Study of VSX1 Mutations in Patients with Keratoconus in Southwest Iran Using PCR-Single-Strand Conformation...
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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\textbf{Key Words} Keratoconus · VSX1 gene · PCR-single-strand conformational polymorphism/heteroduplex analysis · Sequencing · Iran

\textbf{Abstract}

\textbf{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 patients from Iran. Further investigations on other genes are needed to clarify their roles in KC pathogenesis.

\textbf{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. 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. \textbf{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)]. \textbf{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.
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].

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>
<thead>
<tr>
<th>Primer name</th>
<th>Fragment size, bp</th>
<th>Type of primer</th>
<th>Primer sequence (5’ → 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>V2</td>
<td>208</td>
<td>F</td>
<td>ATAGAGGGGATATGATCACC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R</td>
<td>ATAAACCTTTGCGGTTGATC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>FM*</td>
<td>ATAGAGGGGATATGACACC</td>
</tr>
<tr>
<td>V3/1</td>
<td>245</td>
<td>F</td>
<td>TGTGTGTTTGGGGTTCTCT</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R</td>
<td>GTTTGGCTATAGAAGGGGAC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>FM*</td>
<td>TGTGTGTTTGGGGGCGCTT</td>
</tr>
<tr>
<td>V3/2</td>
<td>293</td>
<td>F</td>
<td>CGTGTCATCCTCCACATTCA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R</td>
<td>TCCTTCTAGGCATTTTGTC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>FM*</td>
<td>CGTGTCATCCTCCAGCCTCA</td>
</tr>
<tr>
<td>V4/1</td>
<td>241</td>
<td>F</td>
<td>TACTGCGTTGAAATGACCTG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R</td>
<td>TGAACCTAGACCTTGTTG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>FM*</td>
<td>TACTGCGTTGAAATGACCTG</td>
</tr>
<tr>
<td>V4/2</td>
<td>263</td>
<td>F</td>
<td>TGTGTGCGCTCTGGCTCTCC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R</td>
<td>CACCTCCTACACACCTCTGA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>FM*</td>
<td>TGTGTGCGCTCTGGCTCTCC</td>
</tr>
</tbody>
</table>

F = Forward; R = reverse; FM* = forward mutation.
μM of each primer, 200 μM of each dNTP, 2.0 μM of MgCl₂, 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 (AgNO₃) 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).

<table>
<thead>
<tr>
<th>Reference sequence</th>
<th>Exon</th>
<th>Nucleotide change</th>
<th>Protein change</th>
<th>Controls (n = 50)</th>
<th>Patients (n = 50)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NM_014588</td>
<td>3</td>
<td>c.546A&gt;G (rs12480307)</td>
<td>p.A182A</td>
<td>4 (8%)</td>
<td>6 (12%)</td>
</tr>
<tr>
<td>NM_199425</td>
<td>3</td>
<td>c.650G&gt;A (rs6138482)</td>
<td>p.R217H</td>
<td>3 (6%)</td>
<td>6 (12%)</td>
</tr>
<tr>
<td>NT_011387.8</td>
<td>4</td>
<td></td>
<td>p.H244R</td>
<td>1 (2%)</td>
<td>1 (2%)</td>
</tr>
</tbody>
</table>

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
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%
(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.

Acknowledgements

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VSX1 Mutations in Keratoconus Patients, PCR-SSCP/HA and Sequencing

Acta Cytologica
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46 Dehkordi et al.

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