Genetics and clinical characteristics of keratoconus

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SUMMARY

Keratoconus (KC) is a bilateral, non-inflammatory, and progredient corneal ectasia that mostly occurs as a sporadic disorder, but it has long been recognized that a significant minority of patients also exhibit a family history. In recent years several candidate genes such as *VSX1* and *SOD1* have been proposed, and some disease-causing mutations have been identified. Lately research has also focused on collagen genes, especially those that are differentially expressed in KC cornea. Alterations in *COL4A3* and *COL4A4* genes may be responsible for decreases in collagen types I and III, a feature often detected in KC. To investigate the role of all four genes in 113 Slovenian patients with sporadic or familial keratoconus, DNA extraction, polymerase chain reaction amplification, and sequencing of both genes were performed. No disease-causing mutations were found, but two previously identified single nucleotide polymorphisms were identified (A128A and 627+23G>A) in the *VSX1* gene. D326Y in *COL4A3* and M1237V and F1644F in *COL4A4* were also found to be significantly associated with KC patients. The absence of pathogenic mutations in *VSX1*, *SOD1*, *COL4A3*, and *COL4A4* genes in our large number of unrelated keratoconus patients indicates that other genetic factors are involved in the development of this disorder; nevertheless, a significant correlation of a few polymorphisms indicates that there could be a link between specific polymorphisms and KC disease.

KEY WORDS

keratoconus (KC), polymorphisms, genetic factors

Introduction

Keratoconus (KC) (Online Mendelian Inheritance in Man [OMIM] 148300) is a bilateral, non-inflammatory, and progredient corneal ectasia with an incidence of approximately 1 per 2,000 in the general population (1, 2). The most common presentation of KC is as a sporadic disorder, but it has long been recognized that a significant minority of patients exhibit a family history as an autosomal dominant mode of inheritance (1–3). Most investigators suggest complete penetrance of predisposing factors with variable phenotypic expression. An association with Down syndrome, monosomy X (Turner syndrome), Leber's congenital amaurosis, mitral valve prolapse, Bardet-Biedl syndrome, Ehlers-Danlos syndrome, ichthyosis, nail patella syndrome, neurocutaneous angiomatosis, neurofibromatosis, pseudoxanthoma elasticum, xeroderma pigmentosa, collagenosis, retinitis pigmentosa,

and Marfan syndrome is described (4, 5).

Six loci that are assumed to be responsible for KC have been mapped on locations 2p, 3p, 5q, 15q, 16q, and 20q; however, to date no mutations in any of genes located on these loci have been identified (6). In 2002 the *VSX1* homeobox gene, which 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 (7). Mutations in this gene have been identified in a few families with posterior polymorphous corneal dystrophy (PPCD) and keratoconus (7).

The new mutation in the *VSX1* gene was also described in abnormal craniofacial features, absence of the roof of the sella turcica, and anomalous development of the corneal endothelium (8). In 2005 Bisceglia et al. (9) evaluated the role of the *VSX1* gene in a series of 80 keratoconus-affected Italian subjects and found three previously described missense changes and a novel mutation. The authors concluded that the *VSX1* gene plays an important role in a significant proportion of patients affected by keratoconus inherited as an autosomal dominant trait with variable expressivity and incomplete penetrance. To determine whether keratoconus corneas have more mitochondrial (mt)DNA damage than do normal corneas, 33 normal corneas and 34 KC corneas were studied (10). It was shown that KC corneas exhibit more mtDNA damage than do normal corneas, suggesting that oxidative stress and altered integrity of mtDNA may be related to each other and may be important in KC pathogenesis.

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 (11). The same observation has been published for three other alterations (L159M, R166W, and H224R) in *VSX1* because investigation of 77 KC cases and 71 controls by Tang et al. could not confirm previously reported associations (12). Another alteration that was previously identified as a mutation (D144E) was excluded as a direct cause of the disease by Liskova et al. in 2007, and it was concluded that the lack of possibly pathogenic *VSX1* alterations in familial panels suggested that involvement of the *VSX1* gene in KC disease, if it exists at all, is confined to small number of pedigrees (13). Nevertheless, recent studies published in 2008 and 2009 revealed a few *VSX1* sequence variants that have been observed only in KC patient groups and as such are supportive of the pathogenic role of *VSX1* (14–16). Taken together, all the studies published to date indicate that the involvement of *VSX1* in KC is still not clear.

The next gene proposed as a possible candidate gene for keratoconus was the superoxide dismutase 1 gene (*SOD1*) on chromosome 21. A unique genomic deletion within intron 2 close to the 5' splice junction of the *SOD1* gene was identified in three patients with KC (17). Families with deletion carriers were subsequently genotyped with a set of 7 *SOD1* markers. As a result, intronic deletion in the *SOD1* gene was then proposed as being strongly associated with the KC phenotype in 2009 based on the dissimilarity of disease-associated alleles (18).

The major protein in the cornea is collagen, and several types of collagen have been identified by biochemical and immunochemical methods (19). Corneas from patients with KC contain reduced amounts of total collagen proteins (20) and alterations of the extracellular matrix and basement membrane characterized mostly by a decrease in types I and III (21). KC has not been associated with mutations in type VIII collagen genes (22), although a relation between *COL8A2* mutations and dystrophic corneal disorders has previously been reported (23, 24). Results from imunohistochemistry, in-situ hybridization, and expression arrays show that several other types of collagen are differentially expressed and have an active role in wound healing processes. Upregulation of collagen type XV and downregulation of collagen type IV (alpha 3 and 4) in KC corneas, observed by Bochert et al. and Stachs et al., showed the putative role of these types of collagen in KC (25, 26). *COL4A3* has already been implicated in the pathogenesis of polymorphous corneal dystrophy-3 (27, 28) and both genes are differentially expressed in keratoconus corneas (25, 26). Results from the study by Stachs et al. favored collagen type IV as a candidate gene in keratoconus pathogenesis (26).

This paper reports results of a mutational analysis of the *VSX1*, *SOD1*, *COL4A3*, and *COL4A4* genes in 113 unrelated Slovenian keratoconus patients.

Patients

This study included 113 patients (70 males and 43 females) after they provided informed consent and after determination of the diagnostic and other criteria. Thirty-seven participants with keratoconus belonged to families in which at least one other keratoconus-affected subject was present, whereas the other 76 patients were isolated cases. The patients were 20 to 67 years of age (39.06 ± 10.40 years; Table 1).

Symptoms are highly variable and, in part, depend on the stage of the progression of the disorder. Early in the disease there may be no symptoms, and KC may

Table 1. Characteristics of keratoconus patients studied in this research.

	Gender	Mean (years)	Standard deviation (years)	Standard error mean (years)
Age at diagnosis	Female	27.7	10.5	1.6
	Male	22.3	7.4	0.9
Age at the last visit	Female	41.5	12.2	1.8
	Male	38.3	9.0	1.1
Follow-up in years	Female	14.5	6.5	1.0
	Male	15.9	6.9	0.8

be noted by the ophthalmologist simply because the patient cannot be refracted to clear 20/20 corrected vision. In advanced disease there is significant distortion of vision accompanied by profound visual loss.

Only one patient had a congenital eye disease – amblyopia of the right eye – and she also had polycystic kidneys. Fifteen patients (13%) had high blood pressure, five patients (4.3%) rheumatic diseases, 13 patients (11.3%) allergic diseases, and 10 patients (8.7%) other illnesses. One of them, a 60-year-old male, had Ehlers-Danlos syndrome, which has been described as being connected with KC (1, 29, 30). Ehlers-Danlos syndrome (EDS) (Online Mendelian Inheritance in Man [OMIM] 130000), a heterogeneous group of inheritable connective tissue disorders, is attributed to mutations in connective tissue genes. Ehlers-Danlos syndrome is transmitted through autosomal dominant, autosomal recessive, or X-linked patterns of inheritance (31).

Keratometry was performed using a Humphrey Automatic Refractometer Keratometer model 599, and corneal topography with a Topographic Modeling System (TMS-1, Computed Anatomy, New York, NY) (20–22) and an Orbscan Version 3.0 Bauch & Lomb Surgical (32–34). The mean value of the steepest keratometry of both eyes was 51.8 ± 5.49 D. The cornea was examined with a slit-lamp. The presence of Vogt's striae, Fleischer rings, scars, the location of stromal thinning, and hydrops were noted.

Mutational search

DNA was extracted from peripheral blood by standard phenol-chloroform methodology and amplified in a final reaction volume of 10μL using 100 ng of genomic DNA, 10x PCR buffer with 15 mM $MgCl_2$, 200 μM each dNTPs, 0.10 μM primers, and 1.0 U-Taq DNA polymerase (Ampli*Taq* Gold; Applied Biosystems [ABI], Foster City, CA). PCR cycling conditions consisted of an initial denaturation step at 95 °C for 12 minutes followed by 35 cycles of 94 °C for 30 s, 58 °C (exon 1), 59 °C (exons 2, 4, 5), 62 °C (exon 3) for 30 s, 72 °C for 30 s, and ending with a final elongation step at 72 °C for 7 min. For PCR reactions of *COL4A3* and *COL4A4* exons we used the primers previously described by Heidet et al. (*COL4A3*), Boye et al. (*COL4A4*), and Štabuc-Šilih et al. (35–37).

Screening for changes in PCR products was performed with Single Stranded Conformation Analysis (SSCA) for each PCR fragment of a given set of samples from patients and healthy blood donors. 3.5 μl of PCR product was mixed with 10 μl of loading buffer (95% formamide, 20 mM EDTA, 0.05% bromphenol blue, 0.05% xylencianol, 20 mM NaOH); 4 μl of the denatured mixture was loaded onto a 10% polyacrylamide gel with 2.6% crosslinking and a 6% polyacrylamide gel containing 10% glycerol with 2.6% crosslinking. After the run was completed, samples were visualized by silver nitrate staining. Samples with different migration shifts were chosen for sequencing (37, 38).

DNA sequencing

Sequencing reactions were performed with BigDye Terminator Mix version 3.1 (Applied Biosystems [ABI], Foster City, CA). Samples were denatured at 96 °C for 2 min, then cycled 25 times at 96 °C for 10 s, 50 °C for 5 s, and 60 °C for 4 min. Unincorporated nucleotides were removed using the CleanSeq reagent and a SPRI plate (Agencourt Bioscience Corp., Beverly, MA) according to the manufacturer's instructions and were then analyzed on an ABI-3100 Genetic Analyzer (ABI) after resuspension in 0.1 mM EDTA. The nucleotide sequences listed below and provided by the National Centre for Biotechnology Information ([NCBI], Bethesda, MD) were used for comparison and numbering the alterations found in the genes studied: *VSX1* cDNA sequence (GenBank accession number NM_014588.4), *SOD1* cDNA sequence (GenBank ac-

cession number NM_000454.4), *COL4A3* cDNA sequence (GenBank accession number: NM_000091.3), and *COL4A4* cDNA sequence (GenBank accession number: NM_000092.4).

Statistics

Fisher's exact test (with Woolf's approximation) was used to evaluate statistical differences in polymorphism distribution between keratoconus patient groups and control groups for all the polymorphisms found. Odds ratios (OR) with 95% confidence intervals (95% CI) were calculated using SPSS version 14 software (SPSS Inc., Chicago, USA). A two-sided p value < 0.05 calculated between KC and control group for each polymorphism is considered statistically significant. The polymorphism frequencies found in patients and controls and their significances are summarized in Table 2 for *VSX1* and Table 3 for *COL4A3* and *COL4A4*.

Results

Clinical signs in KC differ widely depending on the severity of the disease. The following signs may be detectable by slit-lamp examination of the cornea: stromal thinning (centrally or paracentrally, most commonly inferiorly or inferotemporally); conical protrusion; an iron line partially or completely surrounding the cone (Fleischer's ring); and fine vertical lines in the deep stroma and Descemet's membrane that parallel the axis of the cone and disappear transiently on gentle digital pressure (Vogt's striae). Other accompanying signs might include epithelial nebulae, anterior stromal scars, enlarged corneal nerves, and increased intensity of the corneal endothelial reflex and subepithelial fibrillary lines. Munson's sign, another useful adjunctive external sign associated with KC, is a V-shaped conformation of the lower lid produced by the ecstatic cornea in downgaze (22–24). Thinning of the corneal stroma, breaks in Bowman's layer, and deposition of iron in the basal layers of the corneal epithelium comprise a triad of the classical histopathologic features found in KC. The epithelium may show degeneration of basal cells and breaks accompanied by epithelial downgrowth into Bowman's layer. Features noted in the stroma are compaction and loss of arrangement of fibrils in the anterior stroma, a reduction in the number of collagen lamellae, normal and degenerating fibroblasts in addition to keratocytes, and fine granular and microfibrillar material associated with the keratocytes. Descemet's membrane is rarely affected except for breaks seen in acute hydrops. The endothelium is usually normal (22, 25).

The entire *VSX1*, *SOD1*, *COL4A3*, and *COL4A4* gene-coding region and the exon–intron junctions were analyzed for mutations in a total of 113 unrelated Slovenian patients with keratoconus. No disease-causing mutation was identified in either of genes. Five previously described non-pathogenic changes in the *VSX1* gene (S6S, D144E, A128A, 504-24C>T, and 627+23G>A; see Table 2) and two previously described SNPs in the *SOD1* gene (V9I in one control, and D16H in one patient) were discovered. None of the polymorphisms found in *VSX1* and *SOD1* genes were statistically significant for KC patients (Fisher's exact test, $p > 0.05$). A pedigree tree of one family affected with KC that carried *VSX1* polymorphisms is shown in Figure 1. Although we detected both polymorphisms in all affected members of this family, the distribution of 627+23G>A and A128A (when compared between unrelated KC patients and the control population) was not significant (Table 2).

In the collagen genes studied we detected eight polymorphisms in the *COL4A3* gene, six of them substitutions (G43R, P141L, E162G, D326Y, H451R, and P574L), and six polymorphisms in the *COL4A4* gene, three of them substitutions (P482S, M1327V,

*Table 2. Frequencies of polymorphisms found in the **VSX1** gene in Slovenian patients with KC and healthy blood donors (control).*

VSX1	KC patients (n = 113)	Polymorphism *frequency*	Controls (n = 100)	Polymorphism frequency	p-value
S6S	21	0.186	15	0.15	0.5834
A128A	35	0.310	39	0.39	0.2498
D144E	1	0.009	1	0.01	1.000
504−24C>T	0	0.000	1	0.01	0.4695
627+23G>A	44	0.389	35	0.35	0.5726

Table 3. Allele frequencies and their significances in COL4A3 and COL4A4 polymorphisms between keratoconus patients and the control population.

Polymorphism	Allele	KC patients (n = 226)	Controls (n = 200)	p-value
COL4A3				
G43R	127G	216	192	
	127C	10	8	1.0000
P141L	422C	187	166	
	422T	39	34	1.0000
E162G	485A	189	167	
	485G	37	33	1.0000
D326Y	976G	216	119	
	976T	10	81	< 0.0001
H451R	1352A	210	186	
	1352G	16	14	1.0000
G484G	1452G	216	190	
	1452A	10	10	0.8220
P574L	1721C	128	106	
	1721T	98	94	0.4949
G895G	2685A	149	145	
	2685C	77	55	0.1723
COL4A4				
P482S	1444C	127	116	
	1444T	99	84	0.7687
G545A	1634G	218	192	
	1634C	8	8	0.8051
G789G	2367G	217	192	
	2367A	9	8	1.0000
M1327V	3979A	80	116	
	3979G	146	84	< 0.0001
V1516V	4548A	129	117	
	4548G	97	83	0.7693
F1644F	4932C	148	104	

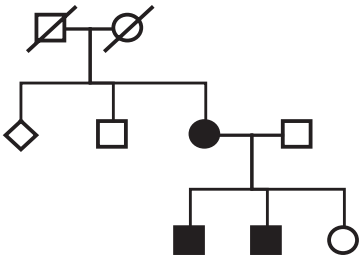

Figure 1. Pedigree tree of a family affected with keratoconus. The 69-year-old woman was diagnosed as having KC at age 28 (black circle). Her first-born son was diagnosed with KC at age 15 (black square) and his brother at age 14 (black square). Both sons began to wear contact lenses at the time of diagnosis; before diagnosis they wore glasses (the first beginning at age 12 and the second at age 3). The first-born son was the only one to have keratoplasty (on the left eye in 1985 and right eye in 2005). Their sister is not affected by KC (white circle). They have no (other) genetic diseases. We discovered 627+23G>A and A128A polymorphisms in all affected family members. The mother's parents were never diagnosed with KC (white crossed symbols); therefore we cannot exclude them as possibly affected.

and M1327V; see Table 3). Allele frequency of these three polymorphisms is significantly associated with KC patients. Allele frequency significant for KC was detected for D326Y (976G/T) in COL4A3, M1327V (3979AG), and F1644F (4932CT), both COL4A4. Odds ratios calculated between KC patients and controls for significant relations are as follows: 976G: OR = 14.703 (CI = 7.34–29.44); 3979A: OR = 0.3969 (CI = 0.27–0.59); and 4932C: OR = 1.751 (CI = 1.19–2.59) (Table 3).

Discussion

Symptoms in familial and sporadic KC are highly variable and depend in part on the stage of progression of the disorder. Early in the disease there may be no symptoms, and KC may be noted by the ophthalmologist simply because the patient cannot be refracted to a clear 20/20 corrected vision. In advanced disease there is significant distortion of vision accompanied by profound visual loss. Because clinical signs in KC differ widely depending on the severity of the disease and it is difficult to distinguish among familial and sporadic KC, genetic analysis of patients with some evidence of familial keratoconus would be beneficial.

Two candidate genes, *VSX1* and *SOD1*, have been proposed and some disease-causing mutations have

been identified (7–9, 17). In two studies the *VSX1* gene mutations assumed to cause KC have already been reported. Heon et al. identified four patients with *VSX1* mutations (7); two (L159M and R166W) were not identified in control subjects and were therefore considered pathogenic, whereas the other two mutations (D144E and H244R) were also identified in subjects without keratoconus and thus were considered only possibly pathogenic. Bisceglia et al. (9) also identified four missense mutations in 7 of 80 patients with keratoconus: one novel L17P and three already described. From these two investigations, four presumably pathogenic mutations in the *VSX1* gene emerged (L17P, D144E, L159M, and R166T). We identified two of these previously reported variations (A128A polymorphism and an intronic change 627+23G>A) in several KC patients and healthy family members, but failed to prove relations between mutations in either of the genes and KC (38). Our results are in accordance with an increasing number of studies in which attempts to associate KC with *VSX1* mutations failed (6, 11–13, 16). On the other hand, our mutational screening of two type IV collagen genes in KC patients is the first according to our knowledge (37). Mutational analysis did not reveal any mutations present in the DNA of KC patients, and most of the polymorphisms found were not significant for the patient group. In the KC cohort we did discover significant representation of following genotypes: 422CC, 422TT, 422CT, and 2685CC in *COL4A3* and 1444TT, 4548AA, and 4548AG in *COL4A4* (37), but their relevance to KC disease needs to be determined. Allele distribution of three polymorphisms already described in previous studies related to Alport syndrome (D326Y (35) in *COL4A3* and M1327V (36, 39) and F1644F (39) in *COL4A4* were significantly differently represented in the KC patient cohort than in the healthy population (37), but we cannot speculate that these polymorphisms in any way alter the collagen assembly or promote KC disease.

The absence of pathogenic mutations of *VSX1* and *SOD1* genes in our large number of unrelated keratoconus patients indicates that other genetic factors are involved in the pathogenesis of this corneal ectatic disorder. Moreover, we can also conclude that mutations in collagen type IV (*COL4A3* and *COL4A4* genes) are not involved in lowering the amount of total collagen in KC, specifically collagen types I and III, nor in promoting the induction or development of KC disease, because no mutations were found in all of the screened KC patients. Our study excluded *VSX1*, *SOD1*, *COL4A3*, and *COL4A4* genes from playing a significant role in KC pathogenesis and we believe the genes that cause KC have yet to be identified. Recent genome-wide linkage analysis based on 18 families with KC provided evidence for a novel gene located on 13q32 that is responsible for KC, but the gene itself is still unknown (6). When considering gene expression in keratoconic corneas, a recent study based on cDNA microarrays identified eight novel genes (*BMP4*, *MRVI1*, *ACTA2*, *GRCC10*, *TIMP3*, *TIMP1*, and *SSTR1*) to be differentially expressed (40). Most of those genes have been associated with apoptosis, wound healing, cell division, and growth control, and have the potential to be involved in stromal thinning (40). Two other studies based on detection of genetic alterations instead of *VSX1* or *SOD1* proposed *IL1B* and *CRB1* as genes involved in KC (41, 42). Considering these studies (6, 40–42), the search for the novel genes involved in the development of KC is already in progress.

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