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Study of *VSX1* Mutations in Patients with Keratoconus in Southwest Iran Using PCR-Single-Strand Conformation Polymorphism/ Heteroduplex Analysis and Sequencing Method

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Key Words
Keratoconus · *VSX1* gene · PCR-single-strand conformational polymorphism/heteroduplex analysis · Sequencing · Iran

Abstract
Objective: Keratoconus (KC) is an eye disorder in which the cornea is swollen, thinned and deformed. Despite extensive studies, the pathophysiological processes and genetic etiology of KC are unknown. The disease incidence is approximately 1 in 2,000, and it is the most common cause of corneal transplantation in the USA. Many genes are involved in the disease, but evidence suggests a major role for *VSX1* in the etiology of KC. This study aimed to determine the frequency of mutations in exons 2, 3 and 4 of the *VSX1* gene in Chaharmahal va Bakhtiari province in the southwest of Iran. ***Study Design:*** In this experimental study, mutations in 3 exons, namely exons 2, 3 and 4, of *VSX1* were investigated in 50 patients with KC and 50 healthy control subjects. DNA was extracted using a standard phenol-chloroform method. PCR-single-strand conformational polymorphism/heteroduplex analysis was performed, followed by DNA sequencing to confirm the identified motility shifts. ***Results:*** H244R mutations were found in 1 patient and also in 1 healthy control subject. Furthermore, 12 polymorphisms were identified in patients with KC and 7 in healthy control subjects [rs6138482 and c.546A>G (rs12480307)]. ***Conclusion:*** Our investigation showed that KC-related *VSX1* mutations were found in a very small proportion of the studied patients from Iran. Further investigations on other genes are needed to clarify their roles in KC pathogenesis.

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Introduction

Keratoconus (KC) is a disorder in which the cornea becomes swollen, thinned and deformed. KC is associated with astigmatism and classically progresses until the third or fourth decade of life. In advanced cases of the disease, corneal scarring causes a further decrease in the visual resolution. Symptoms are different depending on the stage of the disease [1, 2]. Disease prevalence is almost 1 in 2,000. KC is involved in 34.5% of corneal transplant cases. Therefore, it is the most common cause of corneal transplantation in the USA [3]. Despite extensive studies, the pathophysiological processes and genetic etiology of KC are unknown [4]. Some evidence suggests that the gene *VSX1* (located on 20p11.2, MIM 605020) is involved in the etiology of KC. *VSX1* is a developmental gene regarded as significant in ocular development and is usually expressed in the developing cornea. *VSX1* mRNA has been discovered in the outer tier of the inner nuclear layer of the human retina, embryonic craniofacial tissue and the cornea [5]. The gene has 5 exons spanning 6.2 kb of coding sequence [6]. Some genetic variants of the *VSX1* gene [6–8] have been identified in different parts of the world, but a definite pathogenic role of the genetic variants in causation of KC has not yet been confirmed. There is no accurate statistical information regarding the prevalence of KC. However, it has been deduced to have a high prevalence due to its association with spring conjunctivitis. It is more prevalent in men than in women [9]. KC is distinct from other eye diseases in that it affects the individual in young age, leading to decreased quality of life [10–12]. While KC is normally observed as autosomal dominant inheritance with reduced penetrance [13], an autosomal recessive mode of inheritance has been observed in consanguineous marriages [14]. Finally, multifactorial patterns have also been reported [15]. So far, several chromosomal loci and genes have been suggested to be associated with KC [10, 16]. Of course, some genes were eventually excluded [16, 17], some showed no confirmed association with the disease [3, 4] and finally some genes, such as the visual system homebox 1 *(VSX1)* gene, have been proved to cause KC in different studies [18–21]. There are also studies which failed to find any *VSX1* mutations in cohorts of KC patients from different populations [21, 22]. This might suggest that KC is a heterogeneous disorder. Multifactorial inheritance is another explanation.

The present study was aimed at determining the type and frequency of *VSX1* gene mutations in exons 2, 3 and 4 of Iranian patients with KC, using PCR-single-strand conformational polymorphism (SSCP)/heteroduplex analysis (HA) followed by DNA sequencing, because previous studies showed increased probability of *VSX1* mutations in these exons [23].

Table 1. Sequences of primers used in the study

Primer name	Fragment size, bp	Type of primer	Primer sequence (5′ → 3′)
V2	208	F	ATAGAGGGGATATGATCACC
		R	ATAAACCTTGGGCTGTGTC
		FM*	ATAGAGGGGATATGAGCACC
V3/1	245	F	TGTGTGTTTTGGGGTCCT
		R	GTTGGCTATAGAGAAGGGAC
		FM*	TGTGTGTTTTGGGGCCCT
V3/2	293	F	CGTGTCATTCCCACATTCA
		R	TCTCCTCAGGCATTTGTG
		FM*	CGTGTCATTCCCACGCTCA
V4/1	241	F	TACTGCGTTGAATGCCCGT
		R	TGAAACCAGACCTGGTTGG
		FM*	TACTGCGTTGAATGACCGT
V4/2	263	F	TGTGTGCCTTCTGCTTCTCC
		R	CACCTCCTACAACACCTCGA
		FM*	TGTGTGCCTTCTGCTCCTCC

F = Forward; R = reverse; FM* = forward mutation.

Materials and Methods

Subjects

In total, 50 healthy control subjects and 50 sporadic cases of KC, including 30 males and 20 females, were recruited from Shahrekord University Hospital and included in this study. Diagnosis of KC was based on clinical examinations and the presence of characteristic topographic features. Informed consent for participation was signed by all the study subjects. The study protocol was approved by the Clinical Research Ethics Committee of the Shahrekord University of Medical Sciences.

Sampling and Molecular Studies

Five milliliters of whole blood was collected in 0.5 M EDTA-containing tubes. The genomic DNA was extracted with a phenol-chloroform method [24]. Forward and reverse primers for exons 2, 3 and 4 of the *VSX1* gene were designed using Primer 3 software (http://frodo.wi.mit.edu/). The positive control samples for exons 2, 3 and 4 were created using PCR-mediated site-directed mutagenesis using FM* and reverse primers (table 1). Because the PCR product of exons 3 and 4 is great, we would have a maximum efficiency of the PCR-SSCP technique products with sizes of 150–350 bp for exons of the two primer pairs [25–27].

The PCR amplification was performed in a total volume of 25 μl of mixture containing the following: 100 ng of genomic DNA, 1.0

Table 2. Allelic variants in *VSX1* found in this study

Reference sequence	Exon	Nucleotide change	Protein change	Controls (n = 50)	Patients (n = 50)
NM_014588	3	c.546A>G (rs12480307)	p.A182A	4 (8%)	6 (12%)
NM_199425	3	c.650G>A (rs6138482)	p.R217H	3 (6%)	6 (12%)
NT_011387.8	4		p.H244R	1 (2%)	1 (2%)

μM of each primer, 200 μM of each dNTP, 2.0 μM of $MgCl_2$, 1.0 U of Taq DNA polymerase and 10 μl of Taq buffer (Fermentas) using the Astec gradient 96 (Astec, Japan). The thermal cycle profile was as follows: initial denaturation at 95°C for 3 min, followed by 30–35 cycles including 95°C for 1 min, annealing temperature for 30 s for different primers (52–58°C) and extension at 72°C for 30 s. A final extension step followed at 72°C for 8 min. All of the PCR products were subjected to 8% polyacrylamide gel electrophoresis (PAGE). PCR products were visualized by means of silver ($AgNO_3$) staining.

For SSCP, a mixture of 5 μl of PCR product and 4 μl of denaturing buffer (90% formamide, 10 mM disodium EDTA, 1% xylene cyanol and 1% bromophenol blue) was heated at 95°C for 15 min and then immediately placed on ice to prevent renaturation [28].

For HA, 2.2 μl of PCR product from each sample was mixed with 3.2 μl of EDTA (0.5 M) and heated at 95°C for 5 min, then slowly cooled to 37°C using 60 cycles of 30 s. Samples (5 μl) of each denatured PCR product were loaded on nondenaturing PAGE (8%) for 1 h at 50 mA (Merck, Germany). The prepared SSCP product was mixed with the HA product for each sample and was loaded on 8% PAGE. Bands were visualized using a silver staining method. Samples with mobility shifts were verified by a second independent PCR-SSCP.

Subsequent DNA sequencing of the PCR-amplified product with the motility shift on the gel was carried out bidirectionally on an ABI 3130 automated sequencer (Applied Biosystems; Macrogen, South Korea) using the same primers.

Results

The H244R mutation and 2 polymorphisms, namely c.546A>G and c.650G>A, were identified (table 2).

The mutation H244R is a missense mutation in codon 244 in exon 4 of *VSX1*. It was identified in about 2% of the patients and 2% of the healthy control subjects studied. It was found by an altered pattern using SSCP/HA (fig. 1). The DNA sequencing results confirmed the result (fig. 2).

The Synonymous Mutation A182A

In this mutation, a single nucleotide, adenine, was substituted by guanine at g.25059546 (rs12480307; c.546A>G). It affects codon 182, resulting in a synonymous change (fig. 3).

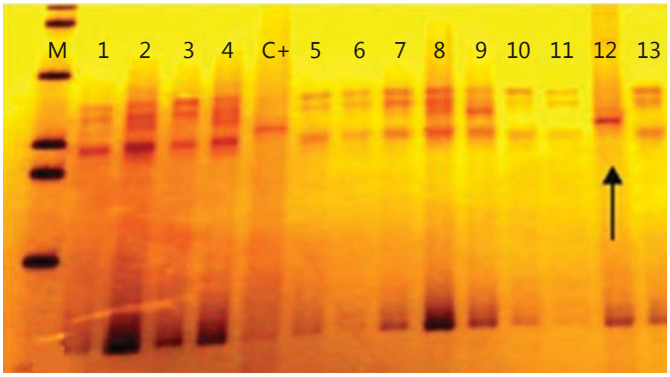


Fig. 1. SSCP and HA of exon 4 part 2 of the *VSX1* gene. The arrow shows a shift in the band motility. Lane M: molecular weight marker (100 bp); lanes 1–13: samples; lane C+: positive control.

R217H

In this mutation, a single nucleotide, thymine, was substituted by adenine at position g.25059442 (rs6138482; c.650T>A), causing codon 217 to change from CGC to CAC, resulting in a nonsynonymous mutation (fig. 4).

This was found by an altered pattern using SSCP/HA (fig. 5).

Discussion

KC is a heterogeneous disorder with variable clinical expression. Several hypotheses regarding the etiology of KC have been suggested, including biomechanical, environmental, genetic and biochemical causes. In spite of an enormous amount of research conducted to explain the etiology and disease progression, *VSX1* is the sole gene indicated as an important genetic factor in determining KC. In this study, 3 sequence variations were detected, all of which have been previously reported [3, 4, 18, 29, 30]. We found the H244R mutation in exon 4 of the gene; it was present in 2% of the patients and 2% of the healthy

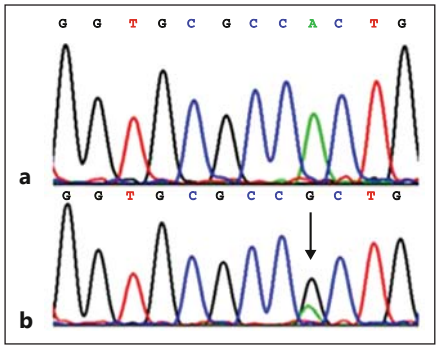


Fig. 2. Electropherogram illustrating the H244R mutation in exon 4 of the *VSX1* gene from subjects' DNA. **a** Nucleotide sequences of a control subject. **b** Partial sequences showing the A>G change in a patient.

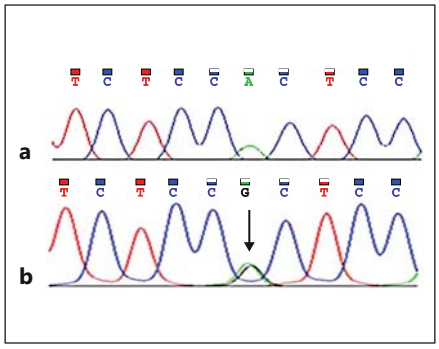


Fig. 3. Electropherogram of the *VSX1* gene corresponding to codons 181–184. **a** The reference sequence derived from a control subject. **b** Sequence derived from a patient showing a heterozygous A>G nucleotide change.

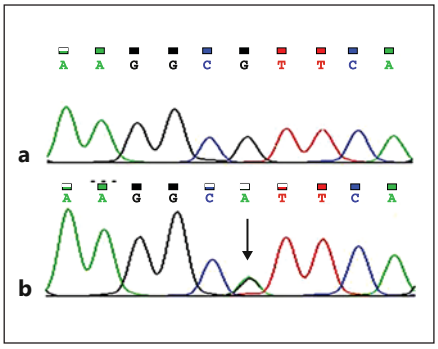
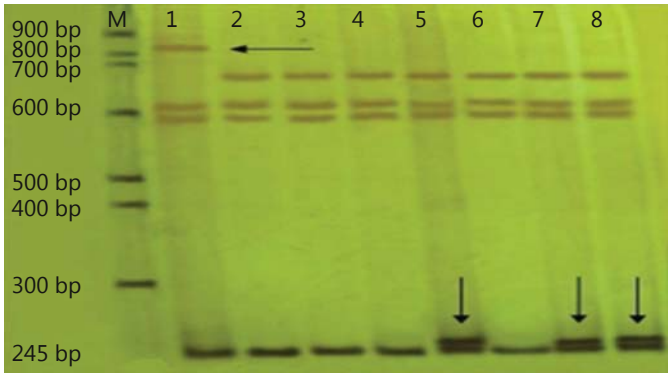


Fig. 4. Electropherogram of the *VSX1* gene corresponding to codons 217–219. **a** The reference sequence derived from a control subject. **b** Sequence derived from a patient showing a heterozygous G>A nucleotide change which is a nonsynonymous mutation (p.R217H).

Table 3. Alterations in the *VSX1* gene in various populations

Study	Population studied	Origin	Alteration	Frequency
Paliwal et al. [35]	66 KC patients	Indian subcontinent	c.525G>C	1.6%
Stabuc-Silih et al. [36]	113 patients	Slovenia	627+23G.A polymorphism	15%
Abu-Amero et al. [12]	55 KC patients, 50 controls	Saudi Arabia	noncoding (g.8326 G>A, g.10945 G>T and g.11059 A>C) synonymous coding sequence changes (g.5053 G>T and g.8222 A>G)	3.6% 5.4%
Paliwal et al. [23]	family	India	Q175H mutation	mutation identified in affected brother and in the mother
Valleix et al. [33]	4 family members	France	H244R mutation	sequence analysis showed that the H244R variant in *VSX1* segregated with corneal and macular disease phenotypes in this family

control subjects, which is concordant with the results of other studies [12, 23, 31–36] (table 3). The H244R variant is important as it is 100% conserved from flies to humans. It is located in the CVC domain, which is functionally essential for the repressive transcriptional action of *VSX1* [21]. Several protein coding changes have been identified in the *VSX1* gene sequence, namely L159M, p.D144Ep, p.L17P, p.P247R, p.R166W, p.H244R and p.G160D. Other mutations, such as Asp144Glu and His244Arg, were observed in healthy relatives of patients and thus should not be pathogenic [37, 38]. Also, a study was conducted in central Iran, indicating that the H244R mutation was co-segregated in affected family members, but not in those that were unaffected [2]. As a result, a defined pathogenic role has not been established for either of these mutations [39]. In our study, 2 polymorphisms were found in the patients and healthy control subjects. PolyPhen and SIFT analyses of p.R217H suggest that it is nonpathogenic (SIFT score >0.05 and position-specific independent counts score <1.5) [4]. Similar results have previously been reported in European populations [18]. *VSX1* mutations have been reported in 4.7% (3 of 63) [40] and 8.75%



Color version available online

Fig. 5. SSCP and HA of exon 3/1 of the *VSX1* gene. The arrows show altered heteroduplex bands in samples 5, 7 and 8, in which variants were observed. Lane M: molecular weight marker; lane 1: positive control; lanes 2–8: samples.

(7 of 80) of unrelated KC patients [31], whereas other groups have failed to identify *VSX1* pathogenic sequence variants in KC [3, 29, 39]. Mutations in *VSX1* have also been reported in posterior polymorphous corneal dystrophy (MIM 122000) [40] and in combination with KC. Although the pathogenic role of *VSX1* is now accepted by many authors, only a small number of patients show mutations in this gene. In addition, several loci for the disease have been mapped [10, 16, 22, 41], and a large number of genes have been shown to be up- or downregulated in KC corneal tissues [42–44]. Reports have confirmed the genetic heterogeneity of the disease and also support the hypothesis that in some pedigrees the defect could be inherited as a multifactorial trait. Some environmental factors that can be involved include use of contact lenses, eye rubbing, spring conjunctivitis, swelling, occupation and even certain seasons of the year [2]. Thus far, mutations reported in this gene such as p.H244R, p.G160D, p.L159M, p.P247R, p.D144E and p.R166W have been reported in different ethnic groups, but no definite pathogenic function has been established for them so far [40, 45].

In summary, in addition to the *VSX1* gene, other genes are likely to be involved in the pathogenesis of KC, which warrants further investigations.

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