Mutation analysis of VSX1 and SOD1 in Iranian patients with keratoconus

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Purpose: To evaluate mutations in the visual system homeobox gene 1 (VSX1) and superoxide dismutase 1 (SOD1) genes with keratoconus (KTCN), direct sequencing was performed in an Iranian population.

Methods: One hundred and twelve autosomal dominant KTCN patients and fifty-two unaffected individuals from twenty-six Iranian families, as well as one hundred healthy people as controls were enrolled. Genomic DNA was extracted from whole blood sample. Then to study the possible linkage between KTCN and six known loci linkage analysis was performed using 12 short tandem repeat (STR) markers. Also, the entire coding region and intron-exon boundaries of VSX1 and SOD1 were amplified by the PCR technique in each proband. Subsequently, PCR products were subjected to direct sequencing. Co-segregation analysis of the identified mutation was conducted in the family members. An Amplification Refractory Mutation System PCR (ARMS-PCR) was additionally employed for detection of the identified mutation in healthy controls.

Results: Linkage analysis of aforementioned loci did not detect evidence for linkage to KTCN. Direct PCR sequencing revealed two single nucleotide polymorphisms (SNPs; g.1502T>G and g.9683C>T), as well as two missense mutations that have been previously reported (R166W and H244R) in VSX1. We also found three undescribed SNPs (g.4886G>A, g.4990C>G, and g.9061T>A) in SOD1. The R166W and H244R mutations were co-segregated in affected family members but not in those that were unaffected. Moreover, the ARMS-PCR strategy did not detect the identified mutations in controls.

Conclusions: Our data suggest a significant association between KTCN patients and VSX1 genetic alterations (p.R166W and p.H244R). Although our findings support VSX1 as a plausible candidate gene responsible for keratoconus, other chromosomal loci and genes could be involved in KTCN development. Taken together, our results suggest that p.R166W and p.H244R could have possible pathogenic influences on KTCN.

Keratoconus (KTCN; OMIM 148300) is a genetically and clinically heterogeneous disease affecting the cornea that causes distortion and reduced vision. KTCN is the most common indication for corneal transplantation in developed countries [1,2]. The estimated incidence is between 1 in 500 and 1 in 2,000 individuals in the general population and the prevalence is estimated to be 54.5 per 100,000 [1]. It occurs in both genders and all ethnicities [3]. Studies suggest that the prevalence and incidence rates are higher in Asians compared to Caucasians [4,5]. Approximately 6% to 23.5% of cases had familial transmission [9] which were inherited in either an X-linked or autosomal recessive or dominant trait. About 90% of pedigrees with familial KTCN exhibit an autosomal dominant inheritance with reduced penetrance, the age of onset in teenage years and variable clinical expression [1,4,6]. In addition, it has been well documented that KTCN is associated with syndromic conditions such as connective tissue disorders (osteogenesis imperfecta, Gapo syndrome, and some subtypes of Ehlers-Danlos syndrome) [1], pigmentary retinopathy, Marfan’s syndrome, Noonan’s syndrome, Apert’s syndrome, Leber congenital amaurosis, and Down syndrome [2,4,10].

Several lines of evidence support the importance of genetic components in the pathogenesis of KTCN [6-8]. It has been shown that the prevalence of KTCN in first-degree relatives is significantly higher than the general population [4,5]. Approximately 6% to 23.5% of cases had familial transmission [9] which were inherited in either an X-linked or autosomal recessive or dominant trait. About 90% of pedigrees with familial KTCN exhibit an autosomal dominant inheritance with reduced penetrance, the age of onset in teenage years and variable clinical expression [1,4,6]. In addition, it has been well documented that KTCN is associated with syndromic conditions such as connective tissue disorders (osteogenesis imperfecta, Gapo syndrome, and some subtypes of Ehlers-Danlos syndrome) [1], pigmentary retinopathy, Marfan’s syndrome, Noonan’s syndrome, Apert’s syndrome, Leber congenital amaurosis, and Down syndrome [2,4,10].

KTCN appears to be a genetically heterogeneous disorder as several chromosomal regions and genes are suggested to be involved in the molecular etiology of KTCN. To date, many
In this study, we conducted mutation detection of the entire VSX1 and SOD1 codon sequences in 112 patients from twenty-six Iranian families. Our results support the possible pathogenic function of VSX1 (visual system homeobox) and SOD1 (superoxide dismutase) have been implicated in KTCN pathogenesis [3,4,18-23]. In addition to genetic factors, environmental factors as well as genetic-environmental interactions could play critical roles in KTCN [9].

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the genotype results, amplification products were resolved on 2% agarose gels stained with ethidium bromide.

**RESULTS**

Probands from 26 Iranian families were identified with KTCN. All affected and controls were born after a normal term pregnancy. Retinoscopy, corneal topography, and Pentacam examinations of probands' first-degree relatives discriminated KTCN from normal individuals. Affected cases were clinically examined and showed no signs and symptoms of any syndromic indication. KTCN was excluded in normal controls by retinoscopy, corneal topography, and Pentacam examination.

**Linkage analysis results:** To investigate the association of six known (20p11-q11, 16q22.3-q23, 3p14-q13, 2p24, 15q22.32–24, and 5q14.3-q21) genetic loci with KTCN, genomic DNA was isolated from 112 affected 52 unaffected family members. First, PCR primers were used to amplify polymorphic markers (Table 1) on chromosome 20p11-q11, 16q22.3-q23, 3p14-q13, 2p24, 15q22.32–24, and 5q14.3-q21 known to be linked to KTCN. Then, PCR products were separated by PAGE. Haplotype analysis could not define linkage between known loci with KTCN in the studied families (as shown in Appendix 1).

**Mutation analysis of VSX1:** Direct PCR sequencing using forward and reverse primers (Table 2) was conducted to evaluate genetic alterations in the coding sequence and exon/intron boundaries of VSX1. We found two single nucleotide polymorphisms (SNPs) (g.1502T>G and g.9683C>T) and two non-synonymous mutations (H244R and R166W) in VSX1 (Figure 1 and Figure 2).

**Table 2. List of VSX1 and SOD1 primers and predicted PCR product sizes (bp) used in this study.**

| Primer | Sequence (5'→3') | PCR product size (bp) | Primer location |
|--------|------------------|-----------------------|----------------|
| **VSX1 primer sequences** |
| V1F    | 5′-GCAGGCAATCCTATAAAGC-3′ | 687 | 1–20 |
| V1R    | 5′-GATTACCGAGTGGAAGA-3′ | 469–486 |
| V2F    | 5′-AGGTCTTCCTCTTCTTCTTGTCACATC-3′ | 800 | 2610–2637 |
| V2R    | 5′-AAAGGACTGTGGCTAAGGCTACCTG-3′ | 3386–3409 |
| V3F    | 5′-ATCGATGGAGGAGAAGA-3′ | 487 | 4178–4197 |
| V3R    | 5′-AAAATGAGGAACCATCGAGA-3′ | 4639–4660 |
| V4F    | 5′-CCTATGCAATCTGCTGTC-3′ | 306 | 5366–5385 |
| V4R    | 5′-CCCAGGATCTTGCAACCTA-3′ | 5652–5672 |
| V5F    | 5′-AGGAAGTGGAAGATAGGTTGCCAG-3′ | 470 | 5640–5662 |
| V5R    | 5′-TAAAGTGCATTAGGAAACG-3′ | 6110–6090 |
| V6F    | 5′-AAAGGCCTTCAAATGGGCACC-3′ | 301 | 6088–6099 |
| V6R    | 5′-TTGGAATATCAAGGCAAGTTCGT-3′ | 6367–6388 |
| V7F    | 5′-ATCGATGGAGGAGAAGA-3′ | 424 | 6312–6337 |
| V7R    | 5′-AGGCCTATCAATGAGCAAGT-3′ | 6964–6984 |
| V8F    | 5′-AGGGAGCTTCTCAGAGGCTTT-3′ | 534 | 9834–9854 |
| V8R    | 5′-AGGTGAGGTAAGCTGCCAGA-3′ | 9321–9340 |
| V9F    | 5′-GCTCAGGTAGCATGTCGC-3′ | 610 | 10272–10292 |
| V9R    | 5′-TGATGGAAGGAGAGAAAGG-3′ | 10861–10881 |
| **ARMS primers** |
| VSM    | 5′-AGTGTGCGAGGAGAAGTGTAG-3′ | 236 | 4392–4411 |
| VSWT   | 5′-ACTGCAATCCCCGTCGAGCAGT-3′ | 43911–4411 |
| VSF    | 5′-GCTATGCAATGCGGAGAAGA-3′ | 4176–4197 |
| **SOD1 primer sequences** |
| S1F    | 5′-CTTACATTCCTCGGTTCT-3′ | 450 | 4850–4868 |
| S1R    | 5′-ACCCCGCTACTAGCAAAAGT-3′ | 5281–5292 |
| S2F    | 5′-CCATCTCCCTTGTGAGGACA-3′ | 426 | 8965–8985 |
| S2R    | 5′-CGACAGGAGCAAGACCTACC-3′ | 9371–9390 |
| S3F    | 5′-TGATGAGGTAAGCTGCCAG-3′ | 344 | 11717–11736 |
| S3R    | 5′-AAAGAGCTTCAGCAGTTTGAG-3′ | 12041–12060 |
| S4F    | 5′-CCATCTCTGCTCAGGTACCTC-3′ | 386 | 12810–12840 |
| S4R    | 5′-GAAACCGCGACTAACAATCAA-3 | 12454–12473 |
| S5F    | 5′-TTTGGATATGTTGGTGGAGA-3′ | 675 | 13780–13799 |
| S5R    | 5′-TGCATGACTGAAGGCTTTC-3′ | 14334–14355 |

In family 1, the H244R mutation was identified in a 21-year-old female. The proband had three affected relatives (Figure 1A). To test the pathologic function of this genetic mutation, co-segregation of H244R (Figure 1B) in other affected family members were performed using PCR sequencing. We also screened the H244 R VSX1 mutation in...
100 controls by ARMS-PCR (Figure 1C). Direct sequencing and ARMS-PCR results confirmed that only affected patients carried p.H244R in the heterozygous state, while Figure 1B (right) indicates the wild-type (WT) variant of VSX1 gene at codon position 244.

Figure 1. Pedigree analysis and molecular study of Family 1. A: DNA sequencing revealed heterozygous missense mutation in the codon 244 VSX1 in which A→G (arrow indicates the position of nucleotide substitution). B: Amplification refractory mutation system (ARMS) for H244R VSX1 genotyping showing the co-segregation of the H244R VSX1 mutation among family members including two KTCN patients (III:1 and II:6) as well as in two individuals without KTCN clinical features (III:2 and II:5). PCR products of the internal control primer pair (383 bp), PCR product of the wild-type (WT) and mutant primer pairs (236 bp) are indicated. M, 50-bp ladder is present. C: The pedigree of Family 1 show four affected patients (arrow indicates the proband) and segregation of p.H244R through the family. Each individual was reported by age (in years), genotype and topography images. Filled symbols represent KTCN patient and open symbols reveal individuals without clinical KTCN.
In family 2, the R166W mutation was detected in an 18-year-old male (Figure 2A). Figure 2B indicates R166W in II-1 (proband) and his father (I-1). Although the proband and his father presented a variable expressivity of KTCN, the R166W co-segregated among KTCN, but not in unaffected his mother.

**Mutation analysis of SOD1:** We additionally evaluated a possible association of KTCN with SOD1 genetic alterations. The full-length SOD1 coding sequence was screened by direct PCR sequencing. In spite of the fact that three novel SNPs (g.4886G>A, g.4990C>G, and g.9061T>A) were identified in non-coding sequences, however, sequencing the coding region did not reveal a sequence variant segregating with disease in any of the families described. The SNPs did not seem to influence the activity of SOD1 protein.

**DISCUSSION**

Various genome-wide linkage analyses and mutation detection studies have reported that six loci and two genes (SOD1 and VSX1) are thought to be associated with KTCN in different ethnic groups [6]. In this study, we performed linkage analysis for six known chromosomal loci as well as a
of pedigrees with familial KTCN are predominately environment interaction, and genetic background. About 90% mode of inheritance, gene-gene interaction, gene-environment interaction, or genetic background, or the presence of this change in the unaffected individual suggests that this could be a causative mutation with incomplete penetrance.

Our results suggest that the H244R VSX1 change may be a pathogenic variant with incomplete penetrance. The R166W VSX1 mutation was also initially found in an isolated case of keratoconus with visual impairment for whom a corneal graft was required in adulthood [11]. We observed this mutation in the proband of a family with two affected patients with KTCN. Our results showed that R166W was co-segregated in two affected family members, but not in unaffected individuals and controls. The R166W alters the highly conserved third amino acid of the DNA binding homeodomain (HD). Dorval et al. [34] suggested that this mutation causes keratoconus in humans by impairing VSX1 DNA binding [34].

Three SNPs were identified in non-coding sequences of the SOD1 gene. This gene is located on chromosome 21 and functions to destroy free superoxide radicals in the body [22]. The association of SOD1 mutation with KTCN was originally reported by Udar et al. [22]. SOD1 mutations were screened in 15 unrelated individuals, each with a family history of KTCN. Results from this study determined an IVS2+50del7 change within intron 2 in two families. Also, they observed that this 7-base deletion segregated with the KTCN subjects in a studied pedigree [22]. However, an independent study failed to define any association between SOD1 mutations and KTCN [34]. Stabuc-Silih and colleagues [33] studied the association of KTCN with VSX1 and SOD1 gene mutations in 113 Slovenian patients with sporadic and familial KTCN by direct sequencing. They found no causative disease mutation in the SOD1 gene but a significant association was detected between a VSX1 polymorphism (627+23G>A) and KTCN [33]. Our findings are in agreement with published data suggesting that other genetic and non-genetic factors are involved in the pathogenesis of KTCN.

We conclude that the R166W and H244R VSX1 variants might play critical roles in the pathogenesis of KTCN. To test
the potential pathogenic relevance of these variants we performed co-segregation analysis in all affected and unaffected family members. To rule out the incomplete penetrance of autosomal dominant KTCN in unaffected family members, we conducted precise clinical examinations of unaffected individuals; a crucial step in an association study involving a molecular genetic analysis of an autosomal dominant disorder. An accurate diagnosis and exclusion of keratoconus in patients and controls (n=100), respectively, was made using corneal topography and Pentacam evaluation. Our findings suggest that the R166W and H244R mutations might be involved in KTCN pathogenesis.

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Appendix 1. Twenty six pedigrees of KTCN.

To access the data, click or select the words “Appendix 1.” This will initiate the download of a compressed (pdf) archive that contains the file.