A novel VSX1 mutation identified in an individual with keratoconus in India

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Purpose: To evaluate the possible role of the VSX1 gene in a group of patients from the Indian subcontinent with keratoconus.

Methods: Molecular analysis of 66 patients with a diagnosis of keratoconus, based on clinical examination and corneal topography, was carried out. DNA extraction from peripheral blood followed by Polymerase Chain Reaction (PCR) amplification of the VSX1 gene was performed. The entire coding region and the exon–intron junctions of the VSX1 gene were analyzed by direct sequencing.

Results: A novel change at c.525G>C, replacing amino acid glutamine at position 175 with histidine, was found in one affected individual. One of the previously reported SNPs (rs12480307) was found with equal frequency in both patients and controls.

Conclusions: This is the first report from the Indian subcontinent exploring the role of VSX1 in the causation of keratoconus. One novel mutation (Q175H) predicted to be a potentially damaging change was seen in an affected individual; this substantiates the importance of this gene but its precise role in disease causation needs further investigation.

Keratoconus is a bilateral, noninflammatory, gradually progressive corneal disorder characterized by progressive thinning and steepening of the central cornea. It usually appears during teenage years, the common symptoms being myopia and astigmatism, and it is one of the major indications for corneal transplantation [1,2]. Keratoconus normally occurs as a sporadic disorder, but both genetic and environmental factors seem to play a role in its causation [3]. A strong family history has been documented in about 6%–10% of patients with both autosomal and X-linked mode of inheritance [4,5]. Its prevalence in first-degree relatives is 13.5% [6] which is between 15 and 64 times higher than for the general population [7]. The etiology of keratoconus is unknown. It arises as an isolated defect but is also known to be associated with conditions such as Ehler–Danlos, Marfan, Apert, Noonan, and Downs Syndrome.

Genome-wide linkage analyses have identified several chromosomal loci and genes that may be associated with keratoconus [8–12]; however, some were eventually excluded [13,14] while for others a conclusive association with the disease is yet to be established. Mutations in AIPL1, CRB1, and CRX have been implicated in patients with Leber congenital amaurosis, rendering them susceptible to keratoconus [15,16].

One of the strongly implicated candidate genes for keratoconus is Visual System Homeobox 1 (VSX1) localized to 20p11-q11. Initially this gene was chosen for screening mutations exploring the association between posterior polymorphous corneal dystrophy (PPCD) and keratoconus [12]. VSX1 is a developmental gene considered important in ocular development and is normally expressed in the developing cornea. VSX1 mRNA has been found in the outer tier of the inner nuclear layer of the human retina, embryonic craniofacial tissue and the cornea [17]. The gene has 5 exons spanning 6.2 kb of coding sequence [12]. Several genetic variants of the VSX1 gene [12,18–23] have been reported from various parts of the world but a definite pathogenic role of the genetic variants in causation of keratoconus is not yet established.

The VSX1 variants include D144E, G160D, P247R, L159M, R166W, H244R, L17P, G160V and N151S (Table 1) which were initially reported as pathogenic but whose pathogenicity could not be confirmed because segregation of these variants was seen in unaffected individuals also. He et al. [12] identified a compound heterozygous change with P247R and G160D and reported G160D to be pathogenic and P247R to be nonpathogenic. Another study [18] reported just the opposite, where P247R was found to be co-segregating with keratoconus. The D144E mutation was reported as pathogenic [18,22] in earlier studies but subsequent studies...
identified its presence in unaffected individuals and suggested this to be a polymorphism [19,20]. The variants R166W, H244R and L159M have been identified in keratoconus patients but these changes did not segregate with the disease phenotype in their family members and hence were not considered sufficiently significant to support a pathogenic role in keratoconus [21]. Similarly, variants G160V and N151S have been identified in patients from the Korean population [23] but these changes have not been reported from other populations.

The present study was undertaken in order to search for genetic variations of the VSX1 gene in the Indian population and to explore its role in causation of keratoconus. Mutational analysis of VSX1 was carried out in 66 unrelated patients affected with keratoconus in comparison to 100 controls.

**METHODS**

Patients affected with keratoconus seen in the Cornea and Refractive Surgeries Services and Contact Lens Clinic at Dr. Rajendra Prasad Centre for Ophthalmic Sciences, All India Institute of Medical Sciences, during the period of March 2007 to 2008 were included in the study. The study adhered to the tenets of the Declaration of Helsinki and was approved by the Institutional Ethics Committee. Informed consent was taken from all the patients before being enrolled for the present study. Detailed family histories up to three generations were taken and pedigree charts were constructed. History of ocular or other hereditary disorders was recorded. The existence of consanguinity and the regional birthplace of patients were also noted.

A total of 66 unrelated patients with keratoconus were recruited in the present study: 23 females and 43 males, with 41 patients (62%) aged between 10 and 20. The patients presented with deterioration of vision and were diagnosed based on clinical features, such as stromal thinning, Vogt’s striae, Fleischer’s ring, Munson’s sign, and corneal topography. Topographical features, such as corneal power (K), inferior–superior dioptric asymmetry (I-S), astigmatism (Ast), and skewed radial axis (SRAX) were used to calculate KISA% (a single index that quantifies the irregularity of corneal shape and presence of astigmatism, typical of keratoconus, with good clinical correlation). A total of 100 healthy volunteers with no ocular or other disorders matched for age and gender formed the controls. A peripheral blood sample (5 ml) was collected from all the patients and control subjects after taking informed consent and explaining the nature and possible consequences of study participation.

**DNA extraction and PCR amplification:** Genomic DNA was extracted from peripheral blood leukocytes using standard protocols. All the five exons and intron/exon boundaries of VSX1 were amplified using custom-synthesized oligonucleotide primers as described previously [12]. Each reaction was carried out in a 25 μl mixture containing 2.5 μl 10X PCR buffer with 3.5 mM MgCl2, 2.5 mM dNTPs, 10 pM of each primer, 0.7 U Taq DNA polymerase (Roche) and 100 ng genomic DNA. Thermal cycling was performed in a thermal cycler (Applied Biosystem 9700) as described: initial
denaturation for 12 min at 95 °C; 35 cycles at 94 °C for 30 s, 62 °C (exons 1–4) or 60 °C (exon 5) for 30 s, 72 °C for 30 s, and a final extension for 10 min at 72 °C.

**DNA Sequencing:** All the amplified products were sequenced bidirectionally using BigDye Terminator Mix version 3.1 (Applied Biosystems [ABI], Foster City, CA) according to the manufacturer’s instructions and were analyzed on an ABI-3100 Genetic Analyzer (ABI). Nucleotide sequences were compared with the published VSX1 cDNA sequence (GenBank NM_014588).

**SIFT and PolyPhen analysis:** The potential impact of the amino acid change was assessed with the SIFT analysis tool (Sorting Intolerant From Tolerant) and PolyPhen analysis. These tools produce a multiple sequence alignment from different species of a gene to assess the positions of conserved amino acids and analyze the effect of missense changes on the conserved structure of proteins. The SIFT tool assigns a score to the mutations and a score of <0.05 is considered potentially damaging. PolyPhen analysis uses PSIC software (Position-Specific Independent Counts) to calculate profile scores (PSIC scores) that are logarithmic ratios of the likelihood of a given amino acid occurring at a particular position to the likelihood of this amino acid occurring at any position (background frequency).

**RESULTS**

The mean age at onset of symptoms among the patients was 18 years. Consanguinity was not present in any of the cases recruited for the present study. The patients were analyzed for the presence of mutations in all the five exons of VSX1. Among the patients under study a novel heterozygous nucleotide change c.525G>C (Q175H; GenBank GU138372; ACZ01961) was identified in one individual. The presence of this change was confirmed by a repeat bidirectional sequencing (Figure 1). No similar change was found in any of the 100 normal controls studied. SIFT tool analysis revealed a score of <0.05 and PolyPhen analysis gave a PSIC score difference of 1.896; both tools predicted that the replaced amino acid would be potentially damaging and would not be tolerated. The amino acid glutamine at position 175 is important and has been conserved throughout the orthologs (Figure 2). A previously reported SNP rs12480307 was also identified in four patients and six controls, segregating with equal frequency in both groups.

**DISCUSSION**

In the present study we identified a novel heterozygous change c.525G>C (Q175H; GenBank GU138372; ACZ01961) in an affected individual. SIFT tool and PolyPhen analyses predicted that the mutation Q175H (replacing amino acid glutamine with histidine) could be damaging. The absence of this change in 100 controls is suggestive of it being pathogenic. VSX1 has previously been implicated in causation of keratoconus according to earlier reports; these established that keratoconus and posterior polymorphic corneal dystrophy are co-localized within the chromosome 20p11-q11 region [12] and identified four sequence variants, two of which were considered pathogenic. To date, several genetic variants of the VSXI gene have been reported (Table 1) but their pathogenicity could not always be confirmed because segregation of these variants was also seen in some unaffected individuals. Q175H is a novel mutation, identified in the present study, adding to the database of mutations in the VSXI gene and supporting its role in keratoconus. However,
its presence in just 1.6% of the patients proves that this may be a minor gene and its exact role during development needs to be documented.

VSX1 expression is detected in adult retinal and corneal tissue and in embryonic craniofacial tissue [24,25]. The gene is a member of the CVC domain containing a paired-like class of homeodomains (HD) which play a role in craniofacial and ocular development [26]. The homeodomain region folds into three alpha helices; the latter two bind DNA in the major groove of the double helix and the third is the recognition helix that is responsible for amino acids making contact with the DNA bases. The Q175H change is present in the HD region of VSX1, where a neutral amino acid is replaced by a polar one. This may affect the binding of VSX1 protein with the DNA and modify the transcription rate. The onset for keratoconus is typically seen around the teenage years and so its role in normal eye development cannot be ruled out. In the light of previous studies, there is still insufficient evidence for the pathogenic role of VSX1 alone in causation of keratoconus. However, in our study potentially damaging mutation was detected, although only in 1.6% of the patients. Recently, CRB1 mutations were found to be associated with keratoconus in patients with Leber Congenital Amaurosis; in this case the authors suggested that the CRB1 mutations could make patients more susceptible to developing keratoconus [15]. Therefore, the VSX1 gene might have a small effect by itself, yet operate in conjunction with other genes or environmental factors in causation of keratoconus.

To conclude, to the best of our knowledge this is the first report from the Indian subcontinent exploring the causative role of the VSX1 gene in keratoconus. We have identified one novel mutation, Q175H, predicted to cause a pathogenic change which would not be tolerated. This study adds one novel pathogenic mutation to the existing repertoire of VSX1 mutations but its wider role still needs to be explored.

ACKNOWLEDGMENTS

We would like to thank the patients and their families for participating in the study and Professor Jaya Tyagi, Department of Biotechnology, All India Institute of Medical Sciences for the help and support provided. The study was supported by an intramural grant by the All India Institute of Medical Sciences (AIIMS). Preeti Paliwal is a recipient of Senior Research Fellowship (SRF) from the Council of Scientific and Industrial Research (CSIR), India. Anuradha Singh has been supported by a grant from the Department of Biotechnology (DBT), India.

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