Two novel missense substitutions in the VSX1 gene: clinical and genetic analysis of families with Keratoconus from India

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Abstract

Background: Visual system homeobox gene (VSX1) plays a major role in the early development of craniofacial and ocular tissues including cone opsin gene in the human retina. To date, few disease-causing mutations of VSX1 have been linked to familial and sporadic keratoconus (KC) in humans. In this study, we describe the clinical features and screening for VSX1 gene in families with KC from India.

Methods: Clinical data and genomic DNA were collected from patients with clinically diagnosed KC and their family members. The study was conducted on 20 subjects of eight families from India. The coding exons of VSX1 gene were amplified using PCR and amplicons were analyzed by direct sequencing. Predictive effect of the mutations was performed using Polyphen-2, SIFT and mutation assessor algorithms. Additionally, haplotypes of VSX1 gene were constructed for affected and unaffected individuals using SNPs.

Results: In the coding region of VSX1, one novel missense heterozygous change (p.Leu268His) was identified in five KC patients from two unrelated families. Another family of three members had a novel heterozygous change (p.Ser251Thr). These variants co-segregated with the disease phenotype in all affected individuals but not in the unaffected family members and 105 normal controls. In silico analysis suggested that p.Leu268His could have a deleterious effect on the protein coded by VSX1, while p.Ser251Thr has a neutral effect on the functional properties of VSX1. Haplotype examination revealed common SNPs around the missense change (p.Leu268His) in two unrelated KC families.

Conclusions: In this study, we add p.Leu268His, a novel missense variation in the coding region of VSX1 to the existing repertoire of VSX1 coding variations observed in Indian patients with the characteristic phenotype of KC. The variant p.Ser251Thr might be a benign polymorphism, but further biophysical studies are necessary to evaluate its molecular mechanism. The shared haplotype by two families with the same variant suggests the possibility of a founder effect, which requires further elucidation. We suggest that p.Leu268His might be involved in the pathogenesis of KC, which may help in the genetic counselling of patients and their family.

Keywords: Visual system homeobox gene (VSX1), Familial keratoconus, Mutation screening, CVC (Chx10/Vsx–1 and ceh–10) domain, In silico analysis, Missense mutation, Haplotype analysis
Background
Keratoconus (KC: OMIM 148380) is a progressive ectatic disorder of the cornea characterized by thinning of the central cornea leading to protrusion and progressive, irregular astigmatism. Though there are several treatment modalities available, severe KC remains an indication for corneal transplantation [1, 2]. The mean age of onset is 15.39 years with a prevalence of 0.0003 %–2.3 % that affects both genders and all ethnicities across the globe [3]. The disease is a complex heterogeneous disorder with risk factors like chronic eye rubbing and atopy playing a significant role besides ultraviolet light induced oxidative stress [4–6]. The genetic basis for keratoconus has always been an accepted theory considering its familial occurrence and high concordance in monozygotic twins [4, 7, 8]. Though most KC cases are sporadic, it has been noted that 6–10 % of cases have a positive family history [9, 10]. Inheritance in KC can be dominant or recessive; with autosomal dominant inheritance, the disease exhibits variable phenotypes with incomplete penetrance [9].

Linkage analysis has identified several genomic loci in KC families thereby establishing genetic heterogeneity [11–15]. Genes with mutations (VSX1, DOCK9, TGFβ1, SOD1, FLG, ZEB1) were found to be responsible for only a small fraction of KC cases in select populations around the world [16–21]. Nevertheless, VSX1 mutations have been identified in two different corneal phenotypes - posterior polymorphous corneal dystrophy (PPCD) and KC [16]. Genetic analysis of KC patients from different ethnic backgrounds has revealed several coding variations in the VSX1 gene [22–26]. So far, four pathogenic VSX1 mutations have been reported in the KC phenotype. Hence, the significance of a genetic basis for KC is still unclear [22–26].

VSX1 is a paired-like homeodomain transcriptional factor gene localized in 20p11.21. It is expressed in the adult cornea and adult retinal cDNA libraries [27], inner nuclear layer of the human retina and embryonic craniofacial tissue [28]. The human VSX1 gene has five exons that encodes for a 365–amino acid protein with homeobox DNA binding domain and a CVC (Chx10/Vsx–1 and ceh–10) domain, which is highly conserved among vertebrates. In this present study, we correlate the genetic, and clinical features of KC patients and their families of Indian origin with VSX1 gene variants.

Methods
Study subjects and clinical examination
Twenty affected individuals from eight unrelated KC families, 11 unaffected family members, and 105 ethnically matched normal controls were included in this study. All patients were examined at the Cornea and Refractive Surgery Department, Narayana Nethralaya Postgraduate Institute of Ophthalmology, Bangalore, India. The study followed the tenets of the Declaration of Helsinki and was approved by the Institutional Ethical Committee (IEC–C/2013/07/01). All patients underwent visual acuity assessment, a detailed slit lamp examination with topographic and pachymetric evaluation on the Pentacam HR (Oculus Inc.) and Orbscan (Orbtek, Bausch, & Lomb). Keratoconus was graded according to the Amsler-Krumeich Classification [29]. If KC was detected in more than one member of the family, the entire family was counselled, detailed informed consent taken, and blood collected for genetic analysis.

Genetic study
A detailed family history was taken including history of ocular and non-ocular hereditary disorders and pedigree charts drawn accordingly. The total genomic DNA was isolated from peripheral blood leukocytes by the salt precipitation method [30] from all study subjects. For mutational analysis, the entire coding exons of VSX1 and their flanking intronic junctions were amplified by PCR in eight probands using the primer reported elsewhere [25]. The PCR products were sequenced on 3130xl Genetic Analyzer (Applied Biosystems) according to the manufacturer’s protocol. Sequencing results were analyzed in chromatogram viewer (FinchTV 1.40), pairwise BLAST (Basic Local Alignment Search Tool) [31] to examine if there were any changes from the normal VSX1 sequence available in the database (NM_014588, ENSG00000100987). The segregation of nucleotide changes were analysed in eight affected and four unaffected individuals from three unrelated families by direct sequencing method. In addition, exon 4 of VSX1 was sequenced in 105 unrelated ethnically matched normal controls to validate the pathogenicity of nucleotide variations.

Bioinformatics analysis
In order to predict the effect of nucleotide change at the protein level, we used in silico prediction servers Polyphen–2 (http://genetics.bwh.harvard.edu/pph2/), SIFT (http://sift.bii.a-star.edu.sg), Mutation Assessor (http://mutationassessor.org/v1/) and PROVEAN (http://provean.jcvi.org/index.php). Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) and multiple sequence alignment programs were used to check the evolutionary conservation of VSX1 protein in other vertebrates. The effect of amino acid changes in the stability of VSX1 was assessed by using the MUpro (version 1.0, http://mupro.proteomics.ics.uci.edu/) prediction server (AAMSPSM).

Haplotype analysis
To examine the disease and mutation associated haplotypes of the eight affected and four unaffected individuals from two unrelated KC families, we analysed four
intragenic SNPs (rs12480307, rs6138482, rs56157240), and (IVS3–24C > T) flanking VSX1 by direct sequencing. Haplotypes were constructed manually.

Results

In this study, we analysed 20 affected individuals of eight families with a clinical diagnosis of KC for mutations in the VSX1 gene (Table 1). A novel missense coding variant (p. Leu268His) was found in five patients from two unrelated KC families (KC_01, KC_02). Another novel heterozygous missense change (p. Ser251Thr) was identified in a third KC family (KC_03) with two affected siblings and their affected father. The clinical features of the affected individuals are summarized in Table 2.

Table 1 Summary of genotype and phenotype characteristics in the study subjects

| Family ID | Individuals | Age at diagnosis/Sex | Keratoconus | Segregation of VSX1 nucleotide changes |
|-----------|-------------|----------------------|-------------|---------------------------------------|
| KC–01     | I:1 40/F    | No                   | -           | rs6157240,                           |
|           | I:2 35/M    | Yes/RE               | L268H       | rs12480307, rs6157240, IVS3–24C > T |
|           | II:1 22/M   | Yes/RE               | L268H       | rs12480307, rs6157240, IVS3–24C > T |
|           | II:2a 19/M  | Yes/BE               | L268H       | rs12480307, rs6157240                |
| KC–02     | I:1 42/F    | No                   | -           | rs12480307, rs6138482, IVS3–24C > T |
|           | I:2 37/M    | Yes/LE               | L268H       | rs12480307, rs6157240, rs6138482     |
|           | II:1a 16/M  | Yes/BE               | L268H       | rs12480307, rs6157240, rs6138482     |
|           | II:2 12/F   | No                   | -           | rs12480307, rs6157240, rs6138482     |
| KC–03     | I:1 45/F    | No                   | -           | rs6157240, rs6138482                 |
|           | I:2 50/M    | Yes/BE               | S251T       | IVS3–24C > T                         |
|           | II:1 20/F   | Yes/BE               | S251T       | IVS3–24C > T                         |
|           | II:2a 18/M  | Yes/BE               | S251T       | rs6157240                            |
| KC–04     | I:1 53/M    | No                   | -           | rs12480307, rs6157240                |
|           | I:2 44/F    | Yes/BE               | -           | IVS3–24C > T                         |
|           | II:1a 29/M  | Yes/LE               | -           | rs6157240                            |
|           | II:2 26/F   | No                   | -           | rs6157240, IVS3–24C > T             |
| KC–05     | I:1 60/M    | No                   | -           | rs6138482, rs6157240, IVS3–24C > T |
|           | I:2 47/M    | Yes/LE               | -           | rs6157240,                           |
|           | II:1 31/M   | Yes/LE               | -           | IVS3–24C > T, rs6138482              |
|           | II:2a 27/F  | Yes/LE               | -           | rs6157240, rs6138482                 |
| KC–06     | I:1 43/F    | Yes/BE               | -           | rs12480307, rs6157240, rs6138482     |
|           | I:2 50/M    | No                   | -           | rs6138482                            |
|           | II:1 29/M   | No                   | -           | rs12480307, rs6138482                |
|           | II:2a 24/F  | Yes/BE               | -           | rs6157240, rs6138482                 |
| KC–07     | I:1 53/F    | Yes/BE               | -           | rs12480307, rs6138482, IVS3–24C > T |
|           | I:2 61/M    | No                   | -           | rs12480307, rs6138482                |
|           | II:1a 34/M  | Yes/RE               | -           | rs6138482, IVS3–24C > T             |
| KC–08     | I:1 39/F    | No                   | -           | rs12480307, rs6138482, rs6157240     |
|           | I:2 45/M    | Yes/RE               | -           | rs6138482, rs6157240                 |
|           | II:1a 18/M  | Yes/RE               | -           | rs12480307, rs6157240                |
|           | II:2 16/F   | Yes/RE               | -           | rs6138482, rs6157240                 |

RE: Right eye, LE: Left eye, BE: Both eye, M-Male, F-Female, The symbol (−) denotes the absence. Symbol (*) indicates the probands.
Table 2 Clinical features of affected individuals from KC families with VSX1 coding variants

| Family ID   | BCVA | Scissors’ retinoscopic reflex | Location of corneal thinning | Corneal topography                                                                 | Thinnest Pachymetry (μm) | Fleisher’s ring | Apical corneal scarring | KC                  |
|-------------|------|------------------------------|-----------------------------|-----------------------------------------------------------------------------------|--------------------------|-----------------|--------------------------|---------------------|
| KC–01_II:2  | 0.15/0.1 + RE: Central LE: Inferior | RE: LE: Infero-superior, asymmetry Infero cone, steepening corresponds with anterior, and posterior elevation. | LE: Normal                    | RE: 447 LE: 450                                                                   | RE: + LE: + RE: +        | Both eyes        |
| KC–01_II:1  | 0/ 0 + RE: Central LE: Normal | RE: Infero-superior, asymmetry, Infero nasal cone. LE: Normal | LE: Normal                    | RE: 482 LE: 493                                                                   | RE: + - RE: + LE: Normal | Both eyes        |
| KC–01_I:2   | 0.47/0 + RE: Infero temporal cone LE: Normal | RE: Infero-Superior, asymmetry, Infero temporal cone, central cone with skewing of axis of 40° LE: Normal | LE: Normal                    | RE: 404 LE: 440                                                                   | RE: + - RE: + LE: Normal | Both eyes        |
| KC–02_II:1  | 0.15/0.15 - RE: Central LE: Central | RE: Posterior elevation LE: Central corresponds with thinnest pachymetry and posterior elevation | LE: Normal                    | RE: 490 LE: 414                                                                   | BE: + - Both eyes        |
| KC–02_I:2   | 0/ 0 - RE: Normal LE: Infero-temporal | RE: Normal LE: Posterior elevation, Infero-temporal cone LE: Normal | LE: Normal                    | RE: 506 LE: 488                                                                   | - - RE: Normal           |
| KC–03_II:2  | 0.47/0.15 - RE: Central LE: Central | RE: Advanced KC, central cone with gross posterior elevation LE: Central cone | LE: Central                    | RE: 333 LE: 443                                                                   | + - Both eyes            |
| KC–03_II:1  | 0/0 RE: Central LE: Inferior | RE: Posterior elevation, LE: Central cone LE: Infero nasal cone | LE: Normal                    | RE: 526 LE: 532                                                                   | - - Both eyes            |
| KC–03_I:2   | 0.15/0.15 - RE: Central LE: Central | BE: Infero cone, Infero- superior asymmetry with similar involvement in both eyes | LE: Central                    | RE: 419 LE: 421                                                                   | - - Both eyes            |

RE: Right eye, LE: Left eye, BE: Both eye, BCVA: Best corrected visual acuity, KC-Keratoconus, The symbols + and - represent present and absent, respectively. M-Male, F-Female

Fig. 1 Pedigrees of the KC families with novel coding variants in VSX1 Legend: A, B, C denotes three unrelated families. Squares and circles indicate males and females, respectively. Black symbols indicate affected members and open symbols indicate unaffected individuals. The black arrow indicates the proband, the sign ‘+’ represents the wild type and the mutations identified are p. Leu268His, p. Ser251Thr
Amino acid conservation analysis revealed that leucine at position 268 was highly conserved in nine vertebrate orthologs and other species (Fig. 2C). This nucleotide change was not present in the 105 normal controls and the unaffected family members.

In another two generation KC family (KC–03) (Fig. 1C), mutation screening of VSX1 revealed a transition at exon 4 and c.751 T > A was found in three affected (II:2, II:1, I:2) individuals (Fig. 2B). The heterozygous T > A substitution at codon 251 (Ser251Thr) converts a highly conserved amino acid serine (TCC) into threonine (ACC). The unaffected mother (I:1) and 105 controls showed wild type alleles of VSX1. None of these identified missense variations have so far been reported in public databases, including NHLBI ESP (http://evs.gs.washington.edu/EVS/), dbSNP (http://www.ncbi.nlm.nih.gov/SNP/) and 1000 Genomes (http://www.1000genomes.org/).

Clinical findings of the three unrelated KC families

**Family KC–01**

There are three affected and one unaffected member (Fig. 1A). The proband (II:2) is a 20 year old male with a refraction of −0.5 D spherical and −2.25 D cylinder in his right eye; −1.25 D cylinder in his left eye. Corneal topography (Pentacam, Oculus Inc) revealed grade 1 KC in both eyes with an inferior cone with inferior-superior asymmetry. He had a thinnest pachymetry of 447 μm and 450 μm in his right and left eye respectively (Fig. 3A). He underwent corneal collagen crosslinking in both eyes and was stable at the end of the first year of follow up. The proband’s male sibling (II:1) had a refraction of −1 D spherical with −2.25 D cylinder in his right eye and −2 D spherical in his left eye. Topography mapping determined grade 1 KC with inferior-superior asymmetry and an inferonasal cone in the right eye with a mean
keratometry of 43.6 D and a thinnest pachymetry of 482 μm. He had a normal corneal topography in the left eye. The right eye of the patient I:2 showed grade 1 KC. His refraction in the right eye was –4.25 D spherical with –4.5 D cylinder and in the left eye it was –2.75 D spherical with –3.25 D cylinder. His topography showed a central cone with inferior-superior asymmetry and skewing of 40° in his right eye and normal corneal topography in the left eye.

Family KC–02
Consists of four individuals with two having the characteristic features of KC (Fig. 1B). The patient (II:1) is a 16 year old male, with a posterior elevation on topography mapping in the right eye with a grade 2–3 KC in the left eye. Refraction in the right eye was –0.75 D sphere with –1.5 D cylinder and in the left eye it was –2.75 D spherical with –3.25 D cylinder. His topography showed a central cone with inferior-superior asymmetry and skewing of 40° in his right eye and normal corneal topography in the left eye.

Family KC–03
This family had one unaffected parent and three individuals with the clinical features of KC (Fig. 1C). The proband (II:2) is an 18 yr old male. Refraction in his right eye was –10 D sphere with –2.25 D cylinder and –3.75 D cylinder in the left eye. The right eye showed advanced KC with a fairly central cone while the left eye showed a grade 2 KC with a mean keratometry of 50.2 D and a thinnest pachymetry of 370 μm (Fig. 3C). He underwent corneal collagen crosslinking in both eyes and was stable on follow up. His sister (II:1) at initial presentation had a normal topography. At the second year follow up (20 years of age), the left eye showed progression to grade 1 keratoconus while the right eye remained stable. Both eyes of the

![Fig. 3](image-url) Corneal topography of the KC probands with VSX1 coding variants. A. Pentacam image of patient II:2 from KC–01, both eyes show an area of inferior steepening on the sagittal curvature map with gross inferior-superior asymmetry, more in the right eye. This area of steepening corresponds to areas of abnormal elevation on both the anterior and posterior elevation maps with values suggestive of Keratoconus (KC) with an inferior cone. B. In the second family (KC–02), patient I:1 has an area of central steepening on the sagittal curvature map. This area of steepening corresponds to areas of abnormal elevation on both the anterior and posterior elevation maps with values suggestive of KC with a central cone in the left eye (OS); right eye (OD) showing the posterior elevation, suggestive of early KC. C. The sagittal curvature on Scheimpflug imaging of patient II:2 from the third family (KC–03) with the left eye showing (OS) an area of central steepening. The anterior and posterior curvature maps show areas of abnormal elevation with values suggestive of KC with a central cone. The corneal thickness map also shows an area of central thinning which is corresponding to the areas of abnormal elevation. The right eye (OD) shows a fairly central area of steepening with features suggestive of advanced KC. There is gross posterior elevation with significant corneal thinning (thinnest pachymetry of 370 μm) in the central 3 mm zone.
patient I:2 exhibited grade 2 KC; his refraction in the right eye was –6 D spherical with a cylinder of –3.5 D and in the left eye, a sphere of –7.5 D with cylinder of –5 D. His topography scans in both eyes showed inferior steepening with a significant inferior-superior asymmetry.

**In silico analysis of the VSX1 missense variants**

*In silico* prediction algorithms of SIFT, Polyphen–2, Provean, and Mutation assessor suggested that the missense change p. Leu268His might negatively affect the function of the coding protein. On the other hand, p. Ser251Thr showed a neutral effect on the functional properties of the protein according to the prediction server results (Table 3). Analysis of amino acid mutation stability for p. Leu268His using the Amino Acid Mutation Stability Prediction Server showed a decrease in the stability of VSX1 protein structure.

**Haplotype analysis**

We carried out haplotype analysis to examine whether the missense change, p. Leu268His, was due to a founder effect or was likely to have arisen independently in two unrelated families with KC. The haplotype of affected and unaffected individuals from the third family (KC–03) showed a different haplotype (Fig. 4C).

**Table 3** The functional classification and score of VSX1 variants are predicted by using various bioinformatics tools

| c. DNA position | Protein change | Location of protein | Polyphen–2 humDiv | SIFT | Mutation assessor | PROVEAN |
|-----------------|----------------|---------------------|-------------------|------|-------------------|---------|
| c.751 T > A     | p. Ser251Thr   | CVC domain          | Benign (0.9)      | Tolerated | Neutral           | Neutral |
|                 |                |                     |                   | (0.17)| (0.485)           | −2.467  |
| c.803 T > A     | p. Leu268His   | CVC domain          | Possibly damaging for function (1.0) | Deleterious | Functional effect on protein | Deleterious |
|                 |                |                     |                   | (0.05)| (1.905)           | −6.831  |

Prediction servers are Polymorphism Phenotyping v2 (PolyPhen–2), Sorting Tolerant From Intolerant (SIFT), Protein Variation Effect Analyzer (PROVEAN), CVC (Chx10/Vsx–1, and ceh–10).

Polyphen–2 scores: 0: benign, 1 possibly damaging for function; 2: Probably damaging for function

SIFT scores: Intolerant or deleterious: score ≤0.05, Tolerant: score >0.05

Mutation Assessor scores: 0–1: no functional effect, 2–3: functional effect on protein function

Provean scores: Cut off threshold = −2.5.

Variants with a score equal to or below −2.5 are considered "deleterious," Variants with a score above −2.5 are considered "neutral"

**Table 4** Details of SNP markers used for the haplotype analysis

| db SNP ID | Physical position | VSX1 transcript ID | cDNA change | Protein change | Allele frequency* | Population frequency* |
|-----------|-------------------|-------------------|-------------|----------------|------------------|----------------------|
| rs12480307 | chr20: 25078910   | NM_014588         | c.546A > G  | p.A182A        | A: 0.748          | A: 75 %              |
|           |                   |                   |             |                | G: 0.252          | G: 25 %              |
|           |                   |                   |             |                | T: 0.252          | T: 25 %              |
| rs56157240 | chr20: 25078745   | NM_014588         | c.627 + 84 T > A | -              | A: 0.748          | A: 75 %              |
|           |                   |                   |             |                | G: 0.735          | G: 74 %              |
| rs6138482  | chr20: 25078806   | NM_014588         | c.627 + 23G > A | -              | A: 0.265          | A: 26 %              |
| (IVS3-24C) | chr20: 25078976   | NM_014588         | c.504-24C > T | -              | C:0.999           | Not available         |
|           |                   |                   |             |                | T:0.001           |                      |

*Allele and population frequencies were determined by 1000 Genomes Project Phase 1, HapMap, and ESP for human
non-polar amino acid while histidine is a basic-polar residue. It may cause abnormal protein folding that may affect DNA binding properties of the VSX1 transcriptional modulation activity. Moreover, this coding variant (p. Leu268His) leads to the replacement of highly conserved amino acid leucine by histidine in the VSX1 protein, perhaps implicating the functional consequence of this region. Interestingly, CVC domain change (p. His244Arg) has been associated with PPCD along with signs of bipolar cell dysfunction and macular degeneration [33]. According to SIFT, Polyphen-2, Provean, and Mutation assessor, the (p. Leu268His) mutation is found to have a deleterious effect on protein function, attributing a pathogenic nature to this missense mutation in VSX1. Furthermore, in our study, this potentially damaging mutation was detected in two families consisting of five affected individuals with a dominant inheritance of KC phenotype [24].

Table 5 Summary of VSX1 coding variants identified in patients with KC and PPCD

| Coding variants | Clinical significance | Phenotype | Unrelated Controls | Ethnic groups | References |
|-----------------|----------------------|-----------|--------------------|---------------|------------|
| p. Leu17Pro     | Pathogenic           | KC        | -                  | Italian       | [26]       |
| p. Leu17Val     | Non-pathogenic       | KC        | +                  | Korean        | [23]       |
| p. Pro58Leu     | Pathogenic           | KC        | -                  | Caucasian     | [22]       |
| p. Asp144Glu    | Unknown              | PPCD      | -                  | Italian, Ashkenazi Jewish, British, European | [16, 26, 34–37] |
| p. Leu159Met    | Unknown              | KC        | -                  | Caucasian     | [16, 38]   |
| p. Asn151Ser    | Pathogenic           | KC        | -                  | Korean        | [39]       |
| p. Gly160Asp    | Non-pathogenic       | PPCD      | -                  | Italian, European | [16, 26, 40] |
| p. Gly160Val    | Non-Pathogenic       | KC        | -                  | Korean        | [23, 39]   |
| p. Val199Leu    | Non pathogenic       | KC        | +                  | Korean        | [23]       |
| p. Arg166Trp    | Unknown              | KC        | +                  | Caucasian, Iranian | [16, 24] |
| p. Gin175His    | Unknown              | KC        | -                  | Indian        | [25]       |
| p. Arg217His    | Non- Pathogenic      | KC        | +                  | Indian, Pakistan, European | [40, 41] |
| p. Gly239Arg    | Pathogenic           | KC        | -                  | Italian       | [42]       |
| p. His244Arg    | Unknown              | KC        | +                  | Caucasian, Iranian | [16, 24, 38, 43] |
| p. Ser251Thr    | Unknown              | KC        | -                  | Indian        | [25]       |
| p. Pro247Arg    | Non-pathogenic       | KC        | +                  | Italian       | [16, 26, 35] |
| p. Leu268His    | Pathogenic           | KC        | -                  | Indian        | Present study |

Coding variants of the VSX1 gene have been reported in present and other studies based on original report and bioinformatics predictions.

KC: Keratoconus, PPCD: Posterior polymorphous corneal dystrophy. The symbols “+” and “-” represent present and absent, respectively.
study has demonstrated the coding variant p. Gln175His in the homeodomain of VSX1 in an Indian family of KC with incomplete penetrance [25].

The probands from KC families showing the variable clinical phenotype, which has been observed in earlier studies as well [34]. On the other hand, it is difficult to establishing a genotype–phenotype correlation in the study subjects due to the presence of inter and even intra-familial clinical variability. Another novel c.751 T > A missense variant was identified in a proband (KC–03_II:2) who exhibited bilateral KC. It is interesting to note that this (p. Ser251Thr) coding variant introduces a missense change that leads to the replacement of highly conserved serine by threonine in the CVC domain of VSX1, probably highlighting the functional importance of this region of the protein. Though serine and threonine have similar properties, threonine is less polar than serine due to the presence of an extra non-polar methyl group. In this context, it may affect the interaction with neighbouring residues that may lead to improper polypeptide folding, thus affecting the protein’s wild-type function. Although this variant was absent in 105 normal controls, in silico studies suggest that p. Ser251Thr might be a benign or neutral variant that may not affect the protein function. At this stage, it is difficult to conclude about the pathogenic nature of variants p. Leu268His and p. Ser251Thr. While some previous studies have concluded that missense substitutions in the VSX1 may or may not be a disease-causing variant [16, 35], others have reported that the mutations were actually non-pathogenic or showed polymorphism [35]. Recent studies have shown that the absence of VSX1 mutations in a large number of unrelated KC patients suggesting a multiple gene involvement with environmental interaction playing a significant role in the pathogenesis of the disease [36, 37]. Haplotype analysis demonstrated a sharing of common SNPs around the missense change (p. Leu268His) in two unrelated KC families, suggesting the possibility of a founder effect, which requires further investigation. The disease causative variants identified in this study were compared to the reported literature of 1–3 % is high (2 probands out of 8, 25 %), probably due to the families belonging to an endogamous community and coincidental selection of study population with high VSX1 mutations. Further screening of the coding variant (p. Ser251Thr) on a large cohort of familial KC cases may reveal the exact pathological role of the VSX1 gene. In the eight families who were analysed for VSX1 mutation screening, we were able to identify a novel missense change (p. Leu268His) in two families and a variant of unknown significance (p. Ser251Thr) in a third family. Screening for other candidate genes including SOD1, ZEBI, TGFBI, FLG in KC families could determine the underlying genetic mechanism of the disease in VSX1 mutation negative patients.

Conclusions
In summary, we add one novel missense variation in the coding region of VSX1 to the existing repertoire of VSX1 coding variants observed in Indian patients with the characteristic phenotype of KC. Another variant p. Ser251Thr that was identified may be a benign polymorphism or a variant of unknown significance. Further biophysical studies are necessary to evaluate the precise molecular mechanism of VSX1 caused by this variant. The variation p. Leu268His may be involved in the pathogenesis of KC and therefore help in the genetic counselling of patients and their family.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
RS, RN, and SGN in recruitment of study subjects and collection of clinical details. VR carried out the molecular genetic studies. JN in research design, data analysis, and drafting of the manuscript. AG, GK, and CI helped in the acquisition of genetic data and critical reading of the manuscript. All authors have read and approved the final manuscript.

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