Original Article

Association of single nucleotide polymorphism rs113420705 of *CASP3* in children with Kawasaki disease from North India

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Abstract

Background: Kawasaki disease is a pediatric, systemic, vasculitic disorder. Its exact etiology is still unknown. Genetic polymorphisms are being investigated as susceptibility factor for this disorder. These are likely to vary among different populations. **Aim:** To investigate the association of single nucleotide polymorphism (SNP) rs113420705 of *CASP3* in Kawasaki disease (KD) from North India. **Settings and Design:** Observational, case–control study. **Methods:** Polymerase chain reaction and bidirectional Sanger sequencing was used for determining genotypes of SNP rs113420705 in 45 cases of KD and 50 healthy age- and sex-matched controls. Allele and genotype frequencies were assessed and compared between the groups. **Results:** Among 45 cases, 32 had TT (71.1%), 13 had CT (28.9%) and none had CC genotype of SNP rs113420705. No significant differences in allele, genotype, or carrier frequencies of rs113420705 were found between the two groups. A comparison was also made between subgroups of KD with coronary abnormality (7 children; 15.5%) and KD with normal coronaries (38 children; 84.4%). The C allele was significantly overexpressed in KD with coronary abnormality group ($P = 0.005$). However, no difference was noted in the genotype frequencies. **Conclusion:** CT genotype of rs113420705 of *CASP3* showed a trend to significance with the occurrence of KD in children in North India. However, we could not establish any association between minor allele C and susceptibility to KD. C allele appeared to be over expressed in children with KD with coronary abnormalities. Larger studies will help us to reach conclusive evidence applicable to all ethnicities.

Keywords: *CASP3* gene, coronary artery abnormalities, Kawasaki Disease, rs113420705, single nucleotide polymorphism

Introduction

Kawasaki disease (KD), also known as mucocutaneous lymph node syndrome, is a medium vessel vasculitis and one of the commonest causes of acquired heart disease in children.[1]

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Incidence of KD is high and continues to increase in countries like Japan, Korea, and Taiwan, whereas the incidence in Western countries has plateaued.[2] Recent data show that the incidence of KD is increasing in India.[3–5] The main concern in KD is the risk of serious cardiovascular complications like coronary artery abnormalities (CAAs), myocarditis and KD shock syndrome.[1]

Diagnosis of KD is based on a set of clinical criteria. At present, there is no laboratory marker for confirmation of diagnosis.[6,7] Modalities like intravenous immunoglobulin (IVIg), anti-inflammatory drugs and biologics have an established value

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in treating KD.[1,8] Though pathophysiology and therapeutics of KD have been explored well, etiology of KD is not understood yet.[9] Many etiological propositions have been studied but none of these are substantiated and verified.

A higher incidence of KD in children of parents with a history of KD and in certain ethnicities like Japan, Korea, and Taiwan are strong pointers towards a possible genetic basis for KD.[10] Evidence for this also comes from the observation that incidence of KD amongst children of Japanese ancestry in Hawaii is similar to the incidence in Japan and is much higher than in native Hawaiian population.

Inositol 1,4,5 triphosphate 3-kinase C (*ITPKC*), Caspase-3 (*CASP3*), B lymphocyte kinase (*BLK*), *CD40*, and HLA locus genes are some of the widely studied genes for possible association with susceptibility to KD.[1,11] Single nucleotide polymorphisms (SNPs) in these genes are found to be associated with risk of developing KD, its complications, and resistance to the IVIg. Genome-wide association studies have helped to identify the possible susceptibility loci and polymorphisms leading to an increased propensity to KD.

CASP3 protein encoded by *CASP3* gene is involved in binding of the nuclear factor of activated T cell (NFAT) and thereby modifies T cell activation. Polymorphisms of this gene may alter the event significantly. There are studies on association of SNP of rs113420705 at *CASP3* gene with susceptibility of KD from Japan and some western countries.[12] However, the genetic association may vary in children of other races and ethnicities. Though there is a study of *ITPKC* gene polymorphisms from India,[13] there is no study on the association of the SNP rs113420705 of *CASP3* gene and susceptibility to KD from India. In this study, we explored the association of this SNP and various aspects of KD among north Indian children at Chandigarh. Though KD is a common childhood vasculitis, it is often missed or misdiagnosed at primary care settings. Such genetic association studies not only describe the validity of the association in studied ethnicities but also helps general pediatricians and primary care physicians better understand of the disease. Genetic association studies will help them explore, diagnose and treat the children in a focused way.

Methods

Study population

This case–control study from North India was conducted in the Advanced Pediatrics Centre, Postgraduate Institute of Medical Education and Research, Chandigarh, India from January 2018 to April 2019. Forty-five children (35 boys and 10 girls, M: F = 3.5) diagnosed and being followed up as KD were enrolled. Control group consisted of 50 age- and sex-matched, afebrile children (34 boys and 16 girls; M: F = 2.15) recruited from a pediatric outpatient clinic. Mean (SD) age of children with KD was 4.18 (±2.90) years whereas that in control group was 3.79 (±2.43) years.

Diagnosis of KD was made as per the American Heart Association (AHA) guidelines, 2017.[1] A coronary artery abnormality (CAA) was considered as coronary abnormality including ectasia, dilatation, and aneurysms (with the coronary z-score value of ≥2.5).[1]

Mean age of controls was 3.79 years. Data were collected in pre-designed case record form. Echocardiographic coronary assessment was carried out during acute stage and at six weeks follow up. Sample for genotyping was obtained from peripheral venous blood. Written informed consent was obtained from parents of all children included in the study. This study was approved by the Institute Ethics Committee (IEC/2020/0075) and Institute Thesis Committee. The manuscript has been approved by Departmental Review Board.

Genetic study

Two milliliters of peripheral blood was taken from cases and controls for genetic analysis. Deoxyribonucleic acid (DNA) extraction was done using QIAmp DNA Blood Mini Kit (QIAGEN, Hilden, Germany). DNA purity was checked using TECAN infinite M200 pro with Nanoquant plate (TECAN group, AG, Switzerland) taking the optical density of 260 nm and 280 nm. Samples were then stored at −80^{0}C.

Genotyping for the SNP rs113420705 was done using polymerase chain reaction (PCR) and Sanger sequencing. PCR amplification reactions were carried out at controlled conditions as per the standard protocol with the help of an automated PCR thermal cycle (Applied Biosystems, Thermo Fisher Scientific, Massachusetts, USA). PCR cycle conditions are available on request.

PCR products were then subject to purification and direct sequencing using the ABI Big Dye Terminator kit and ABI 3500 Gene Analyzer (Applied Biosystems, CA, USA). Sequencing results were analyzed using Codon Code Aligner software (Codon Code Corporation, Centerville, (MA). Primer sequences used for the amplification of the *CASP3* gene comprising the SNP of interest are illustrated in Table 1. Representative pictures of PCR and sequencing are depicted in Figure 1.

Statistical analysis

Frequencies of allele and genotypes in cases and control group were compared and among children with KD and CAA and children with KD without CAA. Categorical variables were analyzed using Chi-squared or Fisher's exact tests. Quantitative variables of cases and controls were compared with independent sample *t*-test (parametric) or Mann–Whitney *U* test (non-parametric). The magnitude of effect size was expressed as risk ratio or odds ratio with 95% confidence interval (CI) for comparison of categorical variables between two groups. An association was considered significant if *P* value was equal to or less than 0.5. Data analysis was done on SPSS software (version 23.0, IBM SPSS, USA).

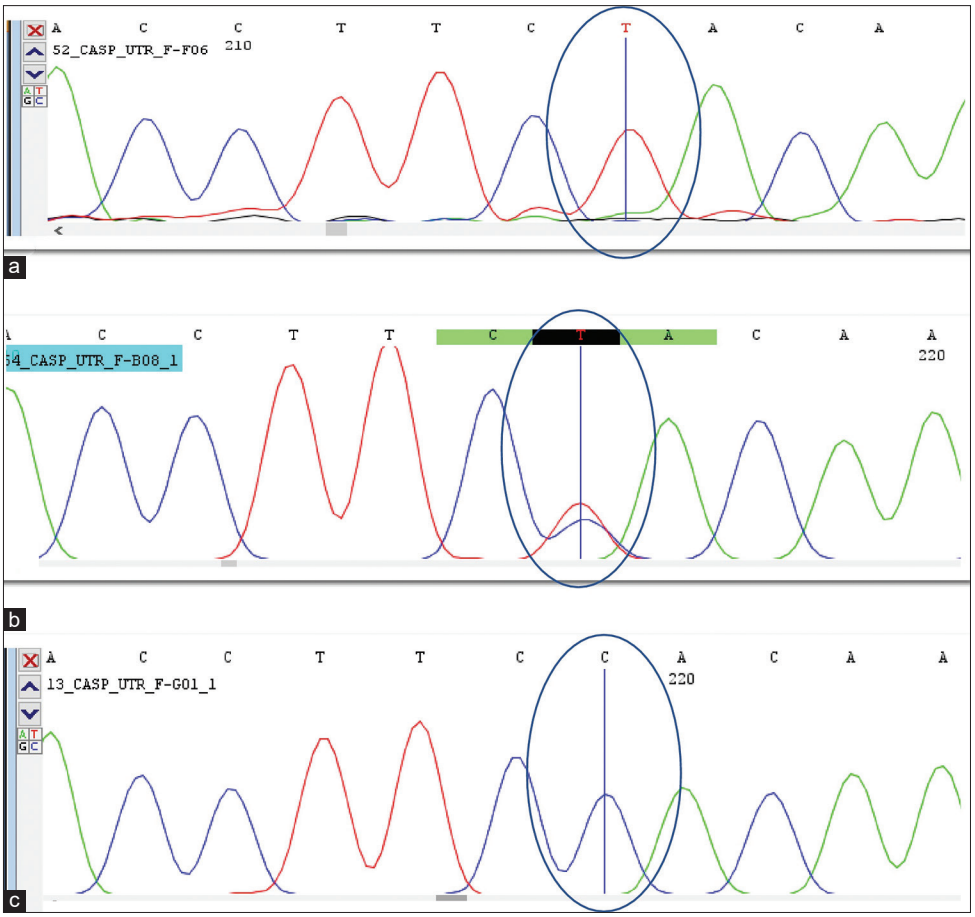

Figure 1: Representative pictures of Sanger sequencing of polymerase chain reaction products of single nucleotide polymorphism rs113420705 of *CASP3*: (a) Wild type TT genotype (b) Heterozygous variant CT genotype (c) Homozygous variant CC genotype

Table 1: The primer sequence used for the amplification of single nucleotide polymorphism rs113420705 of *CASP3* gene

CASP3 gene Primer	Primer Sequence	Tm (°C)	Product size
CASP3 UTR Fwd. (5'-3')	CTCCCTAAATCAAAAGCCTTAACCCGC	64.3	316bp
CASP3 UTR Rev (r5'-3')	CCGCGGAAGCAGTGCAGACG	66.6	

SNP, Single nucleotide polymorphism; UTR, Untranslated region; ™, Temperature; bp, basepair

Results

Genotype distribution among cases and controls were in Hardy–Weinberg equilibrium. We analyzed the association of the SNP rs113420705 in 45 patients with KD and 50 control subjects. Three genotypes namely TT (wild), CT (heterozygous), and CC (homozygous) of the *CASP3* gene were recognized [Figure 1]. T and C allele frequency of rs113420705 were 77 (85.5%) and 13 (14.5%) among cases and 82 (82) and 18 (18) in controls, respectively. Among 45 cases, 32 (71.1%) had the genotype TT, 13 (28.9%) had CT and none had CC genotype of the SNP. Among 50 children in control group, 38 (76%) TT, 7 (14%) CT and 5 (10%) CC genotypes were found. No significant differences in allele, genotype, or carrier frequencies of rs113420705 were found between the two groups [Table 2A]. The odds of finding CT genotype increased by 1.42 times in patients with KD than in controls. However, this trend failed to reach statistical significance.

A comparison was also made between subgroups of 7 children (15.5%) with KD with CAA and 38 children (84.4%) with KD without CAA. No significant differences were noted in the genotype frequencies. However, C allele was found to be expressed more among children with KD with CAA group (P = 0.005) in comparison to those without CAA. C allele carrier state was also significantly higher in the same (P = 0.0022) [Table 2B].

Discussion

Kawasaki disease (KD) is the most common pediatric vasculitic disorder.[1] Diagnosis of KD is based on a set of clinical criteria. Delays in diagnosis and institution of treatment can result in several cardiovascular complications like CAAs, myocarditis and KD shock syndrome.[14,15] Etiology of KD remains an enigma even after 50 years of its first recognition.[7] Higher incidence of

Table 2: Allele, genotype and carrier frequencies of SNP rs113420705 of *CASP3* gene

Table 2A: Allele, genotype and carrier frequencies of SNP rs113420705 of *CASP3* gene between children with KD and controls

Allele/Genotype	KD patients (*n*=45) *n* (%)	Controls (*n*=50) *n* (%)	Odds ratio (95% CI)	*P*
T allele	77 (85.5)	82 (82)	-	-
C allele	13 (14.5)	18 (18)	0.772	0.631
TT genotype	32 (71.1%)	38 (76%)	-	-
CC genotype	0 (0%)	5 (10%)	-	-
CT genotype	13 (28.9%)	7 (14%)	1.42 (0.943-2.144)	0.102
T allele carrier	45 (100%)	44 (88%)	-	-
C allele carrier	13 (28.8%)	12 (24%)	−1.2	1

Table 2B: Allele, genotype and carrier frequencies of SNP rs113420705 of *CASP3* gene between KD with CAA and KD without CAA

Allele/Genotype	KD patients With CAA (*n*=7) *n* (%)	KD Without CAA (*n*=38) *n* (%)	Odds ratio (95% CI)	*P*
T allele	10 (71.5)	67 (88.2)	-	-
C allele	4 (28.5)	9 (11.8)	2.9	0.005
TT genotype	3 (42.8%)	29 (76.3%)	-	-
CC genotype	0 (0%)	0 (0%)	-	-
CT genotype	4 (57.2%)	9 (23.7%)	3.2 (0.850-12.73)	0.093
T allele carrier	7 (100%)	38 (100%)	-	-
C allele carrier	4 (57.2%)	92 (23.7%)	2.4	0.0022

KD, Kawasaki disease; SNP, Single nucleotide polymorphism; CAA, Coronary artery aneurysm

KD in Japanese, Korean, Taiwanese and Hawaiian children of Japanese ancestry points towards some genetic association of KD.[10] Genetic associations of *ITPKC, CASP3, BLK, CD40, HLA* genes have been described in association with KD.[1] A recent study by Buda *et al.*[16] showed that polymorphisms of genes *KIF25*, *SPECC1L* and *RNP2* may also have influence on susceptibility to KD.

CASP3 being one of the effector caspases, has an important role in execution phase of apoptosis. Kuo *et al.*[17] reported the increased pyroptotic expression of caspases including caspase-3 in leukocytes of children with KD. Ferdosian *et al.*[18] found that rs72689236 influences the susceptibility to KD. SNP rs113420705 of *CASP3* gene has been associated with KD as well as development of CAAs.[12]

Onouchi *et al.*[19] found a significant association of rs28493229 of ITPKC gene with KD in Japanese and American patients. Subsequently, studies on Japanese, Taiwanese and European children with KD have shown a significant association of SNP rs113420705 of *CASP3* and occurrence of KD.[20] Onouchi *et al.*[12] first reported that altered *CASP3* expression in immune effector cells influences susceptibility to KD. Marginal association was also found by Kuo *et al.*[20] in their cohort of Taiwanese children.

Phenotype of KD in India is different from KD in East Asian, American, and European countries.[21,22] This may point towards the differences in genotypic association of KD in different ethnicities and races. We have chosen rs113420705 of the *CASP3* gene as there are no genetic association studies on rs113420705 of *CASP*3 gene and KD from India. Our study was an attempt to elicit the association of this SNP in children with KD and controls of Indian ethnicity. In our study, we performed PCR and bidirectional Sanger sequencing for one SNP rs113420705 of *CASP3* gene in children with KD being followed up at our center.

For this SNP, the T allele is the ancestral or wild type allele in the majority of the populations. In our study also, the T allele was over-represented both in KD cases and the controls.

Association of altered *CASP3* gene expression with the occurrence of KD was first described by Onouchi *et al.*[12] It was found that a G to A substitution of rs72689236 (*previous rs ID for rs113420705) located in the 5' untranslated region of *CASP3* abolished binding of nuclear factor of activated T cells to the DNA sequence surrounding the SNP. Metanalysis by Peng *et al.*[23] showed a similar association with the presence of A allele in place of G allele increasing the risk of KD by 59%. Kuo *et al.*[20] from Taiwan demonstrated the frequency of major G and minor A allele to be 69% and 31% in controls and 65% and 35% in KD patients with a borderline significant allelic difference ($P = 0.0575$). No significant association of this SNP was found with occurrence of coronary artery lesions. Peng *et al.*[24] from China found a higher frequency of two haplotypes involving the T allele of rs113420705 in children with KD. In our study, C allele was found to be significantly associated with development of CAAs. However, while studying the effect of a single SNP, one cannot rule out the effect of the various other identified/non-identified genetic/non-genetic risk factors for KD.

It appears that no single SNP may have a uniform association with susceptibility to KD in all ethnicities across the world. KD has multifactorial causation and these SNPs may point towards some predisposition in certain environmental and epigenetic settings. Larger studies involving multiple SNPs and different ethnicities are necessary before definite conclusions can be drawn.

This study contributes significantly to child care specialists, primary care providers and family physicians. KD is one of the commonest childhood vasculitides and a notable cause of acquired heart disease in children. However, KD is often missed or misdiagnosed due to its subtle findings, different symptoms presenting at phases and etiological mystery. Moreover, Indian children with KD are described to have slightly different phenotype.[21,22,25] Genetic association studies will help physicians to explore genotype–phenotype correlation and validate the findings from other ethnicities in Indian children. These studies will help primary care physicians for better understanding of the disease which will ultimately lead to early diagnosis and treatment. KD may result into serious cardiovascular complications like CAA, myocarditis, and thrombosis. Early diagnosis and focused treatment will save them from complications. This

study contributes to etiopathogenetic quest of KD which will be contributory to family physicians in day-to-day practice too.

Bhattarai *et al.*[13] studied the association of SNPs of *ITPKC* gene with KD susceptibility in Indian cohort. This is the first study on association of this *CASP3* SNP and the occurrence of KD in an Indian population. Diagnosis of KD was confirmed in all cases by the same investigator (SS). Analysis with Sanger sequencing and age–sex matching eliminates the analytical blunders and effect of the difference in exposure prevalence, respectively. However, this study has some limitations. This is a hospital-based study and has a relatively small sample size. It represents the dissertation work of the first author. As a result, the study was time bound and had to be completed in a given time frame. We have not conducted the functional effect of studied gene polymorphism and effect of other genes in the studied cohort.

Conclusion

Since KD is one of the commonest childhood vasculitic disorders which still remains undiagnosed or misdiagnosed at times, etiopathogenic study from India like ours will help primary care physician explore more number of patients with KD and add on etiological quest of this vasculitic disorder. This is the first Indian study to observe the association of rs113420705 SNP of *CASP3* gene and susceptibility to KD. We documented that CT genotype of rs113420705 of *CASP3* has a linear association with KD, but this association just shows trend to statistical significance only. We could not establish any association between minor allele C and susceptibility to KD. However, the C allele was found to predispose to coronary complications in patients with KD ($P = 0.005$). Further multicentric larger studies are required to confirm these findings. Besides, functional studies of SNPs and multiple SNPs together with haplotype studies will provide conclusive evidence.

Key points/Highlights

- Genetic predisposition in the form of susceptibility loci and gene polymorphisms for Kawasaki disease is being explored across populations.
- This is the first Indian study on association of rs113420705 SNP of *CASP3* gene and susceptibility to KD.
- We could not establish any association between minor allele C of rs113420705 SNP of *CASP3* gene and susceptibility to KD in our cohort of children from North India.

Take home message

- Association of SNPs of *CASP3* gene with KD susceptibility may be variable among different ethnicities. Multicentric studies will help to establish concluding evidence.

Ethics approval

Approval was obtained from the Institute Thesis Committee and Institute Ethics Committee of Postgraduate Institute of Medical Education and Research (PGIMER), India (IEC/2020/0075). The manuscript has been approved by Departmental Review Board too. The procedures used in this study adhere to the tenets of the Declaration of Helsinki.

Consent to participate and publish

Informed written consent was taken from parents of all subjects and controls for participating in the study and publication of the study results. An assent was obtained from all children above 7 years of age. Parents of the children signed informed consent regarding publishing their data and findings.

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Availability of data and materials

The dataset generated and analyzed during the current study are available from the first author on reasonable request

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Nil.

Conflicts of interest

There are no conflicts of interest.

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