Analysis of VSX1 Variations in Brazilian Subjects with Keratoconus

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Abstract

Purpose: To screen visual system homeobox 1 (VSX1) gene in Brazilian subjects affected with keratoconus (KCN).
Methods: Seventy-three patients with KCN and 106 healthy controls were enrolled in this study. Patients were diagnosed with KCN based on eye examination and corneal topographic features according to Rabinowitz’s criteria (K > 47.2, I-S > 1.4 and KISA > 100%). DNA from blood samples was extracted from donors, and the exons and exon-intron boundaries of VSX1 were sequenced. The potential impact of the identified amino acid changes was assessed with Poly-Phen2, SIFT, and PMUT analysis tools. Genotyping was confirmed by RLFP technique, which was also applied to genotype non-affected individuals.
Results: We found three non-synonymous substitutions (L68H, R131S, and D105E) in VSX1 exon 1, with L68H mutation as a novel variation in this gene. In silico analysis indicated that all variations found were predicted to be probably damaging to VSX1 structure and function. Examination of R131S and L68H variations segregating in one family suggested a strong effect of these variations in increasing disease severity in the proband, which presented bilateral KCN leading to corneal grafting before the age of sixteen. We found a novel synonymous substitution (P79P) and two previously described exonic polymorphisms, with unknown roles in VSX1 pathogenesis.
Conclusion: VSX1 polymorphisms found in the Brazilian population support a genetic component in KCN pathogenesis. L68H is a novel mutation, and the phenotypic data suggest that this mutation might enhance disease severity when combined with other polymorphisms. However, further investigations are needed.

Keywords: Cornea; Keratoconus; Polymorphism; VSX1

INTRODUCTION

Keratoconus (KCN) is a debilitating condition characterized by bilateral progressive corneal ectasia, where the cornea protrudes and assumes a conical shape.[1] This corneal modification leads to visual acuity...
loss due to myopia and astigmatism. The incidence of KCN has been estimated to be approximately 1 in 2,000 individuals, with a prevalence of 50 to 230 cases per 100,000 in western developed countries, which corresponds to its being the major cause of keratoplasty and corneal grafting. Furthermore, KCN affects both genders and all ethnic groups worldwide. The disease onset generally occurs in the second decade of life and progresses until about the fourth decade, after which the disease stabilizes. Other clinical manifestations, such as a decrease in visual acuity and progressive corneal steepening, are often reported in patients with KCN. Disease progression may also lead to corneal scarring and hydrops.

The etiology of KCN is multifactorial and multigenic due to environmental and genetic factors. The most widespread hypothesis suggests that patients with genetic predisposition also require an environmental event (second hit) to elicit disease development. Corneal trauma caused by eye rubbing is a well-recognized risk factor for KCN development. Several investigations also associate the disorder with additional environmental risk factors such as use of contact lenses, atopy of the eye, and oxidative damage. Studies involving monozygotic twins with KCN and families where disease segregates demonstrate a strong genetic predisposition to the disorder. Recent data also suggest that disease prevalence in first-degree relatives is 15 to 67-fold higher than in non-related individuals. In addition, in most cases, both eyes are affected, suggesting a strong genetic influence in the disease pathogenesis. Although most pedigrees exhibit autosomal dominance with variable expression of the phenotype, autosomal recessive inheritance has been also reported, which indicates that KCN is a genetically complex disease.

A multitude of genes are believed to be involved in KCN development, and evidence suggests that the visual system homeobox 1 (VSX1) gene may play an important role in disease etiology. VSX1 is a transcription factor encoded on chromosome 20p11.21, and it is considered a strong candidate gene involved in KCN and posterior polymorphous dystrophy (PPD) development. VSX1 is a member of the paired-like homeodomain transcription factors family, in which its members play a role in craniofacial and ocular development. Expression of VSX1 in keratocytes from injured human cornea is also highly suggestive of its role in response to injuries in cornea-related diseases. Several DNA variations were reported to alter VSX1 homeodomain structure and presumably lead to impaired DNA binding. Although many of these studies have implicated VSX1 in keratoconus susceptibility, it remains unclear how these variations contribute to disease pathogenesis. Remarkably, KCN is a major cause for corneal grafting in the Brazilian population, and considering that no other previous studies have screened VSX1 for genetic variations in this population, it is critical to perform a genetic study to uncover potential gene variants that could be involved in the disease. In the present study, we aimed to perform a mutational screen of the VSX1 gene in Brazilian patients affected by KCN.

**METHODS**

**Patients and Clinical Assessment**

Seventy-three unrelated patients presenting a clinical diagnosis of KCN were recruited from the Vision Hospital and from Dept. of Ophthalmology of Clinical School/UNIFACISA, both located in the city of Campina Grande, state of Paraiba, Brazil. The project was approved by the Human Research Ethics Committee of UNIFACISA, and all protocols adhered to the tenets of the Declaration of Helsinki as well as guidelines from the Brazilian National Health Council (Resolution No. 466/12). All participants gave their written informed consent. KCN diagnosis was based on clinical examinations and the presence of characteristic corneal topographic features based on Rabinowitsz’s criteria, in which affected patients were presented with posterior corneal elevation within the central 3 mm ≥ 20 µm, inferior-superior dioptic asymmetry (I-S value) >1.4 diopters (D), and the steepest keratometry >47.2 D. We also considered KCN-positive patients when KISA, a highly specific and sensitive index for diagnosing KCN, was higher than 100% in at least one of the eyes. Corneal topography and keratometry were performed using an EyetechCT2000SLE Corneal Topographer (Eyetech Equipments, São Paulo, Brazil) and Schwind Sirius Scheimpflug tomography (Eye-tech-Solutions, GmbH and Co. Kleinostheim, Germany). DNA samples from 106 ethnically and age-matched unrelated individuals previously examined and without clinical signs of KCN and no familial history of ocular diseases were used as control. All participants donated 5 mL of venous blood in EDTA-coated tubes, which were then stored at −20°C until processing.

**Mutational Screen**

Total genomic DNA was extracted from peripheral blood leukocytes using HiPura™ Multi-Sample DNA Purification Kit (HiMedia, India) according to the manufacturer’s protocol. The DNA was quantified using Nanovue Plus (GE Healthcare, EUA), diluted in TE buffer (5 mM Tris-HCl, 0.1 mM EDTA, pH 8.5) and stored at −20°C until use. To search for mutations in the VSX1 gene, all VSX1 exons and exon-intron boundaries were amplified using primers described previously. PCRs were carried out in a final volume of 25 µL containing 12.5 µL of GoTaq Green Master Mix (Promega, EUA),...
5 pmol of forward and reverse primers, 1.25 µL of 30% DMSO, and 1 µL of DNA. PCR cycling conditions consisted of an initial denaturation step at 95°C for 5 min, followed by 35 cycles of 95°C for 45 s, 60°C for 45 s, and 72°C for 45 s, with a final extension step at 72°C for 5 min. Two microliters of each PCR product was analyzed by electrophoresis on a non-denaturing 0.8% agarose gel stained with ethidium bromide and directly visualized under UV light illumination using an L-PIX image analyzer (Locus Biotecnologia, Brazil). Amplicon sequencing was performed on an ABI3130 automated sequencer (Applied Biosystems) using BigDyev3.1 and the same primers that were used in the amplification reactions. Electropherograms were analyzed using Sequence Scanner Software v. 2.0 (Applied Biosystems, USA) and CLC Sequence Viewer v. 7.0.2 (CLC Bio, Denmark) and compared with the published VSX1 cDNA sequence (Genbank accession number NM_14588). Detection of the R131S, L68H, and D105E alleles was performed by restriction fragment analysis in all DNA samples (controls and patients). A fragment of 560 bp encompassing the coding sequence of exon 1 was amplified by PCR using the forward primer 5’-GCAGCCCAATCCTATAAAGC-3’ and reverse primer 5’-ctcagagcctaggggacagg-3’, followed by endonuclease digestion with EaeI (R131S), FatI (L68H), and HpyCH4IV (D105E) in separate reactions. Each reaction mixture contained 100 ng of genomic DNA, 1 µL of restriction enzyme, and 5 µL of 10X NEB buffer (New England Biolabs, Ipswich, MA, USA). Fragment analysis was carried out in a 1% agarose gel stained with ethidium bromide.

**Variant Impact Prediction**

To predict the impact of non-synonymous variants on the structure and function of the VSX1 protein, we compared the ancestral and mutant amino acid sequences using the online versions of PolyPhen-2 (www.genetics.bwh.harvard.edu), SIFT (www.sift.jcvi.org), and PMUT (mmb2.pcb.ub.es/PMut/) analytic tools. PolyPhen-2 is based on features comprising the sequence, phylogenetic, and structural information characterizing the substitution by extracting various sequence and structure-based features of the substitution site, and then feeds this information into a probabilistic classifier. The analysis output is provided as a numerical score ranging from 0.0 (benign) to 1.0 (damaging). A prediction of “probably damaging” indicates that the query substitution was predicted to be damaging with high confidence, while a prediction of “benign” indicates that the query substitution was predicted to be benign with high confidence, and a prediction of “possibly damaging” indicates that the query substitution was predicted to be damaging but with low confidence.[32] SIFT evaluates conserved positions, and calculates a score for the amino acid change at a particular position. A score smaller than 0.05 was considered damaging and suggests a pathogenic variation.[33] PMUT calculates the pathological significance of the non-synonymous amino acid substitution using neural networks, where an output >0.5 was considered deleterious.[34] In addition, to assess the impact of synonymous DNA sequence variants close to the splice sites and their possible impact in splicing signals, we employed MutationTaster2 (www.mutationtaster.org), where a score greater than 0.3 was considered significant.

**RESULTS**

Among the 73 KCN-affected patients analyzed, 26 patients (35.6%) also had affected relatives, while the remaining 47 (64.3%) were considered sporadic cases. In order to search for variations that could be related to the disease, we sequenced the entire VSX1 coding region and exon-intron junctions from unrelated KCN-affected individuals. Variations found in affected subjects were assessed in control samples by RFLP genotyping. We were able to detect three missense amino acid changes in VSX1, all of which were located in exon 1 [Figure 1]. The D105E (rs6115023) and R131S (rs6050307) substitutions
were previously described in other populations and implicated in KCN etiology. These substitutions were predominantly found in patients with KCN, but also in healthy individuals that had KCN-positive relatives. The third non-synonymous amino acid change was an L68H substitution, which was detected in three patients with KCN but not in healthy individuals. A database search showed that the L68H variation was not listed in both the 1000 Genomes Project (www.internationalgenome.org) and ExAC (www.exac.broadinstitute.org) databases, and is therefore being considered a novel variation. Additionally, we detected four synonymous amino acid changes in VSX1 exon 1 in affected individuals. A G>T transversion (rs6037016, S6S), a C>T transversion (rs8123716, G113G), and an A>G transition (rs12480307, A182A) were found in previously studied populations, while the c. 337G>A (P79P) variation was novel [Table 1]. All synonymous variations were found in patients with KCN, although their occurrence in control subjects was not assessed. No other previously reported pathogenic or other potential sequence variants were detected in the study cohort.

In an attempt to predict the impact of the potential sequence-altering variants on the structure and function of the VSX1 protein, we used Poly-Phen2, SIFT, and PMUT algorithms to compare the control and mutated VSX1 primary amino acid sequences. The L68H substitution was predicted to be damaging by the SIFT algorithm (score of 0.02), while D105E was considered probably damaging by Poly-Phen2 (score of 0.99) [Table 1]. PMUT scores indicated that all residue changes detected were probably damaging, including R131S, which presented the highest score (0.87) among all of the variants. To highlight the importance of ancestral residues on the normal function of VSX1, we obtained multiple amino acid sequence alignments of VSX1 orthologues from different species. The analysis showed that these amino acids were conserved throughout evolution [Figure 2] and considered essential for VSX1 function.

To evaluate the population frequencies of the VSX1 alleles, we searched the 1000 Genomes Project database for the synonymous and non-synonymous variations detected in our study. The results showed that the frequencies of both L68H and P79P variants were still unknown for those populations, for which genomic data were available. The allelic frequency of R131S (0.0021%) in the European (non-Finnish) population was noteworthy and suggestive of a disease-causing allele. All other allelic frequencies observed for the VSX1 variations obtained in this study are summarized in Table 2.

We employed MutationTaster2 to predict the possible effects of VSX1 variations in creating new acceptor and donor splice sites. The results showed that the L68H and D105E alterations can create ectopic donor and acceptor splice sites with significant scores greater than 0.3. For synonymous substitutions, only the S6S variation was predicted to create one donor and acceptor site with significant scores. The remaining variants were not predicted to abrogate existing splice sites [Table 2].

We also evaluated the segregation of the R131S and L68H variants in KCN families and were able to collect

![Figure 2. DNA sequence electropherograms of L68H (c. 206T>A), D105E (c. 313G>T), and R131S (c. 391G>T) sequence variants in VSX1 (NM_014588). Black arrows indicate nucleotide transversions. Chromatogram for R131S mutation was obtained for the reverse strand.](image)

Table 1. Allelic variants in VSX1 gene found in this study

| dbSNP | Nucleotide change | Protein alteration | Patient (n=73) | Controls (n=107) | PolyPhen | SIFT | PMUT | Prediction |
|-------|-------------------|--------------------|---------------|------------------|----------|------|------|------------|
| (-)   | g. 25081904       | Lys68His (L68H)    | 3 (4.1%)      | 0                | 0.08     | 0.02 | 0.66 | Damaging/Prob. damaging |
| rs615023 | g. 25081782       | Asp105Glu (D105E) | 5 (6.8%)      | 2 (1.8%)         | 0.99     | 0.57 | 0.66 | Damaging/Prob. damaging |
| rs6050307 | g. 25081706       | Arg131Ser (R131S) | 7 (9.5%)      | 4 (3.6%)         | 0        | 0.92 | 0.87 | Probably damaging |
| rs6037016 | g. 25081738       | Gly113Gly (G113G) | 2 (2.7%)      | NA               | NA       | NA   | NA   | NA         |
| rs8123716 | g. 25082081       | Ser76Ser (S6S)    | 5 (6.8%)      | NA               | NA       | NA   | NA   | NA         |
| rs12480307 | g. 25078910       | Ala182Ala (A182A) | 25 (34.2%)    | NA               | NA       | NA   | NA   | NA         |
| (-)   | g. 25081874       | Pro79Pro (P79P)   | 2 (2.6%)      | NA               | NA       | NA   | NA   | NA         |
data from two unrelated families. In the first family, a 17-year-old male proband received a corneal graft for the left eye owing to a prior advanced clinical KCN condition [Figure 3a]. Topographic analysis of his right cornea showed an elevated KISA index of 261% and central dioptry of 57.04 D. VSX1 genotyping showed that this patient carried the R131S and L68H variants. His 16-year-old sister was negative for both VSX1 amino acid changes and was diagnosed with KCN in the right eye (central dioptry, 47.61 D; I-S index, 1.86), and presented a much less severe progression of KCN compared to her brother. Their mother was negative for the mutations and had normal corneas. The second family included one KCN-affected proband that was heterozygous for the R131S variant, presenting a KISA index of 213% for the left cornea [Figure 3b]. This patient also had several related atopy symptoms throughout life, including eye itching and allergic rhinitis. The proband inherited the R131S allele from her mother who did not develop any clinical signs of KCN. The corneal indices for both family members are summarized in Table 3.

DISCUSSION

In this study, we identified a novel non-synonymous variation (L68H) and two previously reported mutations segregating in Brazilian individuals with KCN, and these variants were predicted to impair VSX1 function. KCN represents a major reason of corneal transplantations in developed countries including Brazil, and several genetic and environmental factors are believed to be involved in the disease manifestation, and is thereby classified as a complex multifactorial etiology. Although genetic linkage studies involving families implicated diverse chromosomal regions as containing SNPs and several candidate genes, we decided to screen VSX1 because it was hypothesized to be closely linked to KCN and other related ocular diseases.

We report the identification of three VSX1 amino acid changes (L68H, D105E, and R131S) in a population composed by a cohort of KCN-affected patients. The R131S and D105E variants were identified in previous populations, and have been suggested to be implicated in KCN development, although no experimental approaches to evaluate possible alterations in VSX1 structure and biochemical properties have been

Table 2. Allele frequencies in VSX1 variations and possible impact in splicing signals

| Variation | Population frequencies | Probable effect | MutationTaster analysis |
|-----------|------------------------|-----------------|------------------------|
|           | African | European | Latino | gDNA position | Score |
| L68H      | Unknown | Unknown | Unknown |                   |       |
| D105E     | 0.08511 | 0 | 0 |                   |       |
| R131S     | 0.1726 | 0.002107 | 0.01136 |                   |       |
| P79P      | Unknown | Unknown | Unknown |                   |       |
| S6S       | 0.04516 | 0.1115 | 0.01892 |                   |       |
| G113G     | 0.08511 | 0 | 0 |                   |       |
| A182A     | 0.4464 | 0.2211 | 0.4384 |                   |       |

NA: data not assessed
Table 3. Quantitative video keratographic parameters obtained from keratocone patients and their relatives

| Subject ID | OD K | OS K | OD Ast | OS Ast | OD Abs (I-S) | OS Abs (I-S) | KISA |
|------------|------|------|--------|--------|--------------|--------------|------|
| FI/I.1     | 46.87| 46.99| 0.4    | 0.39   | 0.28         | 0.03         | 9.79 |
| FI/II.1    | 47.42| 46.9 | 2.39   | 1.99   | 1.86         | 0.79         | 1.52 |
| FI/II.2    | 57.04| TR   | 1.73   | TR     | 0.01         | TR           | 261.36 |
| FII/I.1    | 44.53| 44.42| 0.58   | 0.3    | 0.16         | 0.12         | 0.0  |
| FII/I.2    | 46.82| 46.87| 0.67   | 0.95   | 0.21         | 0.98         | 0.0  |
| FII/II.1   | 45.45| 45.5 | 1.35   | 0.44   | 5.91         | 1.05         | 214.8 |
| FII/II.2   | 46.3 | 45.75| 0.14   | 0.38   | 0.19         | 0.01         | 16.33 |

K, central corneal power; Ast, regular corneal astigmatism; Abs (I-S), absolute value of inferior-superior value; KISA%, K X Abs (I-S) X Ast X SRAX/3; OS, left eye; OD, right eye; TR, transplanted cornea (values not obtained)

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We had access to the members of two informative families in which the R131S variation segregates. The proband of family 1 (FI) carried both R131S and L68H alleles, and presented severe corneal dysplasia in his left eye, which justified a corneal graft at the age of 16 years. His right eye showed severe KCN, and another corneal transplant will probably be required. A previous study reported an SNP leading to the R131S mutation, which was previously described (rs6050307, G>T) and strongly associated with KCN at a 5% risk level.[41] The L68H variation is novel and has not been previously associated with KCN in the literature. In the proband, the R131S and L68H polymorphisms were probably arranged in a cis configuration, considering that his mother and sister did not carry any variations. To our knowledge, only one published study has investigated the involvement of VSX1 in KCN pathogenesis, which reported two missense variations in VSX1 in the same patient (G160D and P247R) who required a corneal transplant at 3 months of age owing to an aggressive and bilateral corneal dysplasia.[26] Our data, showing another patient bearing two VSX1 missense variants in highly conserved residues, added to the hypothesis that this genotype results in the KCN phenotype, suggesting a strong involvement of VSX1 in KCN pathogenesis.

Since L68H was predicted to be probably damaging by SIFT and PMUT, we hypothesized that both R131S and L68H alterations may generate a dominant negative VSX1 protein that may lead to a more severe phenotype, although no biochemical data were obtained to support this conclusion. Although the sister of the proband did not carry the R131S and L68H variations, we cannot exclude the possibility that additional mutations in genes related to cornea development previously linked to KCN development, as well as unidentified environmental or atopy factors, can trigger disease onset. Polymorphisms in other candidate genes such as superoxide dismutase 1 (SOD1), which plays a crucial role in cellular antioxidant defense against superoxide radicals, and Flap-endonuclease 1 (FEN1), which is involved in the repair of oxidative DNA damage during base excision repair pathway, have also been implicated in KCN pathogenesis.[25,31,43] In addition, approaches involving linkage analysis in large extended pedigrees have led to the identification of other potential KCN genes such as DOCK9 and MIR184.[36,44] Several other minor genes not mentioned here have also been suggested to be causative...
of KCN. Further studies on mutational analysis of these genes in the Brazilian population will be key to understanding their potential individual contributions for KCN development.

We also studied a second family where both mother and daughter were heterozygous for the R131S variation. In this particular case, familial segregation of the variation clearly occurred, but only the daughter developed KCN (high KISA index value of 214.80 for the right eye only). Although we detected the R131S variant in several patients, we were unable to obtain information on their family members. Further and larger familial cohorts where KCN segregates will be essential to elucidate the role of these variations on disease onset.

Other unrelated patients that were heterozygous for the R131S variant \((n = 3)\), although unaffected, declared having relatives who had the disease. One patient received a corneal graft due to previous debilitating KCN on the right eye, and showed a KISA index of 169% for the left cornea. Two other patients with KCN showed central dioptry higher than 47.2 D for both eyes and related several familiar cases of KCN, although we were not able to reach these individuals for clinical and genetic analyses. We cannot exclude the causative effect imposed by the R131S and L68H variants without considering the existence of genetic background influencing gene expression. It is feasible that other genetic alterations located throughout the genome can modify \(VSX1\) expression, leading to disease manifestation in some individuals but not in others. In this sense, it is absolutely essential to consider this effect when validating the involvement of \(VSX1\) missense mutations in KCN, considering that KCN is considered a complex multifactorial disease. Therefore, studies involving genome analysis through modern next-generation sequencing will be necessary to study causative genetic factors required for KCN development.

In conclusion, we identified a novel non-synonymous variation segregating in Brazilian individuals with KCN as well as two previously reported mutations predicted to impair \(VSX1\) function. We believe that these variations may contribute to genetic predisposition to KCN development, although further molecular and population investigations are necessary. This is the first Brazilian report to explore the causative role of the \(VSX1\) gene in KCN. We believe that genome-wide association studies and whole-genome sequencing approaches, coupled to the analysis of a greater number of affected and non-affected individuals, will be key to untangle modifying environmental factors from genetic elements in order to elucidate the contribution of genes in KCN development.

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Conflict of Interest

There are no conflict of interest.

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