Original Article

# 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*

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## 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



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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,[2] which corresponds to its being the major cause of keratoplasty and corneal grafting. Furthermore, KCN affects both genders and all ethnic groups worldwide.[3] 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.[2] Disease progression may also lead to corneal scarring and hydrops.[4,5]

The etiology of KCN is multifactorial and multigenic due to environmental and genetic factors.[6] The most widespread hypothesis suggests that patients with genetic predisposition also require an environmental event (second hit) to elicit disease development.[7,8] Corneal trauma caused by eye rubbing is a well-recognized risk factor for KCN development.[9-11] Several investigations also associate the disorder with additional environmental risk factors such as use of contact lenses, atopy of the eye, and oxidative damage.[12,13] Studies involving monozygotic twins with KCN and families where disease segregates demonstrate a strong genetic predisposition to the disorder.[14-17] Recent data also suggest that disease prevalence in first-degree relatives is 15 to 67-fold higher than in non-related individuals.[18] In addition, in most cases, both eyes are affected, suggesting a strong genetic influence in the disease pathogenesis.[19] Although most pedigrees exhibit autosomal dominance with variable expression of the phenotype,[20,21] autosomal recessive inheritance has been also reported,[22,23] which indicates that KCN is a genetically complex disease.

A multitude of genes are believed to be involved in KCN development,[24] and evidence suggests that the visual system homeobox 1 (*VSX1*) gene may play an important role in disease etiology.[25-28] *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.[26] *VSX1* is a member of the paired-like homeodomain transcription factors family, in which its members play a role in craniofacial and ocular development.[29] Expression of *VSX1* in keratocytes from injured human cornea is also highly suggestive of its role in response to injuries in cornea-related diseases.[30] Several DNA variations were reported to alter *VSX1* homeodomain structure and presumably lead to impaired DNA binding.[11] 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 Rabinowitz's criteria,[2] in which affected patients were presented with posterior corneal elevation within the central 3 mm ≥ +20 μm, inferior-superior dioptric 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.[6] 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.[31] 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 (Loccus 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 2 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

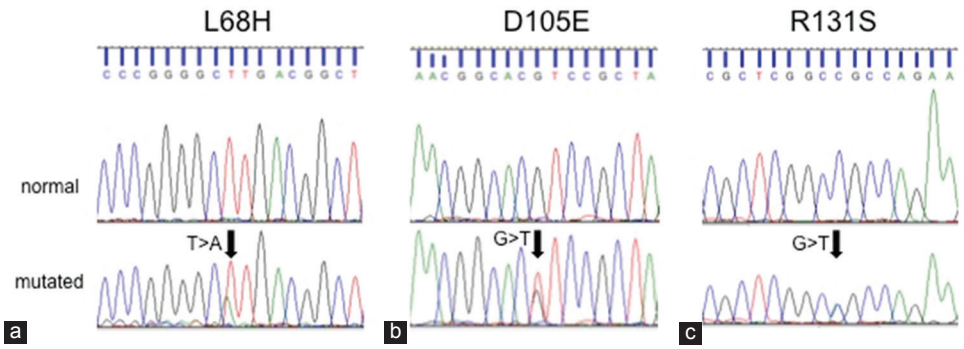


**Figure 1.** Multiple alignment of amino acid sequences of VSX1 from several species. Conserved lysine 68 (a), aspartate 105 (b), and arginine 131 (c) positions in VSX1 exon 1 are shown in bold. Alignment was produced using Clustal Omega online software (www.ebi.ac.uk).

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 obtained for the *VSX1* variations observed 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

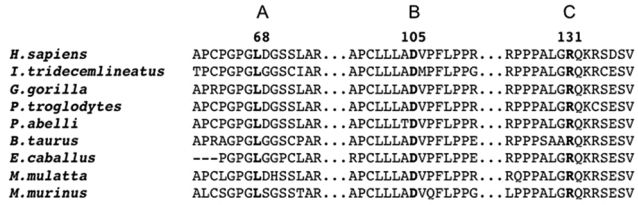


**Figure 2.** DNA sequence electropherograms of L68H (c. 206T>A), D105E (c. 313G>T), and R131S (c. 391G>T) sequence variants in VSX1 (NM_014588). Black arrows indicate nucleotide transversions. Chromatogram for R131S mutation was obtained for the reverse strand.

**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 (c. 206T >A) | Lys68His (L68H) | 3 (4.1%) | 0 | 0.08 | **0.02** | **0.66** | Damaging/Prob. damaging |
| rs6115023 | g. 25081782 (c. 313G >T) | Asp105Glu (D105E) | 5 (6.8%) | 2 (1.8%) | **0.99** | 0.57 | **0.66** | Damaging/Prob. damaging |
| rs6050307 | g. 25081706 (c. 391G >T) | Arg131Ser (R131S) | 7 (9.5%) | 4 (3.6%) | 0 | 0.92 | **0.87** | Probably damaging |
| rs6037016 | g. 25081738 (c. 339C >T) | Gly113Gly (G113G) | 2 (2.7%) | 0 | NA | NA | NA | NA |
| rs8123716 | g. 25082081 (c. 18G >T) | Ser6Ser (S6S) | 5 (6.8%) | 0 | NA | NA | NA | NA |
| rs12480307 | g. 25078910 (c. 815A >G) | Ala182Ala (A182A) | 25 (34.2%) | 0 | NA | NA | NA | NA |
| (-) | g. 25081874 (c. 237G >A) | Pro79Pro (P79P) | 2 (2.6%) | 0 | 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.

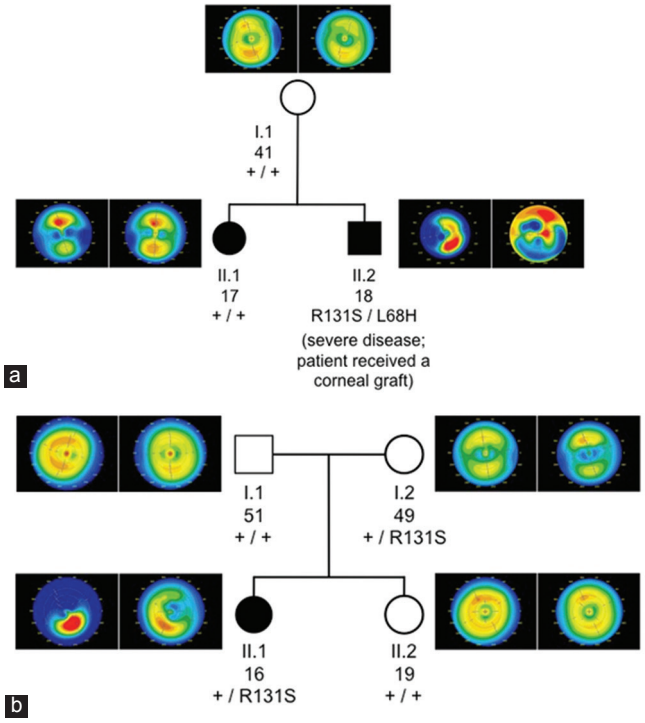


**Figure 3.** Pedigree analysis of KCN patients and segregation of VSX1 variations. Symbols in black represent affected individuals, whereas white symbols correspond to clinically normal subjects. Each member symbol is followed by its age (in years), genotype, and bilateral topography images. (a) Pedigree of family I indicates two affected patients, where patient II.1 bears both R131S and L68H mutations. (b) Pedigree of family II includes one female patient with KCN carrying the R131S variation (II.1).

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,[23] 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,[11,21,35,36] we decided to screen *VSX1* because it was hypothesized to be closely linked to KCN and other related ocular diseases.[37,38]

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[39] and D105E[14,40] 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$^{st}$ructure and biochemical properties have been

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

| Variation | Population frequencies | | | MutationTaster analysis | | |
|---|---|---|---|---|---|---|
| | African | European | Latino | Probable effect | gDNA position | Score |
| L68H | Unknown | Unknown | Unknown | Donor site gained | 463 | 0.52 |
| D105E | 0.08511 | 0 | 0 | Acceptor site gained | 575 | 0.34 |
| | | | | Acceptor site gained | 579 | 0.53 |
| | | | | Acceptor site gained | 581 | 0.7 |
| | | | | Donor site gained | 570 | 0.66 |
| R131S | 0.1726 | 0.002107 | 0.01136 | No abrogation of potential splice sites | NA | NA |
| P79P | Unknown | Unknown | Unknown | No abrogation of potential splice sites | NA | NA |
| S6S | 0.04516 | 0.1115 | 0.01892 | Acceptor site gained | 292 | 0.47 |
| | | | | Donor site gained | 286 | 0.31 |
| G113G | 0.08511 | 0 | 0 | No abrogation of potential splice sites | NA | NA |
| A182A | 0.4464 | 0.2211 | 0.4384 | No abrogation of potential splice sites | NA | NA |

NA: data not assessed

**Table 3. Quantitative video keratographic parameters obtained from keratocone patients and their relatives**

| Subject ID | K | | Ast | | Abs (I-S) | | KISA | |
|---|---|---|---|---|---|---|---|---|
| | OD | OS | OD | OS | OD | OS | OD | OS |
| FI/I.1 | 46.87 | 46.99 | 0.4 | 0.39 | 0.28 | 0.03 | 9.79 | 0.0 |
| FI/II.1 | 47.42 | 46.9 | 2.39 | 1.99 | 1.86 | 0.79 | 1.52 | 1.36 |
| FI/II.2 | 57.04 | TR | 1.73 | TR | 0.01 | TR | 261.36 | TR |
| FII/I.1 | 44.53 | 44.42 | 0.58 | 0.3 | 0.16 | 0.12 | 0.0 | 0.0 |
| FII/I.2 | 46.82 | 46.87 | 0.67 | 0.95 | 0.21 | 0.98 | 0.0 | 3.01 |
| FII/II.1 | 45.45 | 45.5 | 1.35 | 0.44 | 5.91 | 1.05 | 214.8 | 34.01 |
| FII/II.2 | 46.3 | 45.75 | 0.14 | 0.38 | 0.19 | 0.01 | 16.33 | 0.0 |

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)

performed. A considerable number of publications demonstrated that several amino acid changes in the *VSX1*gene were probably implicated in KCN. The L159M and R166W variants have been associated with KCN by affecting a region adjacent to the VSX1 homeodomain, while it has been shown that the VSX1 variant carrying the R166W mutation binds to DNA with lower affinity compared to its wild-type counterpart, thereby impairing homeodomain function.[26] The Q175H mutation has also been suggested to be pathogenic by leading to an internal loss of key amino acid interactions and affecting the DNA binding capacity of VSX1.[41] Another mutation, G160V, was strongly associated with KCN in Korean patients.[37] In our work, we verified that the L68H variation was novel, considering it was not annotated at 1000 Genomes Project database, thus suggesting that this mutation is an endemic variation in the Brazilian population. We also found that the R131S variation, implicated in KCN in previously studies, was present in KCN-affected individuals, in our sample. The population frequency of R131S in the European population was very low (0.0021%), which was suggestive of it being a disease-causing allele. Considering that KCN-affected patients from our study were declared to be natives from Paraíba state, the population genomic structure of which was mainly derived from the European ancestry than African or Amerindian (Dr. Simone Santos, personal communication), we consider R131S to be a strong candidate for KCN pathogenesis in the Brazilian population. Other studies suggesting R131S variations in larger samples are essential to study the involvement of this variant in KCN development. Differences in ethnic contributions in the studied populations, together with increasing evidence that KCN is polygenic and multifactorial, is likely to support the mutational heterogeneity observed for the *VSX1* gene in different countries.

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.[42] THeL68H 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 VSX1protein 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.[24] 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.[24] 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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Nil.

## Conflict of Interest

There are no conflict of interest.

## *REFERENCES*

1. Vazirani J, Basu S. Keratoconus: Current perspectives. *Clin Ophthalmol* 2013;7:2019-2030.
2. Li X, Yang H, Rabinowitz YS. Keratoconus: Classification scheme based on videokeratography and clinical signs. *J Cataract Refract Surg* 2009;35:1597-1603.
3. Gordon-Shaag A, Millodot M, Kaiserman I, Sela T, Itzhaki GB, Zerbib Y, et al. Risk factors for keratoconus in Israel: A case–control study. *Opthalmic Physiol Opt* 2015;35:673-681.
4. Barsam A, Brennan N, Petrushkin H, Xing W, Quartilho A, Bunce C, et al. Case-control study of risk factors for acute corneal hydrops in keratoconus. *Br J Ophthalmol* 2016.
5. Kenney MC, Nesburn AB, Burgeson RE, Butkowski RJ, Ljubimov AV. Abnormalities of the extracellular matrix in keratoconus corneas. *Cornea* 1997;16:345-351.
6. Rabinowitz YS, Rasheed K. KISA index: A quantitative videokeratography algorithm embodying minimal topographic criteria for diagnosing keratoconus. *J Cataract Refract Surg* 2000;26:1327-1335.
7. Patel D, Mcghee C. Understanding keratoconus: What have we learned from the New Zealand perspective? *Clin Exp Optom* 2013;96:183-187.
8. Sugar J, Macsay MS. What causes keratoconus? *Cornea* 2012;31:716-719.
9. McMonnies CW. Mechanisms of rubbing-related corneal trauma in keratoconus. *Cornea* 2009;28:607-615.
10. Jafri B, Lichter H, Stulting D. Asymmetric keratoconus attributed to eye rubbing. *Cornea* 2004;23:560-564.
11. Gordon-Shaag A, Millodot M, Shneor E, Liu Y. The genetic and environmental factors for keratoconus. *Biomed Res Int* 2015;2015:24-32.
12. Davidson AE, Hayes S, Hardcastle AJ, Tuft SJ. The pathogenesis of keratoconus. *Eye (Lond)* 2014;28:189-195.
13. Gordon-Shaag A, Millodot M, Essa M, Garth J, Ghara M, Shneor E. Is consanguinity a risk factor for keratoconus? *Optom Vis Sci* 2013;90:448-454.
14. Tuft SJ, Hassan H, George S, Frazer DG, Willoughby CE, Liskova P. Keratoconus in 18 pairs of twins. *Acta Ophthalmol* 2012;90:482-486.
15. Schmitt-Bernard C-F, Schneider CD, Blanc D, Arnaud B. Keratographic analysis of a family with keratoconus in identical twins. *J Cataract Refract Surg* 2000;26:1830-1832.
16. Weed KH, MacEwen CJ, McGhee CNJ. The variable expression of keratoconus within monozygotic twins: Dundee University Scottish Keratoconus Study (DUSKS). *Contact Lens Anterior Eye* 2006;29:123-126.
17. Bisceglia L, Ciaschetti M, De Bonis P, Campo PAP, Pizzicoli C, Scala C, et al. VSX1 mutational analysis in a series of Italian patients affected by keratoconus: Detection of a novel mutation. *Invest Ophthalmol Vis Sci* 2005;46:39-45.
18. Wang Y, Rabinowitz YS, Rotter JI, Yang H. Genetic epidemiological study of keratoconus: Evidence for major gene determination. *Am*

*J Med Genet* 2000;93:403-409.

19. Rabinowitz YS, Nesburn AB, McDonnell PJ. Videokeratography of the fellow eye in unilateral keratoconus. *Ophthalmology* 1993;100:181-186.
20. Rabinowitz YS. The genetics of keratoconus. *Ophthalmol Clin North Am* 2003;16:607-620.
21. Brancati F, Valente EM, Sarkozy A, Fehèr J, Castori M, Del Duca P, et al. A locus for autosomal dominant keratoconus maps to human chromosome 3p14-q13. *J Med Genet* 2004;41:188-192.
22. Kirby D, Jackson AP, Karbani G, Crow YJ. Mental retardation, keratoconus, febrile seizures and sinoatrial block: A previously undescribed autosomal recessive disorder. *Clin Genet* 2005;67:448-449.
23. Štabuc-Šilih M, Stražišar M, RavnikGlavač M, Hawlina M, Glavač D. Genetics and clinical characteristics of keratoconus. *Acta Dermatovenerol Alp Panonica Adriat* 2010;19:3-10.
24. Abu-Amero KK, Al-Muammar AM, Kondkar AA. Genetics of keratoconus: Where do we stand? *J Ophthalmol* 2014:641708.
25. Bonis *P* De, Laborante A, Pizzicoli C, Stallone R, Barbano R, Longo C, et al. Mutational screening of VSX1, SPARC, SOD1, LOX, and TIMP3 in keratoconus. *Mol Vis* 2011;17:2482-2494.
26. Héon E, Greenberg A, Kopp KK, Rootman D, Vincent AL, Billingsley G, et al. VSX1 : A gene for posterior polymorphous dystrophy and keratoconus. *Hum Mol Genet* 2002;11:1029-1036.
27. Valleix S, Nedelec B, Rigaudiere F, Dighiero P, Pouliquen Y, Renard G, et al. H244RVSX1 is associated with selective cone on bipolar cell dysfunction and macular degeneration in a PPCD family. *Investig Ophthalmol Vis Sci* 2006;47:48-54.
28. Eran P, Almogit A, David Z, Wolf HR, Hana G, Yaniv B, et al. The D144E substitution in the VSX1 gene: A non-pathogenic variant or a disease causing mutation? *Ophthalmic Genet* 2008;29:53-59.
29. Chow RL, Snow B, Novak J, Looser J, Freund C, Vidgen D, et al. Vsx1, a rapidly evolving paired-like homeobox gene expressed in cone bipolar cells. *Mech Dev* 2001;109:315-322.
30. Barbaro V, Di Iorio E, Ferrari S, Bisceglia L, Ruzza A, De Luca M, et al. Expression of VSX1 in human corneal keratocytes during differentiation into myofibroblasts in response to wound healing. *Investig Ophthalmol Vis Sci* 2006;47:5243-5250.
31. Saee-Rad S, Hashemi H, Miraftab M, Noori-Daloii MR, Chaleshtori MH, Raoofian R, et al. Mutation analysis of VSX1 and SOD1 in Iranian patients with keratoconus. *Mol Vis* 2011;17:3128-3136.
32. Adzhubei IA, Schmidt S, Peshkin L, RamenskyVE, Gerasimova A, Bork P, et al. A method and server for predicting damaging missense mutations. *Nat Methods* 2010;7:248-249.
33. Kumar P, Henikoff S, Ng PC. Predicting the effects of coding non-synonymous variants on protein function using the SIFT algorithm. *Nat Protoc* 2009;4:1073-1081.
34. Ferrer-Costa C, Gelpí JL, Zamakola L, Parraga I, de la Cruz X, Orozco M. PMUT: A web-based tool for the annotation of pathological mutations on proteins. *Bioinformatics* 2005;21:3176-3178.
35. Burdon KP, Vincent AL. Insights into keratoconus from a genetic perspective. *Clin Exp Optom* 2013;96:146-154.
36. Czugala M, Karolak JA, Nowak DM, Polakowski P, Pitarque J, Molinari A, et al. Novel mutation and three other sequence variants segregating with phenotype at keratoconus 13q32 susceptibility locus. *Eur J Hum Genet* 2012;20:389-397.
37. MokJW, BaekSJ, Joo CK. VSX1 gene variants are associated with keratoconus in unrelated Korean patients. *J Hum Genet* 2008;53:842-849.
38. Wheeler J, Hauser MA, Afshari NA, Allingham RR, Liu Y. The genetics of keratoconus: A review. Reprod Syst Sex Disord Curr Res 2012;(Suppl. 6):1-19.
39. Dehkordi FA, Rashki A, Bagheri N, Chaleshtori MH, Memarzadeh E, Rashki A, et al. Study of VSX1 mutations in patients with keratoconus in southwest Iran using PCR-single-strand conformation polymorphism/heteroduplex analysis and sequencing method. *Acta Cytol* 2013;57:646-51.
40. Aldave AJ, Yellore VS, Salem AK, Yoo GL, Rayner SA, Yang H, et al. No VSX1 gene mutations associated with keratoconus. *Investig Ophthalmol Vis Sci* 2006;47:2820-2.
41. Paliwal P, Singh A, Tandon R, Titiyal JS, Sharma A. A novel VSX1 mutation identified in an individual with keratoconus in India. *Mol Vis* 2009;15:2475-9.
42. Wang Y, Jin T, Zhang X, Wei W, Cui Y, Geng T, et al. Common single nucleotide polymorphisms and keratoconus in the Han Chinese population. *Ophthalmic Genet* 2013;34:160-6.
43. Wojcik KA, Synowiec E, Polakowski P, Głowacki S, Izdebska J, Lloyd S, et al. Polymorphism of the flap endonuclease 1 gene in keratoconus and Fuchs endothelial corneal dystrophy. *Int J Mol Sci* 2014;15:14786-802.
44. Hughes AE, Bradley DT, Campbell M, Lechner J, Dash DP, Simpson DA, et al. Mutation altering the miR-184 seed region causes familial keratoconus with cataract. *Am J Hum Genet* 2011;89:628-33.