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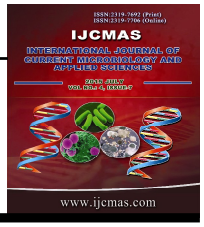

Original Research Article

Molecular genetic analysis of VSX1 Gene Mutations Associated with Keratoconus at Taif governorate, KSA

Adel E. El-Tarras[1,2*], Tallal Al Thomali[3] and Abdlhamid Al Ghamdi[3]

[1]High Altitude Research Center (HARC), Taif University, Saudi Arabia Biotechnology and Genetic Engineering Unit, Scientific Research Deanship, Taif University, Saudi Arabia
[2]Genetic Dep., Fac. of Agric., Cairo University, Cairo, Egypt
[3]Faculty of Medicine , Taif University, Saudi Arabia
**Corresponding author*

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A B S T R A C T

Keratoconus (KTCN) is a noninflammatory thinning and anterior protrusion of the cornea that results in steepening and distortion of the cornea, altered refractive powers, and reduced visual acuity. Several loci responsible for a familial form of KTCN have been mapped, however; no mutations in any genes have been identified for any of these loci. There is also evidence that visual system homebox 1 (VSX1) and superoxide dismutase 1gene (SOD1) may be involved in the etiology of KTCN. The purpose of this study was to verify the available data and to identify a new keratoconus susceptibility locus in west region of KSA (Taif governorate). Three to five milliliters of peripheral blood were collected in EDTA tubes from all participating individuals. DNA was extracted using the (QIAamp DNA Mini Kit spin protocol; Qiagen, Valencia, CA). PCR amplification of the VSX1 coding region were performed using the specific primers, The purified PCR products were sequenced bidirectionally using Big Dye Terminator ready reaction mix and analyzed on an ABI-3130 genetic analyzer (Applied Biosystems, Fostercity, CA).The results showed that **:** In the VSX1 gene screening we could find the presence of four reported single nucleotide polymorphisms; g.7512 A>T, g.7555 A>C, g. 7810 C>A and g. 8326 G>A mutation. These variations were observed in similar frequency between cases and controls. The lack of VSX1 pathogenic variations in a large number of unrelated sporadic keratoconus patients tend to delete its role, and enhance the involvement of other genetic, environmental or behavioral factors in the development of this complex disorder.

Introduction

Keratoconus (KTCN; [OMIM] 148300) is a corneal ecstatic disorder characterized by progressive thinning of the central cornea, which acquires a conical shape rather than its normal dome-shaped curve. This results in optical aberrations leading to distorted blurred vision, progressive high myopia and irregular astigmatism (Ambekar et al., 2011). KC is one of the most common cause for corneal transplantation.

Inheritance of KC does not usually follow a simple Mendelian inheritance in majority of cases and appears to be sporadic, but positive family history is reported in 6% to 10% of patients (Edwards et al., 2001). The prevalence of keratoconus is estimated to be 1- 4 in 2000 in the general population (Rabinowitz et al., 1990). KC is a heterogeneous disorder believed to be caused by both genetic and environmental factors (Rabinowitz 2003).

Six loci responsible for a familial form of KTCN have been mapped to 16q22.3-q23.1 (KTCN2; MIM [Mendelian Inheritance in Man] 608932), 3p14-q13 (KTCN3; MIM 608586), 2p24 (KTCN4; MIM 609271), 5q14.3-q21.1, 15q23-24, and 20q12. (Hutchings et al., 2005) However, to date, no mutations in any genes have been identified for any of these loci. There is also evidence that *VSX1* on 20p11.2 (KTCN1; MIM 605,020) and *SOD1* (MIM 147,450) on 21q22.11 are involved in the etiology of KTCN (Héon et al., 2002)

Visual system homeobox 1 is a protein that in humans is encoded by the VSX1 gene, plays a role in craniofacial and ocular development. VSX1 gene missense mutations (R166W and L159M) were identified in KC patients. (Héon et al., 2002), VSX1 is a member of the paired-like homeodomain transcription factors (TFs) which may regulate expression of the cone opsin genes during the embryonic development., Although it plays a role in the development of retinal bipolar interneurons, (Hayashi et al., 2005) no expression has been detected in the mouse and human.

The VSX1 homeobox gene contains a paired-like homeodomain and binds to the core of the locus control region of the red/green visual pigment gene cluster, was described, Mutations in this gene have been identified in a few families with posterior polymorphous corneal dystrophy (PPCD) and keratoconus (Héon et al., 2002).Heon et al.,2002 , Bisceglia et al.,2005, and Paliwal et al.,2009 found that one of the well-studied genes in genetic association with keratoconus is VSX1.

HumanVSX1 [OMIM 605020] is a member of the VSX1group of vertebrate paired-like home domain transcription factors localized to human chromosome 20p11-q11. Heon et al., first identified VSX1mutations in patients with either keratoconus or posterior polymorphous corneal dystrophy (PPCD). This led to the assumption that mutations in the VSX1gene may be involved in pathogenesis of keratoconus. A number of other stud-ies, further showed the presence of VSX1variants in keratoconus patients from different ethnic populations.

Among them, several variants were found in highly conserved residues of VSX1, (11) and predicted to be pathogenic by the bioinformatics tools like PolyPhen, SIFT, etc. (Paliwal et al.,2009).

The advances in computerized topographic diagnostic techniques enable higher accuracy in the diagnosis of keratoconus, including forme fruste, which eases the identification of extensive families affected by keratoconus (Brancati et al., 2004)

Recently, direct sequencing of the VSX1 gene was performed in 100 unrelated patients with diagnoses of clinical and topographic features of KC, revealing no disease-causing mutations in the VSX1 gene (11). The absence of pathogenic mutations in the VSX1 gene in a large number of unrelated KC patients indicates that other genetic factors are involved in the development of this disorder (Aldave et al., 2006).

In recent application, molecular genetic markers can be used to study the relationship between an inherited disease and its genetic cause (for example, a particular mutation of a gene that results in a defective protein). It is known that pieces of DNA that lie near each other on a chromosome tend to be inherited together. This property enables the use of a marker, which can then be used to determine the precise inheritance pattern of the gene that has not yet been exactly localized (El-Tarras el al., 2012 a,b and Awad et al., 2014).

Materials and Methods

Ethics statement: All patients provided informed consent before participation in this study. All study subjects were self identified of Saudi Arabian ethnicity. The study was approved by High Altitude Research Center (HARC) , Project 15 Med / 2012.

Clinical examination and selection of cases: In this study we screened Keratoconus patients and ethnically matched unrelated controls for mutations in the VSX1 gene. The study consisted of 48 unrelated sporadic keratoconus patients, including 26 males and 22 females with an age range between 12 to 46 years old. A total of 40 ethnic-matched healthy blood donors with age range between 17 to 65 years old, including 27 males and 13 females, (during April 2012 to April 2014) , Patients were selected from clinic of King Abdulaziz Hospital at Taif after examination or at the Tadawy Centre for Ophthalmic Sciences (Taif, KSA). Clinical evaluation involved Ultrasonic Pachymetry, videokeratography (VKG), Orbscan, visual testing, fundos copy, slitlamp-biomicroscopy, and retinoscopy.

Diagnosis of keratoconus involved the presence of characteristic topographic features, such as inferior or central corneal steepening, or an asymmetric bowtie pattern with skewing of the radial axes, and the presence of one or more of the following characteristic, clinical features in one or both eyes: conical corneal deformation, munsen sign, corneal stromal thinning, a Fleischer ring or Vogt striae. Family history up to three generations was collected and pedigrees were drawn. All 48 cases were sporadic without any family history. All keratoconus cases secondary to causes like trauma, surgery, Ehlers Danlos syndrome, osteogenesis imperfecta, and pellucid marginal degeneration were excluded from the study.

Blood collection and DNA extraction: Forty ethnically matched normal individuals without any ocular disorder were enrolled as controls. Health information was obtained from controls through the questionnaire; all underwent ophthalmological examination. Five milliliters of blood was collected by venipuncture in EDTA (EDTA) vaccutainers (Greiner Bio-One GmbH, Frickenhausen, Germany) from both patients and controls. DNA was extracted from whole blood samples by the inorganic method. For the population study, controls were taken from published data defining the lineage of the Indian population.

PCR Amplification

The Two exons and intron/exon boundaries of the VSX1 gene were amplified with the use of custom-designed oligonucleotide primers (Table 1). Each reaction was carried out in a 25-_L mixture containing 12.5 _L premix (Failsafe PCR 2_ PreMix D; Epicenter, Madison, WI), 0.2 _M each primer, 0.5 U genomic DNA polymerase (RedTaq; Sigma- Aldrich Corp., St. Louis, MO), 2.5 _L 10_ PCR buffer (RedTaq; Sigma- Aldrich Corp.) with 2.5 mM MgCl2, and approximately 100 ng genomic DNA.

Thermal cycling was performed in a thermal cycler (iCycler; Bio-Rad, Hercules, CA), under the following conditions: initial denaturation for 3 minutes at 95°C; 35 cycles of 94°C for 30 seconds, 58°C for 35 seconds, 72°C for 60 seconds; and a final extension for 10 minutes at 72°C.

DNA sequencing

The purified PCR products were sequenced bidirectionally using Big Dye Terminator ready reaction mix and analyzed on an ABI-3130 genetic analyzer (Applied Biosystems, Fostercity, CA). The sequence data analysis was done using BLAST software and compared with the published nucleotide sequence of the VSX1 gene [Gen Bank accession number NM_014588].

Results and Discussion

A total of 48 sporadic KTCN patients and 40 unrelated controls were analyzed for coding and flanking intronic regions of VSX1 through bidirectional DNA sequencing analysis. Of the 48 Keratoconus patients there were 26 males and 22 females with a mean age of 28.9. Of the 40 controls there were 19 males and 21 females with a mean age of 50. The full coding region, exon-intron boundaries of VSX1 was sequenced in all subjects.

We detected four nucleotide changes in both patients and Controls (Table 2). one of them were previously reported (G8326A) and the sequences change in this mutation is heterozygous , all the sequences changes detected were pathogenic and non coding also intronic as showing in Table 2. The PCR product size for exon 1 and 2 were shown in fig 1 and 2 after using specific primers.

Although the VSX1 gene is expressed in the retina, where it is thought to play a role in the development of retinal bipolar interneurons, (Hayashi et al., 2005 , Ohtoshi et al., 2004) no expression has been detected in the mouse cornea25,26 or in one of two studies using RT-PCR performed on RNA isolated from adult human cornea. (Paliwal et al., 2009 , Hayashi et al., 2005 and Chow et al., 2004), Heon et al.2002 selected the VSX1 gene for screening as a positional candidate gene for posterior polymorphous corneal dystrophy (PPCD, MIM, 122,000) because it is positioned within the chromosome 20p11-q11 PPCD1 candidate region. Although the PPCD candidate gene region is not one to which keratoconus has been linked previously, the investigators chose to screen the VSX1 gene in keratoconus patients because of previous reports of the coexistence of the two corneal disorders.(Blair et al., 1992 and Weissman et al., 1989).

Our resulted showed that females were more affected than males. But in the literature, it is unclear whether significant differences between males and females exist. In the same time some studies have not found differences in the prevalence between genders (Krachmer et al., 1986 , Li et al., 2004) ; others have found a greater prevalence in females (Krachmer et al., 1984 , Stein et al., 2006) ; while other investigators have found a greater prevalence in males..

Recently.Khaled Abu-Amero et al., 2014 found that mtDNA mutation may be considered as a genetic risk factor contributing indirectly through the oxidative stress mechanism to the development and/or progression of KTCN.

Table.1 Primer Sequences used for VSX1 Amplification

Exon	Forward Primer (5_-3_)	Reverse Primer (5_-3_)	Product Size (bp)	Coding Region bp)	Annealing Temp.
1	CTTAAGTACCCAAGA GGTTCATAACT	GAAACCACTGGGCCT GCTATCAT	297	79	60
2	AAGCAGGCACGGTGG TCCTTA	AAGGGACTGCTGATTG GCTCACT	294	124	60

Table.2 VSX1 sequences changes observed in keratoconus patients

I D	Loc. Snp. id	Clinical	Germline / somatic	Independent observation	Alternate designations
605020	VSX1-TU-A7512T	Pathogenic	Inherited/ Germline	39	Non coding (Intron 1)
605020	VSX1-TU-A7555C	Pathogenic	Non tested	2	Non coding (Intron 1)
605020	VSX1-TU-C7810A	Pathogenic	Non tested	4	Non coding (Intron 2)
605020	VSX1-TU-G8326A	Pathogenic	Inherited/ Germline	12	Non coding (Intron 3)

Fig.1 PCR product Size (297 bp) after using exon 1primer

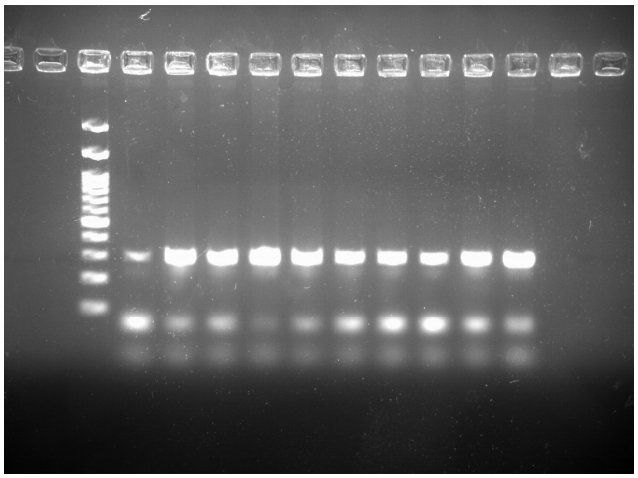
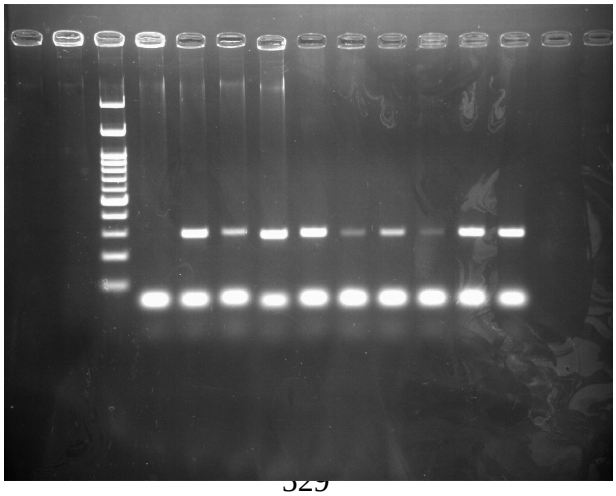


Fig.2 PCR product Size (294 bp) after using exon 2 primer



Kennedy et al., 1996 , Adel Alhayek and Lu 2015 demonstrated that Tiny fibers of protein in the eye called collagen help hold the cornea in place and keep it from bulging. When these fibers become weak, they cannot hold the shape and the cornea becomes progressively more cone shaped. The high altitude region play an important role as environmental factor for the incidence of keratoconus due to the effect of ultraviolet radiation.

In conclusion, an early onset and increased severity of keratoconus was found in Taif, Saudi Arabia. This may be related to a combination of genetic and/or environmental factors specially ultraviolet radiation. Clinically, contact lens correction should be considered earlier to maximise visual performance. The results have implications for keratoconus screening in Saudi Arabia, to improve early detection and treatment.

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