chain reaction-restriction fragment length polymorphism, and fractalkine and hs-CRP levels were analyzed by enzyme-linked immunosorbent assay. MM genotype of T280M was absent in CKD patients, while in controls it was seen in 1% of the individuals. The allele frequencies in both the groups were similar ($P = 0.059$). Compared to HC, M280M + T280M genotype was more frequent in CKD ($P = 0.041$). The frequency of II genotype of V249I was 0.8% in CKD, whereas in HC, it was 2%. I249I + V249I genotype was more frequent in CKD as compared to HC ($P = 0.034$). No difference in allelic frequency of V249I was noted between the two groups ($P = 0.061$, odds ratios = 1.74, 95% confidence intervals = 0.96–3.12). Plasma fractalkine and serum hs-CRP levels were higher in CKD subjects ($P = 0.004$ and $P < 0.0001$). No association of either genotype was found with fractalkine and hs-CRP levels. Polymorphisms at I249 and M280 genotype in *CX3CR1* gene are associated with CKD; however, there was no association of fractalkine or inflammatory marker with these genotypes.

Key words: Chemokine receptor 1, chronic kidney disease, fractalkine, inflammation, single-nucleotide polymorphism

Introduction

Chronic kidney disease (CKD) is associated with persistent inflammatory activation. The adverse outcomes of CKD include progression to end-stage renal disease (ESRD) and increased risk for cardiovascular disease (CVD).^[1] Increased circulating inflammatory proteins are powerful predictors of all-cause mortality and cardiovascular death in ESRD patients.^[1,2]

Inflammation involves complex interactions among immune cells and soluble proteins such as cytokines, chemokines, and co-stimulatory molecules in response to several stimuli.^[3]

Chemokine fractalkine (*CX3CL1*), a member of the CX3C chemokine subfamily, is a transmembrane molecule expressed on endothelial cells. It is activated by pro-inflammatory cytokines^[4] and serves as an adhesion protein promoting the retention of monocytes and T-cells. It also exists in a soluble form, which resembles a conventional chemokine and induces chemotaxis. CX3C chemokine receptor 1 (*CX3CR1*), a 7-transmembrane

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domain G-protein-coupled receptor, is expressed by natural killer cells, monocytes, and CD8⁺ T-cells and was recently shown on CD4⁺ T-cells.^[5,6] Both chemotaxis and adhesion are mediated by CX3CR1-fractalkine signaling. Accumulating evidence indicates that CX3CL1-CX3CR1 pathway is involved in the pathogenesis of inflammatory diseases,^[7] including atherosclerotic CVD in CKD.^[6,8,9]

Fractalkine and its receptor CX3CR1 have been suggested to play a key role in human and animal model of renal disease. Fractalkine expression and cells expressing CX3CR1 have been shown in renal abnormalities.^[10-14] A report by Feng *et al.*^[15] shows that anti-CX3CR1 antibody treatment inhibits leukocyte infiltration into the glomeruli, improving renal function. Another study^[16] showed that CX3CR1 inhibition is protective against ischemic acute renal failure in mice. These studies suggest a role for fractalkine/CX3CR1 axis in pathogenesis of human renal disease.

The two most common single-nucleotide polymorphisms (SNPs) in the CX3CR1 gene (V249I and T280M) lead to changes in conserved amino acids in the receptor protein. Both polymorphisms are in strong linkage disequilibrium, forming a common I249M280 haplotype.^[17] An association of several inflammatory diseases including arteriosclerosis and coronary artery disease and ESRD with these SNPs has been demonstrated.^[18-21] The early genotyping of CX3CR1 polymorphism may play a role in identifying advanced ESRD progression and potential use of anti-CX3CR1 therapy.^[18]

In view of the existing studies suggesting a role of this pathway in complications in CKD, we studied the frequency of V249I and T280M polymorphisms in the CX3CR1 gene and evaluated its association with circulating fractalkine level and highly sensitive C-reactive protein (hs-CRP).

Materials and Methods

Patients

A total of 123 CKD patients with estimated glomerular filtration rate (eGFR) <30 ml/min were recruited for

the study from Nephrology Department of Postgraduate Institute of Medical Education and Research, Chandigarh. Patients below 18 and above 70 years of age, those with malignancy, or infection in the last 3 months were excluded. The Institute Ethics Committee approved the study protocol and all subjects provided a written consent. One hundred healthy age-, sex-, and ethnicity-matched North Indians were selected as controls. All study subjects underwent a thorough clinical examination. Hypertension and diabetes were diagnosed according to the standard criteria. Details of clinical features and laboratory investigations were recorded.

Genetic analysis

Genomic DNA was extracted from cells in the buffy coat isolated from samples collected after overnight fasting using a QIAamp DNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. V249I and T280M polymorphisms were detected using polymerase chain reaction (PCR) followed by restriction fragment length polymorphism. Details of the location of the SNPs, primer sequences, PCR conditions, and restriction enzymes (New England BioLabs Inc., USA) with product sizes are given in Table 1. The digested PCR products were resolved on 2–3% agarose gels stained with ethidium bromide [Figure 1].

Highly sensitive C-reactive protein and fractalkine level

Sandwich enzyme-linked immunosorbent assay was used to determine hs-CRP (Diagnostics Biochem Canada Inc., London, Ontario, Canada) and circulating fractalkine (Ray Biotech Inc., Norcross, GA, USA)

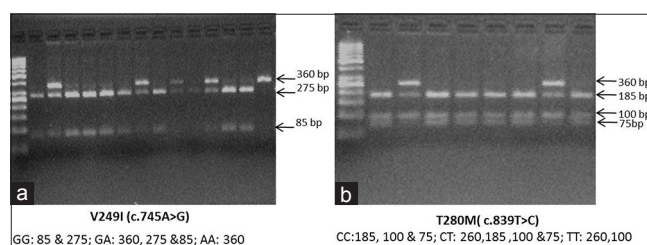


Figure 1: Representative image of agarose gel electrophoresis of (a) CX3CR1 V249I and (b) CX3CR1 T280M polymorphism

Table 1: Polymorphisms in the CX3CR1 gene, their location, primer sequences, polymerase chain reaction conditions, and restriction enzyme and restriction digestion product size

Gene and polymorphism	Primer	PCR condition (°C)	Restriction enzyme	Restriction digestion product (base pair)
CX3CR1-V249I (rs3732379)	F: 5'-CCTGCAAGAACCACAAGAAAGC-3'	95°C - 10 min	Ac1I	VV: 85 and 275
	R: 5'-GGCATGGAAGTGTCTGAGCA-3'	94°C - 30 s		VI: 360, 275 and 85
CX3CR1-T280M (rs3732378)		65°C - 45 s	Bsmb1	II: 360
		72°C - 30 s		TT: 185, 100 and 75
		72°C - 10 min		TM: 260, 185, 100 and 75
		30 cycles with step 2-4		MM: 260, 100

PCR: Polymerase chain reaction, CX3CR1: CX3C chemokine receptor 1

levels in plasma according to the manufacturer's instructions.

Statistical analysis

Data are expressed as mean \pm standard deviation. Statistical analysis was performed using SPSS 16.0 (SPSS Chicago, IL, USA). Differences between groups were analyzed with the Student's *t*-test when the variable was normally distributed. Mann-Whitney U-test was used for data with skewed distribution. Hardy-Weinberg equilibrium (HWE) was tested for each SNP for cases and controls. Allelic and genotypic associations of SNPs were evaluated by Pearson's χ^2 -test; odds ratios (OR) and 95% confidence intervals (CI) were determined. Haplotype analysis was done using online software SHEsis.^[22] All *P* values were calculated two-sided, and a value of <0.05 was considered significant.

Results

General demographic and clinical characteristics of the study population are shown in Table 2. A majority of the patients were males. As expected, the blood pressure was higher among the patients. About 33% of the CKD patients were diabetic and 85% had hypertension.

Plasma fractalkine and serum hs-CRP levels were increased in subjects with CKD as compared to healthy controls (438.6 ± 490.6 vs. 239.7 ± 73.1 pg/ml, $P = 0.004$ and 94.4 ± 58.9 vs. 48.4 ± 52.9 μ g/ml, $P < 0.0001$ respectively).

Genotype frequencies in cases and controls were in HWE (for T280M; $P = 0.097$, $\chi^2 = 2.750$ in CKD and $P = 0.528$, $\chi^2 = 0.397$ in HC), for V249I; $P = 0.181$, $\chi^2 = 1.784$ in CKD and $P = 0.201$, $\chi^2 = 1.630$ in HC).

The distribution of genotype and allele frequencies for CX3CR1 T280M and V249I polymorphisms are shown in Table 3. For the 280 locus, the allele frequencies in both groups were similar ($P = 0.059$, OR = 1.84, 95% CI = 0.96–3.51). Compared to HC, the MM and TM variants were more frequent in CKD ($P = 0.041$, OR = 2.16, 95% CI = 1.07–4.32). At the 249 position, II and VI variants were more frequent in CKD as compared to HC ($P = 0.034$; OR = 2.1, 95% CI = 1.09–4.01). No difference in allelic frequency, however, was noted between the two groups ($P = 0.061$, OR = 1.74, 95% CI = 0.96–3.12).

The haplotype distribution is shown in Table 4. The V249T80 haplotype was more frequent in healthy subjects. CKD subject shows increased IT and IM haplotypes but the difference was not significant ($P = 0.08$, OR = 2.7 and $P = 0.128$, OR = 1.7, respectively).

We also compared the clinical characteristics of CKD subjects according to their genotypes, but found no significant association with any parameter except serum albumin [Table 5]. IV + II and TM + MM had lower serum albumin levels as compared to VV and TT, respectively ($P = 0.043$ and $P = 0.042$).

Discussion

Persistent inflammatory activation has been identified as the key factor behind development and progression of CVD in patients with CKD. Why some patients show more intense inflammatory activation and others do not is not well understood. One possibility is differential activation secondary to genetic variations in pro-inflammatory cytokine/chemokine and their receptors. We have shown an increase in CD4⁺ CX3CR1⁺ T-cell population and plasma fractalkine level in advanced renal failure patients'. In another study, the CX3CR1 V249I SNP was shown to be associated with advanced CKD.^[18]

Epidemiological and clinical study reports have investigated the association between polymorphisms in the CX3CR1 and coronary artery disease,^[21,23] risk of acute rejection in renal transplant recipients,^[24] cancer rates in renal transplant recipient,^[25] age-related macular degeneration,^[26] and HIV.^[17] The presence of V249 and T280 SNPs in CX3CR1 gene leads to its overexpression by activated inflammatory cells and with higher binding affinity for fractalkine.^[17,27] Although the exact mechanism is not clear, it is important that CX3CR1 has been reported

Table 2: Baseline characteristic of study subjects

Parameters	CKD	HC	<i>P</i>
Number of cases	123	100	
Age (years)	51.7 \pm 12.5	48.9 \pm 11.2	0.08
Gender: Male/female	91/32	78/22	0.5
Body mass index (kg/m ²)	22.9 \pm 4.1	22.7 \pm 3.3	0.7
Systolic blood pressure (mmHg)	140.0 \pm 17.6	110.3 \pm 22.0	0.001
Diastolic blood pressure (mmHg)	84.7 \pm 8.7	80.6 \pm 6.4	0.001
Diabetes (%)	41 (33.3)	-	
Hypertension (%)	105 (85)	-	
Hemoglobin (mg/dl)	10.1 \pm 2.1		
Serum creatinine (mg/dl)	6.7 \pm 3.7		
Serum urea (mg/dl)	128.7 \pm 58.8		
eGFR (ml/min/1.73 m ²)	13.3 \pm 10.1		
Serum albumin (g/dl)	4.02 \pm 0.7		
Total cholesterol (mg/dl)	164.1 \pm 44.2		
LDL cholesterol (mg/dl)	94.0 \pm 40.3		
HDL cholesterol (mg/dl)	44.3 \pm 12.2		
Triglycerides (mg/dl)	154.5 \pm 82.2		
Calcium (mg/dl)	8.1 \pm 1.5		
Inorganic phosphate (mg/dl)	6.3 \pm 2.2		
Fractalkine (pg/ml)	438.6 \pm 490.6	239.7 \pm 73.1*	0.004
hs-CRP (μ g/ml)	94.4 \pm 58.9	48.4 \pm 52.9*	<0.0001

Data are presented in mean \pm SD, *Fractalkine and hs-CRP were analyzed in 60 HC only. CKD: Chronic kidney disease, eGFR: Estimated glomerular filtration rate, LDL: Low-density lipoprotein, HDL: High-density lipoprotein, hs-CRP: Highly sensitive C-reactive protein, SD: Standard deviation, HC: Healthy controls

Table 3: Genotype and allele frequencies of CX3CR1-V249I and T280M polymorphisms in CKD and HC groups

Gene polymorphism	Genotype	CKD (n=123)	HC (n=100)	OR (95% CI)	P
V249I	VV	86 (69.9)	83 (83)	2.1 (1.09-4.01)	0.034 (II + VI vs. VV)
	VI	36 (29.2)	15 (15)		
	II	1 (0.8)	2 (2)		
T280M	TT	91 (73.9)	86 (86)	2.16 (1.07-4.32)	0.041 (MM + TM vs. TT)
	TM	32 (26.1)	13 (13)		
	MM	0 (0)	1 (1)		
Allele V249I	V	208 (84.5)	181 (90.5)	1.74 (0.96-3.12)	0.061 (I vs. V)
	I	38 (15.5)	19 (9.5)		
T280M	T	214 (86.9)	185 (92.5)	1.84 (0.96-3.51)	0.059 (M vs. T)
	M	32 (13.1)	15 (7.5)		

CKD: Chronic kidney disease, CI: Confidence interval, OR: Odds ratio, HC: Healthy controls

Table 4: Estimated haplotype in study subjects

Haplotype	CKD (frequency)	HC (frequency)	χ^2	Pearson's P	OR (95% CI)
V249-T280	0.820	0.905	5.214	0.022	0.498 (0.272-0.914)
I249-T280	0.050	0.020	2.924	0.087	2.669 (0.851-8.373)
I249-M280	0.104	0.075	2.321	0.127	1.710 (0.852-3.432)
V249-M280	0.026	0	-	-	-

CKD: Chronic kidney disease, CI: Confidence interval, OR: Odds ratio, HC: Healthy controls

Table 5: Comparison of clinical and laboratory characteristics in CKD subjects according to their genotype

Parameters	CX3CR1-V249I polymorphism			CX3CR1-T280M polymorphism		
	VV genotype	IV + II genotype	P	TT genotype	TM + MM genotype	P
Age (years)	52.3±12.2	50.1±11.7	0.286	53.2±11.5	47.2±12.6	0.015
Gender: Male/female	64/22	27/10	0.870	65/26	26/6	0.251
Body mass index (kg/m ²)	23.1±4.0	22.5±4.4	0.443	23.2±3.6	22.3±3.7	0.318
Systolic blood pressure (mmHg)	139.1±17.6	141.8±16.9	0.446	138.0±16.8	145.0±17.9	0.068
Diastolic blood pressure (mmHg)	84.1±8.6	86.0±9.1	0.295	84.2±8.5	86.1±9.4	0.346
Hemoglobin (mg/dl)	10.2±2.2	9.8±2.2	0.287	10.1±2.2	9.8±2.3	0.498
Serum urea (mg/dl)	131.9±64.2	122.2±45.5	0.428	127.5±59.3	132.9±58.2	0.695
eGFR (ml/min/1.73 m ²)	13.7±10.5	12.6±9.4	0.566	13.6±10.5	12.7±9.6	0.654
Serum albumin (g/dl)	4.3±0.6	3.9±0.7	0.043	4.3±0.7	3.9±0.7	0.042
Total cholesterol (mg/dl)	165.0±45.5	161.9±40.9	0.770	165.6±42.7	158.6±49.9	0.598
LDL cholesterol (mg/dl)	98.1±44.3	84.1±26.7	0.104	97.7±41.9	82.2±33.1	0.122
HDL cholesterol (mg/dl)	44.5±12.9	43.7±10.5	0.771	43.8±9.6	45.7±18.3	0.682
Triglycerides (mg/dl)	150.0±72.3	165.5±103.4	0.525	153.3±66.7	158.6±124.1	0.865
Calcium (mg/dl)	8.1±1.5	8.1±1.6	0.891	8.2±1.5	7.9±1.8	0.250
Inorganic phosphate (mg/dl)	6.1±2.3	6.8±2.1	0.112	6.2±2.2	6.8±2.3	0.496
Fractalkine (pg/ml)	439.5±513.8	436.4±437.6	0.974	440.9±501.2	431.6±466.1	0.926
hs-CRP (µg/ml)	94.2±59.6	94.9±58.1	0.953	95.4±59.5	91.5±57.9	0.749

Data are presented in mean±SD. CKD: Chronic kidney disease, eGFR: Estimated glomerular filtration rate, LDL: Low-density lipoprotein, HDL: High-density lipoprotein, hs-CRP: Highly sensitive C-reactive protein, SD: Standard deviation, CX3CR1: CX3C chemokine receptor 1

to function *in vitro* in CD4 as an HIV co-receptor, and the M280 allele is associated with reduced receptor expression on PBMCs from HIV-positive subjects.^[17] In contrast to HIV, in which disease progression was faster in M280 homozygotes, the association of CX3CR1 with CKD was noted for I249 and M280 heterozygotes, whereas only I249 was associated with ESRD in previous report.^[18] These report suggest a differential effect of the two polymorphisms on CX3CR1 function.

The association between fractalkine level and genetic variants of its receptor CX3CR1 has not been investigated so far. Although patients with CKD exhibit increased levels of plasma fractalkine, no association with CX3CR1 V249I

and T280M was noted. Further, there was no association of these polymorphisms with other parameters including hs-CRP. However, decrease in serum albumin was noted in genotypes with I249 and M280. However, in relation to coronary artery disease, M280 and I249 alleles seem to be protective, perhaps due to a reduced number of receptor binding sites and a decreased fractalkine binding affinity.^[19-21]

There are some limitations to this study. The number of cases is relatively small. Also, it is likely that other genetic factors might play a role in inflammatory activation. Ideally, therefore, interaction between the various factors needs to be evaluated. On the plus side, it is well-selected

homogenous population with well-characterized subjects, reducing phenotyping errors and bias. The study also includes well-matched controls of the same ethnic background, which reduced the risk of population stratification that is one of the major causes of false results in case-control studies. To conclude, our results suggest that CX3CR1 V249M and T280M polymorphisms may influence the risk of CKD development. These polymorphisms could be added to the list of potential markers to enable physicians in determining the CKD risk profile. The study, however, needs to be replicated in a larger population with longitudinal follow-up study. The ongoing Indian chronic kidney disease study will provide such an opportunity.

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Conflicts of interest

There are no conflicts of interest.

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