Original Article

# *VSX1* and *SOD1* Mutation Screening in Patients with Keratoconus in the South of Iran

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## Abstract

**Purpose:** To investigate mutations of visual system homeobox 1 (*VSX1*) and superoxide dismutase 1 (*SOD1*) in 20 patients with keratoconus in the south of Iran.

**Methods:** Twenty patients with keratoconus who had a positive familial history were enrolled in this study and gave informed consent for DNA analysis. Genomic DNA was extracted from peripheral blood lymphocytes. Polymerase chain reaction (PCR) was carried out to amplify exon 2 of *SOD1* and its exon-intron boundary for the detection of a seven-base deletion in intron 2 of *SOD1*, and also all five exons of *VSX1* and their exon-intron boundaries. Amplified samples were then subjected to direct DNA sequencing.

**Results:** Sequencing data were compared against reference sequences using NCBI basic local alignment search tool (BLAST), which revealed that our patients had no mutations in *SOD1* and *VSX1*. Two single-nucleotide polymorphisms (SNPs), namely in *VSX1* (rs58752432 and rs59089167) were found in six patients.

**Conclusion:** Mutations in *VSX1* and *SOD1* genes associated with keratoconus were not identified in our patients. Therefore, it will be necessary to investigate other chromosomal loci for potential causal mutations of keratoconus using next generation sequencing (NGS) methods in our population.

**Keywords:** Keratoconus; Superoxide Dismutase 1; Visual System Homeobox 1

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## INTRODUCTION

Keratoconus (KCN), which is a bilateral, noninflammatory corneal ectasia, is associated with a progressive increase in corneal curvature, apical thinning, and irregular corneal astigmatism. KCN is often asymmetric and an obvious cone-shaped protrusion of the corneal surface may develop in this condition.[1-3] Its worldwide prevalence has been estimated to be 5.4 cases per 10,000 individuals in the general population and the disease affects both genders and all ethnicities.[4,5]

The etiology of KCN is unknown and is believed to be multifactorial.[4] The hereditary form of KCN was

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estimated to account for between 6% and 23.5% of cases and its prevalence among first-degree relatives is up to 68 times higher compared to the general population.[4,6,7] It has been reported that KCN is associated with several systemic disorders, including atopy,[8,9] Down's syndrome,[10,11] floppy eyelid syndrome,[12] congenital hip dysplasia,[13] Rieger's syndrome,[14] focal dermal hypoplasia,[15] Crouzon's syndrome (craniofacial dysostosis)[16] and Marfan's syndrome.[4,17] Several studies have been conducted to identify the biochemical, histological, and genetic bases of KCN.[18,19]

Several genes with key roles in corneal development have been proposed as potential candidate disease-causing genes for KCN. Some studies have shown that mutations in a specific exon of visual system homeobox 1 (*VSX1*) are among the molecular bases of this disease.[20-22] However, other studies have not been able to find a disease-causing mutation in *VSX1*.[23] *VSX1* is located on chromosome 20p11.21 and has important roles in craniofacial and ocular development. It encodes a protein consisting of a paired-like homeodomain that may regulate cone opsin expression during the early stages of ocular development.[24-27]

Another proposed candidate gene responsible for KCN is superoxide dismutase 1 (*SOD1*). This gene is located on chromosome 21q22.11 and provides instructions for making superoxide dismutase enzyme.[28,29] The enzyme, which binds zinc and copper ions, is responsible for destroying free superoxide radicals that can cause damage to cells.[30,31] Mutations in *SOD1* are associated with amyotrophic lateral sclerosis.[29,32] Some studies identified a variant in *SOD1*, namely a seven-base deletion in intron 2 (IVS2+50del7bp), to be associated with KCN,[28] while other studies have not found any pathogenic mutations in *SOD1* in patients with KCN.[33]

By the fact that some mutations in different exons of VSX1[21,22,34,35] and only in intron 2 of *SOD1* were identified to be associated with KCN,[28] the purpose of this study was to investigate previously identified mutations of these two genes in 20 KCN patients in the south of Iran.

## METHODS

This study was designed as a mutation detection analysis in patients with KCN from the south of Iran. Twenty unrelated KCN patients with a familial history of the disease were recruited for this study. The study was approved by the Ethics Committee of Shiraz University of Medical Sciences, Shiraz, Iran. All patients gave informed consent before undergoing DNA tests for previously reported mutations in *SOD1* and *VSX1*. The patients were diagnosed by a group of ophthalmologists and the definitive diagnosis of KCN was made based on slit-lamp biomicroscopy, retinoscopy, and corneal topography. Additionally, other members of every affected patient were examined for KCN using imaging techniques such as corneal topography and Pentacam® (OCULUS, Wetzlar, Germany) scans. All 20 selected patients had at least one operation for KCN.

Three mL of whole blood samples from the patients were collected into ethylenediaminetetraacetic acid tubes (Vacutainer™ EDTA K3 Tubes) and genomic DNA was then extracted from peripheral blood lymphocytes using a CinnaPure® DNA extraction kit (SinaClon, Tehran, Iran) according to the manufacturer's instructions.

Polymerase chain reaction (PCR) oligonucleotide primers were designed to amplify exon 2 of *SOD1* and its exon-intron boundary for the investigation of a genomic seven-base deletion in intron 2 of *SOD1* (IVS2+50del7bp), and also all five exons of *VSX1* and their exon-intron boundaries. All critical primer parameters were analyzed using online bioinformatics software programs (Primer 3, OligoAnalyzer, and OligoCalc) and compared against the NCBI database (BLASTn). All PCR primers used in the current study are listed in Table 1.

The PCR reactions were carried out in a total volume of 50 µl containing 1 µl of each primer (20 pmol/µl), 5 µl DNA template (50–200 ng), 5 µl PCR buffer (CinnaGen, Tehran, Iran), 0.5 µl dNTPs (10 mM), 1.5 µl $MgCl_2$ (50 mM, CinnaGen), 0.2 µl Taq DNA Polymerase (CinnaGen), and 35.8 µl $dH_2O$. The PCR reactions were performed using

**Table 1. Polymerase chain reaction (PCR) primers used in this study**

| *VSX1* primers | Sequence (5′→3′) | Product size (Base pairs) | Annealing temperature (°C) |
|---|---|---|---|
| E1 | FVSX1-E1: AGAGTCTGGAAGGAAGGAG<br>RVSX1-E1: ATGAGAGGCAGGGATTTAG | 1019 | 54 |
| E2-3 | FVSX1-E2-3: TCATAACTTCAATCCTCACAT<br>RVSX1-E2-3:<br>CAGATAATATACTCCACAAAGTA | 1140 | 52 |
| E4 | FVSX1-E4: TCCTGACTCTATGGAAACTTC<br>RVSX1-E4: GTTCTGGACCTGAATCTCA | 585 | 54 |
| E5 | FVSX1-E5: AGATAGGCACTGACAAGGACA<br>RVSX1-E5: TGTATGGAGTCTTCACTATGATG | 851 | 57 |
| *SOD*1 primers | Forward: CACTCCCAAGTCTGGCTGC<br>Reverse: GCGACAGAGCAAGACCCTTTC | 326 | 61 |

*VSX*1, visual system homeobox 1; *SOD*1, superoxide dismutase 1; E, exon; F, forward; R, reverse

a Veriti™ Thermal Cycler (Applied Biosystems, Foster City, CA, USA) according to the Taq DNA polymerase protocol (CinnaGen) and the amplification products were then subjected to direct DNA sequencing.

## RESULTS

Twenty unrelated patients (14 females and 6 males, mean ages: 22 years [females] and 45 years [males]) were evaluated using clinical and molecular approaches. DNA was extracted from peripheral blood lymphocytes, amplified, and subjected to direct DNA sequencing. The sequencing data were then compared against reference sequences (*VSX1*: NG_008101, *SOD1*: NG_008689) using NCBI BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi). None of the patients showed mutations in these two genes. Only two single nucleotide polymorphisms (SNPs) were found in *VSX1*: g. 8326G>A (c.627+23G>A, rs58752432) and g. 7898 G>A (c.503+202G>A, rs59089167) [Table 2 and Figure 1].

## DISCUSSION

Keratoconus has been described as a complex multifactorial disorder with involvement of both environmental and genetic elements. It has been shown that dizygotic twin discordance, familial inheritance, and association with other genetic diseases can provide insights into its genetic etiology. However, environmental conditions that are responsible for some clinical presentations of KCN have also been reported, including chronic eye rubbing, eye atopy, and wearing contact lenses. It is worth noting that genetic heterogeneity, reduced penetrance, and interactions between genes may also influence KCN.[4,36]

Until now, various loci have been proposed to be related to the molecular mechanism of KCN. Two main methods that have been used to investigate the disease-causing genes related to KCN are association studies and linkage analysis.[37,38] In the case of KCN, linkage analysis is usually difficult because this method of gene mapping is a model-based approach that requires inputs including the disease allele's frequency, penetration, and phenocopy. However, linkage analyses conducted for KCN have reported some loci related to the disease,[39] and one KCN locus, 5q21.2, was identified in two separate studies.[40]

The association study method has been used in several studies on KCN.[41,42] This method, which investigates the relationship between genotype and phenotype, is applied in two ways, namely direct association using SNPs as the causative factor or indirect association that reveals linkage disequilibrium with the causative SNP. In addition, a genome-wide association study was conducted to investigate candidate genes for KCN and a candidate gene, *RAB3GAP1*, was identified at locus 2q21.3.[42]

Different modes of inheritance have been identified in patients with KCN; for instance, 95% of cases with familial KCN have been reported to show autosomal dominant inheritance.[7] Other modes of inheritance such as autosomal recessive have also identified in the children of consanguineous parents.[43] Some studies have also reported that the relatives of KCN patients showed an increased prevalence of the disease.[44]

To date, a large number of loci have been identified in KCN, such as 16q22.3–q23.1 (autosomal dominant locus), 15q22.33–24.2, 17p13, 3p14–q13, 5q14.3–q21.1, 2p24, and 13q32.[45] However, until now, there have been

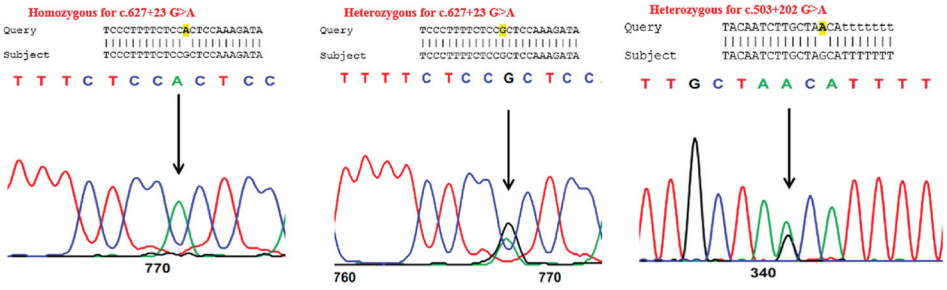


**Figure 1.** Sequencing results obtained in the current study.

**Table 2. Sequencing results for *VSX1***

| Patient number | Variant name (nucleotide) | Variant type | Exon/Intron |
|---|---|---|---|
| 2 | Homozygote NG.8326 G>A (c.627+23 G>A) | dbSNP: rs58752432 | Intron 3 |
| 7 | Homozygote NG.8326 G>A (c.627+23 G>A) | dbSNP: rs58752432 | Intron 3 |
| 15 | Heterozygote NG.8326 G>A (c.627+23 G>A) | dbSNP: rs58752432 | Intron 3 |
| 16 | Homozygote NG.8326 G>A (c.627+23 G>A) | dbSNP: rs58752432 | Intron 3 |
| 17 | Heterozygote NG.7898 G>A (c.503+202 G>A) | dbSNP: rs59089167 | Intron 2 |

SNP, single-nucleotide polymorphism; dbSNP, single nucleotide polymorphism database

no reports on the identification of mutations within genes at those loci.[45]

The only major genetic factor reported in the pathogenesis of KCN to date is *VSX1*. This gene encodes a protein that contains a paired-like homeodomain. The protein, which binds to the red/green visual pigment gene cluster region of genomic DNA, may play an important role in the regulation of cone opsin expression during the initial stages of development.[25,46] Posterior polymorphous and corneal dystrophies are two abnormalities that can result from mutations in *VSX1*.[47]

Several genetic variations (p.L17P, p.D144E, p.R166W, and others) in *VSX1* have been identified to be deleterious in KCN patients [Figure 2].[22,48,49] The mutation frequency of *VSX1* has been shown to be different in KCN patients, compared to general population, but the pathogenicity of these mutations has not been fully confirmed. It has been found that *VSX1* plays a role in corneal wound healing by influencing the differentiation of corneal keratocytes into myofibroblasts.[50] This function of *VSX1* may be connected with its involvement in the pathogenesis of KCN. In several studies, *VSX1* has been shown to be responsible for causing KCN in some ethnicities and countries. For instance, *VSX1* mutations were first identified in about 9% of 63 unrelated KCN patients in a study conducted by Heon et al in the United States.[20] In Iran, mutations of *VSX1* were reported in two out of 26 unrelated patients with KCN.[49] In many studies performed to investigate *VSX1* mutations, the variants responsible for KCN have not been found.[23,51]

To understand the pathogenic mechanism of KCN, it is essential to identify genes that may be associated with this disorder. It has been shown that various mutations in *VSX1* have caused distinct disorders that may be due to disrupted interactions between this protein and its predicted partners in a complex protein network. The initial step in drug discovery research is to identify essential proteins or drug targets for a biological process. To identify interactions between this protein and other partners that may play important roles in the pathogeneses of KCN and other eye disorders, we used STRING software (Search tool for the Retrieval of Interacting Genes/Proteins: string.embl.de/) and we identified that VSX1 protein had interactions with several essential proteins expressed in eyes according to the National Eye Institute (https://neibank.nei.nih.gov/) such as ubiquitin-conjugating enzyme E2I (UBE2I) and NK2 homeobox 1 (NKX2-1) [Figure 3]. One of the predicted partners of VSX1 is UBE2I, which is crucial for nuclear architecture and chromosome segregation SUMOylates p53/TP53 at 'Lys-386'. NKX2-1, which is another predicted partner of VSX1, is a transcription factor with a major role in the maintenance of the thyroid differentiation phenotype (data about predicted protein partners of VSX1 were extracted using the network analysis functionality of STRING software). Disruption of the interactions between VSX1 and these predicted partners may result in different clinical manifestations in patients with disease-causing mutations of *VSX1* and should be investigated in future studies.

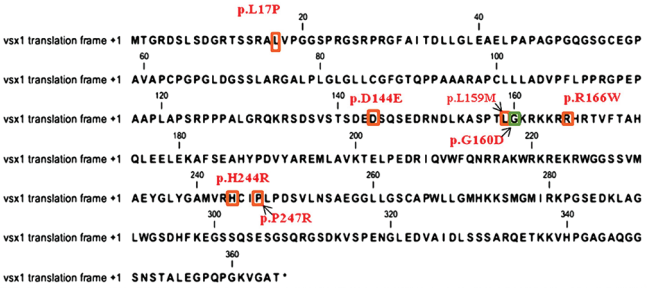


**Figure 2.** Variations reported in *VSX1* protein.

Another reported candidate disease-causing gene for KCN is *SOD1*.[28] The protein encoded by *SOD1* is responsible for destroying superoxide free radicals in the body[52] and mutations of *SOD1* result in postcholecystectomy syndrome, ocular colobomas, ichthyosis, brain malformations, and endocrine abnormalities.[53,54]

In a study conducted by Udar et al, mutation screening of *SOD1* was carried out using DNA sequencing in 15 families with KCN and a seven-base deletion in intron 2 of the gene was identified in two families. Based on this observation, a pathogenic role was proposed for variants of *SOD1* in KCN.[28] In Iran, a mutation analysis of *SOD1* was performed in 26 unrelated families; however, no mutations were identified.[49]

To determine whether interactions between SOD1 protein and its partners may have important roles in the pathogeneses of KCN and other eye disorders, we conducted an analysis of protein-protein interactions using STRING software. This analysis revealed that

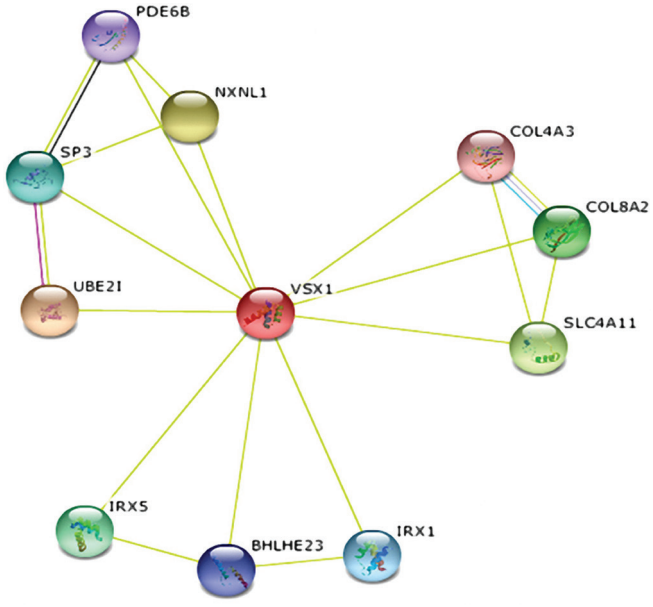


**Figure 3.** Functional and physical protein interactions of *VSX1* identified using STRING9 software.

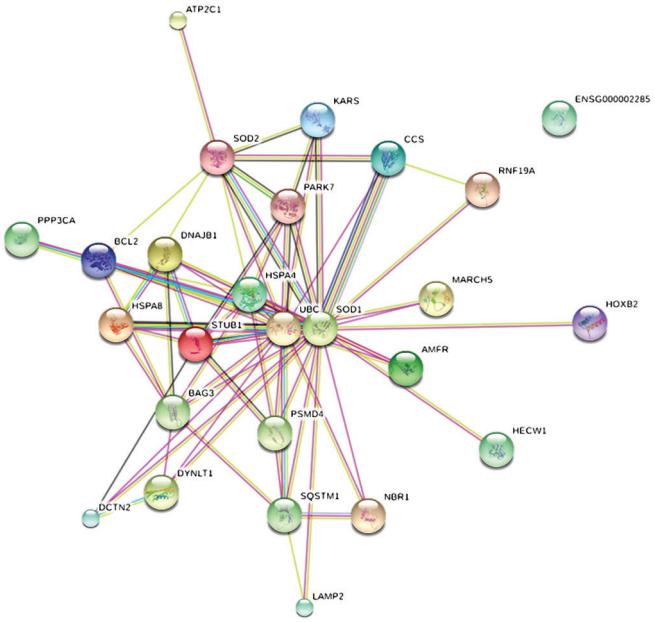


**Figure 4.** Functional and physical protein interactions of *SOD1* identified using STRING9 software.

SOD1 protein had essential interactions with several crucial partners [Figure 4] expressed in eyes according to the National Eye Institute. Identification of the main roles of these proteins may help to determine the mechanism by which a specific mutation in SOD1 can cause KCN, while other SOD1 variants do not have this consequence.

As described above, some studies have identified mutations of *VSX1* and *SOD1*; however, other subsequent investigations have not been able to confirm the roles of these variants. These studies support the notion that several genes are involved in KCN pathophysiology, rather than a single gene. Therefore, more studies should be conducted to investigate the roles of other possible genes such as *MIR184* which its mutations were reported in families affected by KCN.[55] However, in a study conducted By Farzadfard et al, they could not find any pathogenic mutations in their Iranian patients with KCN.[56] In conclusion, the present study showed that there were no mutations in *VSX1* and the previously reported mutation of *SOD1* was also not present in our patients. Therefore, we suggest that other genes may have essential roles in the pathogenesis of KCN in this population and *VSX1* and *SOD1* should be investigated in a large number of KCN patients in Iran.

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Nil.

## Conflicts of Interest

There are no conflicts of interest.

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