Polymorphism in the promoter of the *CCL5* gene (*CCL5* G-403A) in a cohort of North Indian children with Kawasaki disease. A preliminary study

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ABSTRACT

Objective. *Kawasaki disease (KD) is a common vasculitic disorder and a leading cause of acquired heart disease in children. However, there is a paucity of information on KD from developing countries. The clinical phenotype of KD in India is different from that in the West. In this study, we investigated the association of a promotor gene variant of chemokines like chemokine ligand 5 (CCL5) and a deletion in chemokine receptor CCR5 (which is a common receptor for CCL5, macrophage inhibitory protein 1α and 1β), in a cohort of North Indian children with KD.*

Methods. *CCL5 G-403A and CCR5Del 32 gene variants were genotyped in the KD cohort (n=40) and in healthy controls (n=100) using the PCR-RFLP assay. Logistic regression analysis was performed in order to examine the association of these variants with KD, with special reference to those with direct (on echocardiography) or indirect (on myocardial scintigraphy) evidence of coronary involvement.*

Results. *No significant difference in genotype or allele frequency of CCL5 G-403A variant was observed between patients and controls. However, patients with evidence of coronary involvement had a higher frequency of the minor allele CCL5 -403A (p<0.004; OR- 2.25, 95%CI: 1.13–4.46). CCR5 Del 32 variant was found to be monomorphic (minor allele frequency <0.05) in our cohort.*

Conclusion. *CCL5 -403A variant may be associated with coronary involvement in North Indian children with KD. Our results, however, have to be replicated on a larger sample before any definitive conclusions can be drawn.*

Introduction

Kawasaki disease (KD) is a common vasculitic disorder predominantly affecting infants and young children (1, 2). It involves medium-sized arteries, especially the coronaries. It is now the leading cause of acquired heart disease in children in several developed countries (3). KD can result in the development of coronary artery abnormalities (CAA) in 15%–25% of untreated children (4, 5). Though the etiology of KD is unknown, clinical and epidemiological features strongly suggest an infectious cause in a genetically predisposed individual. The initial infectious trigger results in massive stimulation of the immune system through T lymphocytes (6). Furthermore, there is evidence of endothelial cell activation through chemokines-like RANTES (Chemokine ligand 5; CCL5) and up-regulation of adhesion molecules (ICAM1, VCAM 1, E-selectin) (7-10).

Striking differences in the incidence of KD among various ethnic populations, and an increased risk of KD among siblings and twins of index patients, both suggest a possible genetic component in the etiopathogenesis of this condition (3, 4). Recent studies also indicate that genetic factors could influence the development and progression of KD (11, 12). Genetic polymorphisms in several genes, *e.g.* TNF-α, and IL10 amongst others, (13) have been found to be associated with KD. The associations that have been validated in independent cohorts are IL-4, ITPKC, Caspase 3, and CCR5 Del 32 (14).

Since genetic variations in cytokine genes may influence levels, it has been suggested that common genetic variants in these genes such as the *CCR5*, *CCR3* and *CCR2* gene cluster could contrib-

Table I. Standard PCR conditions used in genotyping *CCL5* (-403) G/A and *CCR5* (Del 32) variants.

SNP (rs#)	Primers	Amplicon (bp) (mmol)	Annealing temp.(°C) / ($MgCl_2$)	Restriction enzyme/allele size
CCL5 (-403)G>A (rs2107538)	P1: 5'- GCC TCA ATT TAC AGT GTG -3' **P2: 5'- TGC TTA TTC ATT ACA GAT gTT -3'	135	50/1. 5	Mae III (New England Biolabs Inc.) A = 135 bp G = 112, 23 bp
CCR5 Del 32* (I/D) (rs333)	P1: 5'- GAA GTT CCT CAT TAC ACC TGC AGC TCT C -3' P2: 5'- CTT CTT CTC ATT TCG ACA CCG AAG CAG AG -3	174/142	63.5/3.5	I allele = 174 bp D allele = 142 bp

*Primers for this variant have been designed using Primer 3.
**This primer has a substitution depicted by the letter 'g' to create a restriction site.

ute to the susceptibility to KD (15, 16). Burns *et al*. proposed that genetic variations in *CCR5* could play an influential role in the KD susceptibility and may influence coronary artery outcomes in affected children (9). The aim of our study was to determine if common genetic variants in the *CCL5* (G-403A) and *CCR5* (Del 32) are associated with the susceptibility to KD and the involvement of coronary arteries in a cohort of North Indian children with KD.

Patients and methods

Subject selection

The study population included 40 children diagnosed with KD and registered at the Pediatric Rheumatology Clinic, Advanced Pediatrics Center, Postgraduate Institute of Medical Education and Research (PGIMER), Chandigarh, between January 2007 and December 2008. Diagnosis of KD was made on the basis of guidelines given by the American Heart Association1. Study-group children were treated with intravenous immunoglobulin, 2gm/kg bolus. This was accompanied by aspirin, initially in high doses (75–80mg/kg/day) followed by an antiplatelet dose (3–5mg/kg/day) for 4–6 weeks.

For the purposes of this study, a child with KD was said to have coronary artery involvement if there was direct evidence based on standard echocardiography criteria (17) or if there was indirect evidence based on perfusion abnormalities on the myocardial scintigraphy examination. Echocardiographic evaluations were carried out in all children during the initial admission and then subsequently on follow up after approximately 4–8 weeks. Myocardial scintiscanning was carried out in the follow-up period, approximately 5–6 months after the diagnosis. Age-, sex- and ethnicity-matched healthy children (n=100) were enrolled as controls. The study protocol was approved by the Institute Ethics Committee and informed consent was obtained from the parents of all the patients.

DNA isolation and genetic analysis

Genomic DNA was extracted from peripheral blood using the phenol-chloroform method (18). *CCL5* G-403A (rs2107538) polymorphism was genotyped using the PCR-RFLP method as previously performed by Makki *et al*. (19). The *CCR5* Del 32 polymorphism (rs333) is characterised by the presence (Insertion) or absence (Deletion) of a 32bp fragment in exon 3. Primers were designed using Primer 3 software, where a PCR product of 174bp confirmed an insertion allele, and a product of 142bp confirmed the deletion allele. Primer sequences and detailed PCR conditions are given in Table I. The genotyping results were validated to check for false positives, through random sequencing of samples.

Statistical analysis

All the statistical tests were performed using the SPSS version 13.0 (SPSS Inc. Chicago, IL, USA). Differences in frequencies of the various genotypes between KD patients and healthy controls were computed with the Pearson's χ^2 test. The Hardy-Weinberg Equilibrium was determined for each polymorphism in accordance to our previous study (20).The relationships between genotypes as well as several known risk factors and coronary artery involvement (yes/no) were studied using logistic regression analysis. Furthermore, the variables showing a statistically significant association were evaluated in a multivariate logistic regression model to assess their independent prognostic value for coronary artery involvement. Prognostic values of the variables were expressed as odds ratios (OR) with their 95% confidence intervals (95% CI). *P*-values were subjected to Bonferroni's correction and considered significant when $p<0.05$. Parameters with skewed distribution were compared using the Mann-Whitney U-test.

Results

Demographic profile

The demographic profile of our patients is given in Table II. The mean age did not differ significantly between cases and controls ($p>0.05$). Seventy-three percent of the cases and 71% of controls were males. Ten children (25%) were diagnosed as having coronary artery involvement based on the findings of echocardiography (these included children with transient coronary artery dilatation) and myocardial scintiscanning. Of these, 7 had abnormalities on echocardiography while 4 had an abnormal scintiscan. One patient had abnormalities on both echocardiography and scintiscanning.

Genotype analysis

Study subjects were genotyped for *CCL5* (G-403A) and *CCR5* Del 32 variants. Both the polymorphisms were in Hardy-Weinberg equilibrium. No significant differences were observed in the allele and genotype frequencies of *CCL5* G-403A polymorphism between the patient and control groups (Table IIIa).

The minor allele (D allele of CCR5 Del 32) frequency (MAF) was ob-

served to be less than 5%, indicating this CCR5 variant to be monomorphic (MAF<0.05), and thus was not studied further (Table IIIb).

Polymorphisms and other risk factors in relation to coronary artery involvement

The frequency of *CCL5* (G-403A) genotypes in patients with and without coronary artery involvement are shown in Table IV. Frequency of minor allele 'A' was significantly higher among cases with coronary artery involvement as compared to cases without such involvement (p<0.02; OR: 2.25, 95%CI: 1.13–4.46). A comparison of demographic and inflammatory parameters in patients with and without coronary artery involvement showed no significant difference in any of the variables between the two groups (Table II).

Discussion

India has a lower prevalence of KD than Japan and several western countries (4, 5, 21-25). Clinical presentation of KD in our country is also different from that in the West (4, 21-25). For instance, KD occurs in a relatively older age group in Indian children (21-25). Also, in India, thrombocytosis and the peeling of extremities tend to occur within 10 days of onset of fever, unlike the usual course of KD described in the West where this usually occurs after 10 days during the convalescent phase of KD (21-25). It is possible that these differences in phenotype could have a genetic basis. In view of the fact that there is no information available on genetic aspects of KD from India (or from other developing countries), we embarked on this study.

KD is accompanied by hyper-reactive immune responses resulting in vascular inflammation. Polymorphisms in the genes encoding chemokines have been described as affecting chemokine production or function, and thus could contribute to susceptibility and sequelae of KD (9, 15). In the present study, we tested the potential associations between functional polymorphisms of two inflammatory genes (*CCR5* and *CCL5)* and the susceptibility to KD with special reference to coronary ar-

Table II. Demographic profile of Kawasaki patients and controls.

Characteristics	KD Patients[a] (n=40)	Controls[b] (n=100)	p-value (a *vs*. b)
*Age (years)	5.25 ± 2.5	5.13 ± 1.07	0.98[c]
Age (<5 years) (%)	45	40	0.75[c]
Gender (male/female)	72.5/27.5	71/29	0.92[d]
Oral lesion – n (%)	26 (65)	–	–
Conjunctival injection – n (%)	16 (40)	–	–
Rash – n (%)	31 (77.5)	–	–
Change in extremities – n (%)	15 (37.5)	–	–
Cervical adenopathy – n (%)	26 (65)	–	–
Peripheral desquamation – n (%)	32 (80)	–	–
Cardiac abnormality – n (%)	10 (25)	–	–
Irritability – n (%)	14 (35)	–	–
Jaundice – n (%)	1 (2.5)	–	–
TLC (>12000 / mm^3)	12042.50 ± 3614.41 (16)	–	–
Platelet count (>4.5×10^5 / mm^3)	459.78 ± 225.9 (23)	–	–
ESR (mm /1st hour)	30.05 ± 13.79 (40)	–	–

*Value is Mean ± Standard deviation; [a] Kawasaki patients, [b] Controls, [c] Student's t-test; [d] Pearson's Chi square test, n: number of subjects.

Table III. Allele and genotype frequencies of the **a**) the *CCL5 G(-403)A* and **b**) *CCR5 (Del 32)* variants.

a) *CCL5* (G-403A)*

	Genotype frequency n (%)		Odds ratio (95% CI)	p-value[Ψ]
	Patients (n=40)	Controls (n=100)		
GA + AA	14 (0.35)	28 (0.28)	1.38 (0.63–3.02)	0.54
GG	26 (0.65)	72 (0.72)		
	Allele Frequency 2n (%)			
G	64 (0.8)	172 (0.86)	1.53 (0.77–3.02)	0.27
A	16 (0.2)	28 (0.14)		

*Pearson's Chi Square Test, n= number of subjects.

b) *CCR5* (Del. 32)**

	Genotype frequency n (%)		Odds ratio (95% CI)	p-value[Ψ]
	Patients (n=40)	Controls (n=100)		
ID+DD	1 (0.023)	2 (0.02)	0.837 (0.07–9.48)	0.65
II	41 (0.977)	98 (0.98)		
	Allele frequency 2n (%)			
D	1 (0.011)	2 (0.01)	1.193 (0.11–13.3)	0.65
I	83 (0.99)	198 (0.99)		

**Fisher's exact test.

Table IV. Association of *CCL5* (G-403A) polymorphism with coronary artery involvement

*CCL5 (*G-403A) Polymorphism				
Coronary artery involvement	Positive 2n (%)	Negative (%)	OR (95%CI)	p-value
G	14 (0.7)	50 (0.84)	2.25 (1.13–4.46)	<0.02
A	6 (0.3)	10 (0.16)		

[φ]Pearson's Chi square test, 2n = number of alleles.

tery involvement. We studied two key polymorphisms, the -403G>A SNP, which lies in the promoter region of *CCL5* gene, and the Del 32 variant in the *CCR5* gene, which is linked with a frame-shift and premature termination of transcript translation, thus preventing expression of the receptor molecule on cell surface (26, 27).

Our study demonstrated that patients with KD were more likely to have the *CCL5* homozygous mutant genotype (AA). This genotype was only present in KD patients, indicating that this mutant genotype may be associated with a susceptibility to KD. A comparison of *CCL5* G-403A allele frequency between KD patients who showed evidence of coronary artery involvement (direct or indirect) and those without such involvement, revealed a significantly higher frequency of mutant A allele in the former group. The relative risk of coronary artery involvement in A allele carriers was 2.5 times greater. This suggests that the presence of the mutant *CCL5* -403A allele may increase genetic susceptibility to coronary artery involvement in children with KD from North India. Such patients may need to be followed up closely for the development of premature arteriosclerosis. Our results support the observations by Simeoni *et al*. who reported that *CCL5* -403A was associated with coronary artery disease and that this association was enhanced in conditions where inflammation predominates (26). The probable biological mechanism underlying this genetic association has been proposed to be an increased *CCL5* expression in -403A allele carriers. This may lead to increased deposition of CCL5 on the surface of inflamed endothelium. CCL5 is a potent chemoattractant for monocytes, lymphocytes, eosinophils and basophils. To the best of our knowledge, ours is the first study suggesting a possible association of *CCL5* G-403A polymorphism with KD from a developing country.

Burns *et al*. (9) have also reported a trend towards an inverse relationship between the presence of the *CCR5* HHG*2 haplotype that includes the *CCR5* Del 32 allele and the risk of CAL in KD patients. However, Breunis *et al*. could not confirm this trend (27). We observed that CCR5 ID polymorphism was monomorphic (MAF <0.05%) in our study population, and hence was not studied further. The frequency of Del 32 deletion mutation varies in different populations. In Japan, where KD is far more prevalent, the 32-bp deletion is almost absent, whereas in northern Europe this deletion is more common. CCR5 has very low frequency in the Indian subcontinent (28, 29), but the prevalence of KD in the Indian population appears to be much lower than in Japan and several western countries (5, 23, 25).

The originality of this work lies in the fact that it has been conducted in an Indian population, whilst most work on KD has been done on Caucasian populations, thereby raising the profile of this disease more globally. Furthermore, there is no information about the genetic aspects of KD from a developing country. Another strength of our study is that we had ethnically homogenous subjects who were enrolled from a single centre, thus avoiding phenotype errors and bias.

We are also aware of some limitations of our study. Given that KD is a complex multifactorial disease and multiple genes are implicated, samples sizes in the order of several hundred to >1000 are required. The small size of our study cohort could introduce a type I error and make the interpretation of the results somewhat difficult. However, for a condition like KD in India, it is very difficult to access a larger sample size, especially at a time when the condition is just emerging in the country (5, 23, 25). Although it would be imprudent to extrapolate our data, the present study could point towards genetic trends. Further replication through multicentric studies in a larger cohort of KD patients may be required to validate these results among Indian children with KD. However, this may not be easy because of the significant genetic heterogeneity amongst different races in the Indian subcontinent.

For the purposes of this study, a child with KD was said to have coronary artery involvement if there was direct evidence based on standard echocardiography criteria (17) or if there was indirect evidence based on perfusion abnormalities on myocardial scintigraphy. Although myocardial scintigraphy is generally not considered to be part of the standard testing procedures in children with KD, it is now being increasingly used in the assessment of this condition (30-33).

In conclusion, our results suggest that the *CCL5* G-403A polymorphism does not appear to be associated with KD. However, an association may exist between a promoter SNP in CCL5 and coronary artery abnormalities associated with this condition. Also, the *CCR5* Deletion 32 polymorphism is monomorphic and is non-existent in our cohort of North Indian children with KD.

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