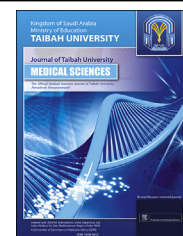




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Original Article

Sequence analysis of the *VSX1* and *SOD1* genes in families with Keratoconus and a review of the literature



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المخلص

أهداف البحث: تعتبر القرنية المخروطية اضطراباً غير التهابي للقرنية، يصبح فيه الجزء المركزي من القرنية رقيقاً ومخروطياً الشكل، مما يؤدي إلى قصر النظر اللاابورية غير المنتظمة الذي يتسبب في ضعف من بسيط إلى متوسط في الرؤية. صممت الدراسة الحالية لفحص اثنين من الجينات المرشحة بأنها مسؤولة عن مرض القرنية المخروطية بهدف تحديد تغيرات في التسلسل الجيني، قد تكون هي سبب حدوث هذا المرض في الأسر السعودية.

طرق البحث: تم جمع عينات من عدد خمس عائلات سعودية من المنطقة الشمالية مصابة بالقرنية المخروطية. عُزل الحمض النووي الجيني وأجري التسلسل ثنائي الاتجاه لجميع إكسونات التشفير الجيني *SOD1* & *VSX1* باستخدام طريقة سانجر.

النتائج: أظهرت جميع الأسر المصابة بالقرنية المخروطية نمطاً من التوريث المتنحي، تم تحديد الصفات الظاهرية لهذه العائلات من قبل طبيب عيون ذي خبرة، إلا أن تحليل تسلسل جيني *VSX1* & *SOD1* أخفق في الكشف عن أي تغير في التسلسل قد تعزي له مسؤولية الإصابة بالقرنية المخروطية في الأفراد المصابين.

الاستنتاجات: إن إخفاقتنا في الكشف عن متغير تسلسلي في اثنين من الجينات المعروفة في العائلات المصابة بالقرنية المخروطية أثار اهتمامنا لفحص جينات أخرى معروفة ومرشحة في العائلات المصابة منها *ILIRN* & *SLC4A11* . *DOCK9* . *miR-184* . سيكون التنميط الجيني باستخدام المصفوفات الكثيفة لتعدد أشكال النيوكليوتيد الواحد متبوعاً بتسلسل الإكسوم في هذه العائلات، وسيكون ذلك نهجاً مفيداً لتحديد الجينات المتسببة في القرنية المخروطية، في السعوديين، التي قد تختلف عن تلك المسجلة في أماكن أخرى.

الكلمات المفتاحية: القرنية المخروطية؛ *VSX1*؛ *SOD1*؛ العائلات السعودية

Abstract

Objective: Keratoconus (KC) is a non-inflammatory disorder of the cornea in which the cornea becomes thin and conical, inducing myopia and irregular astigmatism and resulting in mild to marked impairment of vision. The present study was designed to screen two candidate KC genes to identify pathogenic sequence variants responsible for KC in Saudi families.

Methods: Peripheral blood samples from members of five Saudi families with KC from the Northern region were collected. Genomic DNA was isolated, and bidirectional sequencing was performed of all coding exons of *VSX1* and *SOD1* genes using Sanger sequencing.

Results: All five of the KC families showed a pattern of autosomal recessive inheritance. Phenotyping of these families was performed by a senior ophthalmologist. Sequence analysis of the *VSX1* and *SOD1* genes failed to reveal any pathogenic sequence variant that could account for KC in the affected individuals.

Conclusion: Our failure to detect sequence variants in two of the known KC associated genes triggers an interest in other known KC candidate genes, including *miR-184*, *DOCK9*, *ILIRN* and *SLC4A11*. Future genotyping with dense SNP arrays followed by exome sequencing in these families will be a useful approach to identify the gene(s) underlying KC in this Saudi cohort, which may be different from those reported elsewhere.

Keywords: Keratoconus; *VSX1*; *SOD1*; Saudi families

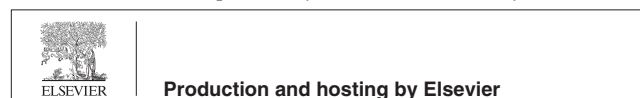
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Introduction

The cornea is the window of the eye. Light travels through the cornea past the lens to the retina and then to the brain to form a visual image.¹ The normal corneal surface is smooth and aspheric. Keratoconus (KC) is caused by bilateral and asymmetric corneal degeneration characterized by localized corneal thinning, which leads to protrusion of the thinned cornea.² This abnormal shape of the cornea prevents the light entering the eye from being focused correctly on the retina and thus causes distorted vision.³

Keratoconus usually occurs in the second decade of life, affects both genders and shows no ethnic bias.

Signs and symptoms vary depending on disease severity. Early cases usually go unnoticed unless corneal topography is performed. Disease progression manifests with the loss of visual acuity which cannot be compensated for with spectacles. In moderate and advanced cases, characteristic signs include both Fleischer's ring and Vogt's striae, fine vertical lines produced by compression of Descemet's membrane. Most patients develop corneal scarring. Munson's sign, a V-shape deformation of the lower eyelid in the downward position, and fractures in Descemet's membrane causing acute stromal edema, known as hydrops, are observed in advanced stages.⁴

The management of KC varies depending on the disease severity. Incipient cases can be managed with spectacles, mild and moderate cases with contact lenses and severe cases with keratoplasty. Other new surgical treatment options include intra-corneal ring segments, corneal cross-linking, laser procedures (i.e., photorefractive keratectomy, phototherapeutic keratectomy, lasik in situ keratomileusis), intraocular lens implants or a combination.⁵

The estimated incidence of KC varies between 1 in 500 and 1 in 2000 individuals in the general population, and the estimated prevalence is reported to be 54.5 per 100,000.^{6,7} Keratoconus is the leading cause of corneal transplant in KSA. Various studies have been conducted in KSA to measure the prevalence of KC. A retrospective study undertaken in a tertiary eye care hospital (KKESH) on patients referred from 1999 to 2009 to evaluate the prevalence of KC in KSA, estimated the prevalence to be 0.81 per 100,000 citizens.⁸ Interestingly, the prevalence estimated by this study is lower than what had been previously reported in the Saudi population.⁸ The lower prevalence of KC observed at KKESH may be the result of patients being referred to other ophthalmic facilities or hospitals, suggesting that the results of this study may not be a true representation of the prevalence of KC in KSA. The potential for under diagnoses and under treatment therefore exists.

Keratoconus segregates as either autosomal dominant with reduced penetrance, or autosomal recessive but the majority of cases are sporadic.^{14,17,18} Several chromosomal loci and genes have been reported to be associated with KC.^{6,14} Mutations in the *VSY1* and *SOD1* genes have been found to be associated with KC.³⁴ However, several studies have failed to detect *VSY1* and *SOD1* mutations in cohorts of KC patients.³⁵ Improved screening and diagnostic strategies could identify currently unrecognized KC cases and lead to their treatment. In addition, the determination

of the genetic defects underlying KC could lead to the development of molecular therapies. The present study was undertaken to recruit familial cases of KC and to screen the *VSY1* and *SOD1* candidate genes for sequence variants to determine the genetic basis of the KC in KSA.

Materials and Methods

Study subjects

Five consanguineous Saudi families with KC segregating in an autosomal recessive form from different regions of KSA were evaluated, including two families from the northern region (Al-Wajh and Dheba) and three families from the western region (Almadinah Almunawwarah). Peripheral blood samples were collected for genetic analysis, and pedigrees were determined (Figure 1). At the time of the study, the families had a total of 13 affected individuals. All affected individuals underwent careful clinical examination. A complete ophthalmic evaluation of all subjects was performed. Affected individuals and individuals with a suspected corneal abnormality underwent topographic evaluation using a computer-assisted videokeratoscope. Children under the age of 16 years and unaffected individuals were scored as "unknown phenotype status"; children were scored as unknown because the age of onset of KC is at puberty.

The family pedigrees (Figure 1) provided convincing evidence of the autosomal recessive inheritance of the phenotype, and consanguineous marriages accounted for all of the affected individuals being homozygous for the mutant allele. Approval for this study was obtained from the Institutional Review Board (IRB) of the Center for Genetics and Inherited Diseases (CGID), Taibah University Almadinah Almunawwarah, KSA. All family members provided informed consent after the purpose and possible consequences of the study were explained.

Extraction of nucleic acid and sequencing

Peripheral blood samples were collected from the 13 affected and 29 unaffected members of the five families in EDTA Vacutainer tubes. Genomic DNA was extracted using the Qiagen Midi Genomic DNA Extraction Kit (QIAGEN GmbH, Strasse 1, 40724 Hilden, Germany). PCR was performed in 0.2-ml tubes (Axygen, Inc., CA, USA) in a total volume of 50 μ l, which contained 40 ng of human genomic DNA, 25 μ l of GoTaq[®] Green Master Mix (Promega Corporation, 2800 Woods Hollow Road, Madison, WI 53711, USA) and 20 pmol of each forward and reverse primer (Macrogen Inc., GaSan-Dong, 153-801 GeumCheon-Gu, Seoul, Korea). The thermal cycling conditions used included 95[°]C for 5 min, followed by 40 cycles of 95[°]C for 1 min, 55–58[°]C for 1 min, 72[°]C for 1 min and a final extension at 72[°]C for 10 min. Thermocycling was performed using a Veriti[®] Thermal Cycler (Thermo Fisher Scientific Inc., 81 Wyman Street Waltham, MA USA), and the products generated were resolved on 2% agarose gels.

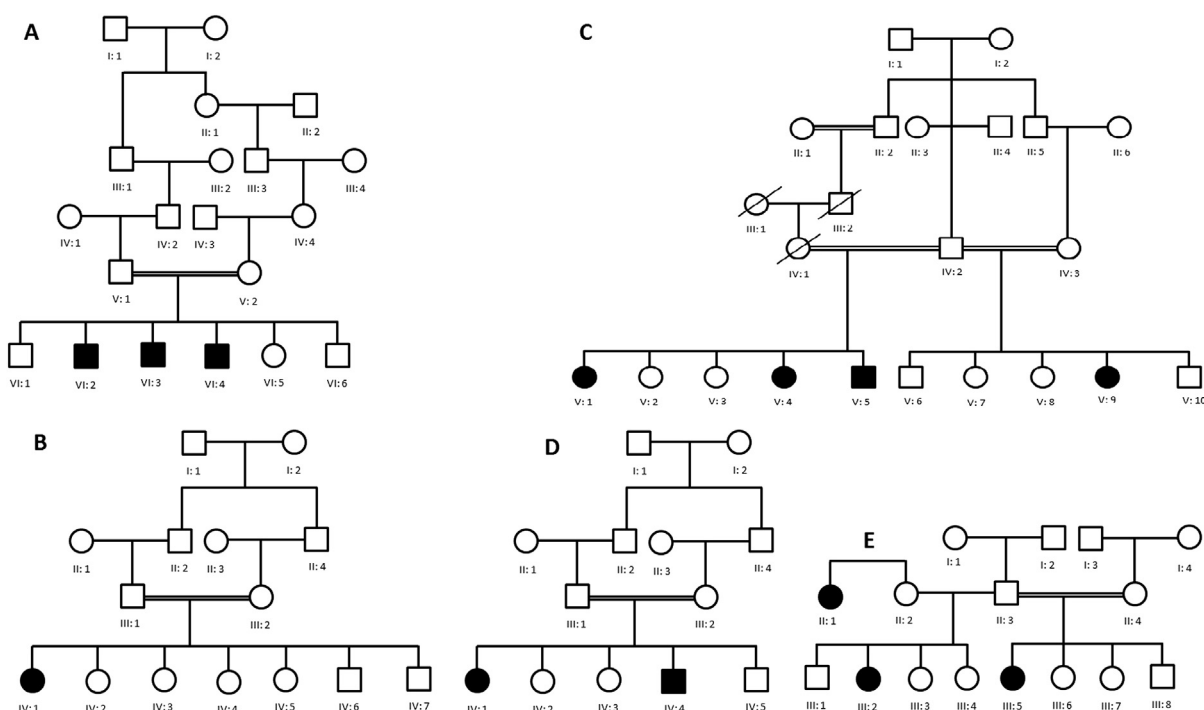


Figure 1: Pedigrees of five Saudi families segregating Keratoconus (KC) in autosomal recessive fashion. Circles represent females, squares represent males, and filled symbols represent affected individuals.

Results

Clinical description of families

Family A

Family A was recruited from Almadinah Almunawwarah. This is a six generation autosomal recessive KC family with 20 members having no history of any other genetic disease. In this family, 17 individuals are unaffected (I:1, I:2, II:1, II:2, III:1, III:2, III:3, III:4, IV:1, IV:2, IV:3, IV:4, V:1, V:2, VI:1, VI:5, IV:6), and three individuals are affected. Interestingly, all affected individuals are males. Affected individual VI:2 is a 34-year-old male who was diagnosed with KC of both eyes at the age of 33. Corneal cross linking was performed on both of his eyes. The second affected (VI:3) is a 27-year-old male diagnosed with KC of both eyes at the age of 26. He received a corneal transplant for one eye, and MyoRing insertion was performed for the other eye. The third affected individual (VI:4) is a 21-year-old male diagnosed with KC of both eyes at the age of 19, and MyoRing insertion was performed for both eyes. Blood samples were collected from three affected (VI:2, VI:3, VI:4) and three unaffected members (V:1, V:2, VI:5), including the parents of the affected members for DNA extraction.

Family B

Family B was recruited from Al-Wajh. It is a four-generation autosomal recessive KC family with 15 members. The family showed history of atopy without any other associated genetic disease. In this family, 14 individuals are unaffected (I:1, I:2, II:1, II:2, II:3, II:4, III:1, III:2, IV:2, IV:3, IV:4, IV:5, IV:6, IV:7), and one individual is affected. The affected individual (IV:1) is a 20-year-old female with history

of allergic conjunctivitis, she was diagnosed with KC of both eyes at the age of 17 and MyoRing insertion was performed for both eyes. Blood samples were collected from the one affected (IV:1) and seven unaffected members (III:1, III:2, IV:2, IV:3, IV:4, IV:5, IV:7).

Family C

Family C was recruited from Al-Madina. It is a five-generation autosomal recessive KC family with 22 members and no history of any other genetic disease. In this family, 18 members are unaffected (I:1, I:2, II:1, II:2, II:3, II:4, II:5, II:6, III:1, III:2, IV:1, IV:2, IV:3, V:2, V:3, V:6, V:7, V:8, V:10), and four members are affected. One affected individual V:1 is a 37-year-old female diagnosed with KC of both eyes at the age of 22. Corneal cross linking was performed for both eyes. The second affected case (V:4) is a 36-year-old female diagnosed with KC of both eyes at the age of 20. Corneal cross linking was performed for both eyes. The third affected case (V:5) is a 35-year-old male diagnosed with KC of both eyes at the age of 21. Corneal transplant was performed for both eyes. The fourth affected individual (V:9) is a 32-year-old female diagnosed with KC of both eyes at the age of 18. Corneal transplant was performed for the both eyes. Blood samples were collected from three affected (V:1, V:5, V:9) and six unaffected members (IV:2, IV:3, V:2, V:3, V:6, V:7) for genetic studies.

Family D

Family D was recruited from Dheba. It is a five-generation autosomal recessive KC family with 13 members, there is a family history of atopy. In this family, 11 individuals are unaffected (I:1, I:2, II:1, II:2, II:3, II:4, III:1, III:2, IV:2, IV:2, IV:3, IV:5), and two individuals are

affected. One affected individual (IV:1) is a 34-year-old female with history of allergic conjunctivitis diagnosed with KC of both eyes at the age of 31. Corneal cross linking was performed for both eyes. The second affected individual (IV:4) is a 28-year-old male with history of allergic conjunctivitis diagnosed with KC of the left eye at the age of 27 and MyoRing insertion was performed. Blood samples were collected from the two affected (IV:1, IV:4) and five unaffected members (III:1, III:2, IV:2, IV:3, IV:5), including the parents of the affected for genetic analysis.

Family E

Family E was recruited from Almadinah Almunawwarah. It is a three-generation autosomal recessive KC family with 16 members and no history of any other genetic disease. This family consists of three affected individuals (II:1, III:2, III:5) and 13 normal individuals (I:1, I:2, I:3, I:4, II:2, II:3, II:4, III:1, III:3, III:4, III:6, III:7, III:8). Interestingly, all affected individuals are females. Affected individual II:1 is a 34-year-old female diagnosed with KC of both eyes at the age of 33. Corneal cross linking was performed for both eyes. The second affected (III:2) member is a 20-year-old female diagnosed with KC of both eyes at the age of 19; corneal transplant was performed for one eye, and MyoRing insertion was performed for the other eye. The third affected individual (III:5) is an 18-year-old female diagnosed with KC of both eyes at the age of 16, and MyoRing insertion was performed for both eyes. Blood samples were collected from three unaffected (II:2, II:4, III:1) and three affected members (II:1, III:2, III:5), including the parents of the affected for genetic study. In all of the families with affected and unaffected siblings described above, the parents were asymptomatic.

Sequence analysis of *VSX1* and *SOD1* genes

Primers flanking the coding exons of *VSX1* and *SOD1* were designed using Primer3 software. To identify the mutations responsible for the KC in the five Saudi families, the coding exons and splice-junctions of the two candidate genes (*VSX1* and *SOD1*) were screened by sequencing in two affected and one unaffected individual of each family. Sequence analysis of the exons and splice junctions of the two genes (http://www.ensembl.org/Homo_sapiens) failed to discover any potential sequence variant, which could have been responsible for the disease phenotype.

Discussion

Genetic studies in keratoconus

A review of the literature shows that multiple genomic approaches have been used to identify chromosomal loci and genes involved in KC. Linkage analysis is a powerful tool to map susceptible genetic loci and has been utilized at the genome-wide level in KC. With the advent of high density SNP arrays and whole-genome/exome sequencing using the next-generation sequencing technologies, it may now be possible to identify causal genetic variants in chromosomal regions exhibiting linkage in families of KC. Using linkage studies in various populations at least 17 different

chromosomal regions segregating with the KC phenotype have been identified, including 1p36.23-36.21,⁹ 2p24,¹⁰ 3p14-q13,¹¹ 5q14.3-q21.1,¹² 5q23.2,¹³ 5q32-q33,¹⁴ 8q13.1-q21.11,⁹ 13q32,¹⁵ 14q11.2,¹⁴ 14q11.2,¹³ 14q24.3,¹⁶ 15q2.32,¹⁴ 15q22.33-24.2,¹⁷ 16q22.3-q23.1,¹⁸ 17p13¹⁹ and 20q12.²⁰ Although 17 different loci have been reported, only a few genes have been identified including *VSX1* (visual system homeobox 1),^{21,22} *miR-184*^{17,23} and *DOCK9* (dedicator of cytokines 9).^{15,24} A potential contribution of *SOD1* (superoxide dismutase 1) in KC has also been documented.²⁵ More recently, linkage analysis identified a substitution in the *IL1RN* gene and a deletion in the *SLC4A11* gene that segregated with the KC phenotype in an Ecuadorian family.²⁶ Mutations in the *IL1RN* gene, a member of cytokine family and modulator inflammatory response, and *SLC4A11*, which encodes a membrane-bound sodium–borate co-transporter, have been associated with corneal endothelial dystrophy (CHED2) and Fuchs endothelial corneal dystrophy (FECD), respectively.²⁷

Genome-wide association studies (GWAS) in case–control cohorts provide a powerful platform to identify common risk variants in complex genetic diseases. These studies identify SNP(s) that are in linkage disequilibrium (LD) with relevant variants and require a huge number of population-based samples to achieve genome-wide significance ($p < 5 \times 10^{-8}$). GWAS has also been performed in KC cases, although in a relatively small number of patients. Li and colleagues recently conducted a GWAS study in KC from a Caucasian population of 222 patients and 3324 controls using Illumina 370k beadchips, containing 370,000 SNPs but failed to detect variants with genome-wide significance (p -value 5×10^{-8}).²⁸ They then selected a group of SNPs with p -value $< 10^{-4}$ and performed meta-analysis but again failed to identify any SNP with significant genome-wide association. The most significant association (p -value 1.6×10^{-7}) in this study was with SNP rs4954218, located near the *RAB3GAP1* (*RAB3* GTPase activating protein subunit 1 (catalytic)) gene on chromosome 2q21.3.²⁸ Interestingly, mutations in the *RAB3GAP1* gene are reportedly associated with Warburg Micro Syndrome, a rare autosomal recessive syndrome characterized by ocular and neurodevelopmental abnormalities, especially microphthalmos, microcornea, congenital cataracts and optic atrophy.^{29,30} In similar studies, Burdon and colleagues pooled the DNA of 97 Australian KC patients and 216 controls, performed GWAS and identified a significant association with one SNP (rs1014091) located upstream of the *HGF* (hepatocyte growth factor) gene.³¹ It is noteworthy that the *HGF* gene has been associated with refractive error in several populations including the Han Chinese and Caucasians.^{32,33} The association of *HGF* with KC suggests that inflammatory pathways may play a role in KC.

The KSA population is highly consanguineous, and many autosomal recessive disorders are prevalent in the population. Large Saudi families with multiple affected individuals having rare disorders are easy to locate. In these disorders, linkage analysis becomes a fruitful strategy to identify pathogenic genes responsible for disease phenotypes. Keratoconus families are rare worldwide; however, we have identified five Saudi KC families with multiple affected individuals from the Almadinah Almunawwarah region of

KSA. Although we did not find any mutations in the two KC genes sequenced, we aim to perform genome wide linkage analysis using dense SNP arrays to identify chromosomal regions linked with the disease in this Saudi cohort. Genes for sequencing in the candidate intervals will be prioritized using functional and expression data. Pathogenic variants in these genes will be screened in all isolated (sporadic) cases of KC. Identification of the underlying mutations in the KC-associated genes will help understand the pathobiology of KC. Moreover, it will also help in early diagnosis, disease management and genetic counseling of afflicted families. Thus, the patient samples we have collected will be very useful resources for future genetic studies on Saudi KC showing recessive inheritance.

Conclusion

Despite our inability to detect mutations in either the *VSX1* or *SOD1* genes, the samples collected from this Saudi cohort remain a valuable resources for further molecular studies. As functional sequence variants were not discovered in *VSX1* or *SOD1*, their involvement in causing KC in any of these five Saudi families is unlikely. However, we cannot exclude the possibility of the presence of functional sequence aberrations in the regulatory regions of these genes.

Recommendation

Screening additional candidate genes, performing SNP genotyping and locus identification and performing exome sequencing will be required to identify the genes underlying the autosomal recessive KC in the present five Saudi families.

Conflict of interest

All authors declare that they have no conflict of interest.

Authors' contribution

HSA and MAA recruited families and performed the clinical evaluation. EA, AMA and SB performed the DNA extraction, PCR, sequencing and data analysis. MIS provided funding for this project. SB drafted the manuscript. All authors have seen and agreed to the content of the manuscript.

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