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Corneal perforation following corneal cross-linking in keratoconus associated with potentially pathogenic *ZNF469* mutations

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Abstract

Purpose: To report a case of bilateral and repetitive corneal perforations following corneal cross-linking (CXL) for keratoconus in a woman harboring potentially pathogenic variants in the *ZNF469* gene, and to characterize the keratoconus phenotype in this woman and her daughter who shared the same *ZNF469* mutations.

Methods: Clinical characterization of the proband and her daughter followed by sequencing of the genes associated with brittle cornea syndrome, *ZNF469* and *PRDM5*, in both individuals.

Results: An Ashkenazi Jewish woman in her sixth decade presented with diffuse corneal thinning and progressive steepening consistent with keratoconus. Following CXL, epithelium-off in the first eye and epithelium-on in the second, she developed spontaneous corneal perforations in each eye. Her daughter in her fourth decade demonstrated a similar pattern of diffuse corneal thinning and progressive corneal steepening but did not undergo CXL and did not develop corneal perforation. Screening of the *ZNF469* and *PRDM5* genes revealed three missense *ZNF469* variants (c.2035G>A, c.10244G>C, and c.11119A>G) *in cis* arrangement on one allele of *ZNF469* in both the proband and her daughter. Although the three variants share low (< 0.01) global minor allele frequencies (MAF), each has significantly higher MAFs (0.01 ~0.03) in the Ashkenazi Jewish population, leading to uncertainty regarding a pathogenic role for the identified variants.

Conclusion: Corneal cross-linking may be associated with the development of corneal perforation in particular at-risk individuals with keratoconus. Identifying clinical and genetic risk factors, including screening of *ZNF469* and *PRDM5*, may be useful in the prevention of significant complications following CXL.

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Keywords

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INTRODUCTION

The corneal ectasias are progressive non-inflammatory corneal thinning disorders in which compromised stromal collagen matrix leads to the loss of corneal biomechanical integrity and subsequent corneal protrusion. Common examples of corneal ectasia include keratoconus including posterior keratoconus, pellucid marginal degeneration, and post-LASIK ectasia.[1] Rare conditions classified as corneal ectasia include keratoglobus, posterior polymorphous corneal dystrophy,[2] brittle cornea syndrome,[3] and Ehler-Danlos syndrome type VI.[4]

Corneal cross-linking (CXL) is a photopolymerization technique to increase the biomechanical strength of the cornea.[5, 6] The main indication for CXL is to retard the progression of keratoconus,[7] while CXL has also been used prophylactically or as treatment for iatrogenic ectasia after LASIK,[8–10] PRK,[8] and radial keratotomy.[11, 12] CXL can be performed using the standard Dresden protocol after epithelium debridement (epi-off CXL)[13] or via a trans-epithelial approach (epi-on CXL).[14] The therapeutic CXL procedure is considered relatively safe given currently available data with complication rates between 1–10%.[15] While the most common complication of epi-off CXL is delayed re-epithelialization resulting in corneal haze, more significant complications have been observed, including perforation.[15, 16] Perforation has been reported in only nine eyes following CXL: three cases of microbial keratitis after CXL for keratoconus[17, 18]; and six cases of sterile keratolysis: both eyes of an individual with Down syndrome in which baseline thickness was less than 400 μm; two cases with an acute inflammatory response after CXL; and two cases without significant inflammation following CXL.[19–23]

We report a case of a woman in her sixth decade with progressive keratoconus who developed recurrent and spontaneous corneal perforations involving both eyes after epi-off CXL in right eye and epi-on CXL in left eye. Extensive phenotypic and genotypic characterization of the proband and her affected daughter were performed to identify risk factors that may be used to identify individuals at increased risk of corneal perforation following CXL.

MATERIALS AND METHODS

The authors followed the tenets of the Declaration of Helsinki in the treatment of the subjects reported herein. This study was approved by the Institutional Review Board at The University of California, Los Angeles (UCLA IRB # 11–000020) and was performed after obtaining informed, written consent from the proband, her daughter and her daughter's father.

Clinical Evaluation

Serial slit lamp biomicroscopic and corneal tomographic (Oculus Pentacam®, Wetzlar, Germany) evaluations of the proband and her daughter were performed to asses for abnormalities of corneal curvature, clarity and thickness and to detect progression. Central corneal thickness was measured in each eye, while paracentral and peripheral thickness measurements were obtained as the mean value of the corneal thickness measured at radii of 4mm and 8mm from the center, respectively.

Sanger Sequencing of *ZNF469* and *PRDM5*

Saliva samples were collected using the Oragene saliva collection kits (DNA Genotek, Inc., Ontario, Canada) from the proband, her affected daughter and her daughter's father. Genomic DNA was extracted from saliva samples using Oragene Purifier (OG-L2P, DNA Genotek, Inc.) and purified with QIAamp DNA micro kit (#56304, Qiagen, Valencia, CA) according to the manufacturer's instructions. PCR amplification of the 2 exons of *ZNF469* and 16 exons of *PRDM5* was performed using previously described primers.[24, 25] PCR amplification reactions were performed using the KAPA2G Robust PCR Kit (KAPA Biosystems, Wilmington, MA) with annealing temperature of 65 °C (*ZNF469* primers) and 63 °C (*PRDM5* primers).[24, 25] Purified PCR products were purified using 0.5 U of Shrimp Alkaline Phosphatase and 5 U of Exonuclease I (USB Corp., Cleveland, OH) and underwent Sanger sequencing (Laragen, Inc., Culver City, CA). The resulting sequencing reads were compared to the wild-type *ZNF469* transcript (NM_001127464.2) and *PRDM5* transcript (NM_018699.3). Minor allele frequencies (MAF) were obtained for each identified variant from Trans-Omics for Precision Medicine (TOPMed) Program, Exome Aggregation Consortium (ExAC) database, 1000 Genomes Project and Genome Aggregation Database (gnomeAD). A variant was determined to be rare if the TOPMed MAF was ≤ 0.01.

In silico analysis of identified rare variants

In silico analysis of identified rare coding region variants for their potential effect on ZNF469 protein function was performed using PROVEAN, SIFT and PolyPhen-2.[26–28] The Conserved Domain Database from the National Center for Biotechnology Information (NCBI) was used to identify the location of the putative Zinc-finger motifs on the ZNF469 protein and Consurf was used to calculate the evolutionary conservation of the ZNF469 amino acid sequence.[29]

RESULTS

Clinical Features

The proband, a woman of Ashkenazi Jewish descent, had a history of rapid myopia progression in her teens and then relatively stable vision with mild astigmatism until her 50s. Her vision was corrected with spectacles and rigid gas permeable (RGP) contact lenses for cosmetic reason in her teens and twenties, and then with glasses and soft contact lenses for the next 30 years. In her 50s, she developed increasing topographic astigmatism in the right eye, which was not fully correctable with glasses. She was diagnosed with keratoconus versus pellucid marginal degeneration at age 58 and when the topographical astigmatism in

the right eye reached 6 D, she was referred for evaluation as a candidate for CXL. At that time, corrected distance visual acuities (CDVA) were 20/80 in the right eye (−2.50 −8.00 × 28°) and 20/20 in left eye (−4.25 −5.00 × 130°). Corneal tomographic imaging in the right eye demonstrated significant inferotemporal steepening with a high oblique astigmatism (40.80D/49.00 D × 46.8°, 8.2 D) and diffuse stromal thinning with centrally located minimum thickness of 404 μm (Fig. 1). Corneal tomographic imaging in the left eye demonstrated mild diffuse steepening with low against-the-rule astigmatism (45.50D/46.30 D × 82.5°, 0.8 D) and diffuse stromal thinning with a centrally located minimum thickness of 399 μm (Fig. 1). Based on the corneal tomographic findings, the patient was diagnosed with keratoconus. Neither corneal topographic or tomographic imaging performed prior to referral for CXL were available to assess for progression.

At age 59, the patient underwent epi-off CXL for progressive keratoconus in the right eye. One month and four months after the CXL procedure, spontaneous micro-perforations developed in the right cornea. Slit-lamp examination after the second perforation demonstrated a diffusely thin cornea with a temporal paracentral perforation (Fig. 2A, B). Both perforations were managed conservatively with bandage contact lens placement and aqueous suppression. One year after the second perforation, the vision in the right eye was correctable to 20/60 (−9.00 −6.00 D × 10°) on refraction and to 20/30 with a KeraSoft® IC lens.

The following year, the patient noted worsening of vision secondary to progressive myopia and astigmatism in the left eye. The topographic astigmatism had progressed to 4.7 D although the CDVA and refractive astigmatism remained relatively stable (20/25 with −4.50 −5.50 D × 136°). Given the history of perforation following epi-off CXL for the right eye, epi-on CXL was performed for the left eye. Eight days after the epi-on CXL, a spontaneous corneal perforation developed in the left eye, which was managed with sutures, amniotic membrane transplantation, and fibrin sealant (Fig. 2C). Seven weeks after CXL, following suture removal in left eye, a leak developed in the area of the initial perforation, which was managed conservatively. Nearly two years after the CXL was performed for the left eye, a spontaneous cornea perforation developed in the right eye that required suturing and amniotic membrane transplantation for closure (Fig. 2D). After recovery, the right eye was fit with a BostonSight® PROSE device, providing 20/40 CDVA. Review of systems and continued follow-up of the proband reveal no history or evidence of immunologic or rheumatologic conditions.

Review of the patient's 37-year-old daughter's medical records revealed high myopia with soft contact lens CDVA of 20/25 in both eyes (−9.50 −2.00 × 90° OD, −10.75 sphere OS). Corneal tomographic imaging demonstrated inferior paracentral steepening with low astigmatism in both eyes (OD 48.50D/48.90D × 94.0°, 0.4D, K_{max} 50.0D; OS 48.80D/49.20D × 121.5°, 0.4D, K_{max} 50.3D) (Fig. 3). Both corneas demonstrated diffuse thinning with centrally located minimum thicknesses of 405 μm (OD) and 410 μm (OS). Serial corneal tomographic imaging performed over the course of five years revealed no progression in the K_{max} or topographic astigmatism, although evidence of pachymetric progression was observed in both eyes.

Although the global corneal thinning extended to the limbus in the proband and her daughter, the corneal thickness profiles were within the 95% (± 2 SD) interval around the mean reported for keratoconic corneas at central, paracentral (4 mm) and peripheral (8 mm) locations (with the exception of the peripheral corneal thickness in the proband) with maximal thinning at the apex in both the proband and her daughter (Table 1).[30, 31] Based on this, both the proband and her daughter were diagnosed with keratoconus as opposed to keratoglobus.

Genetic Analysis

The repetitive spontaneous perforations after CXL in both eyes of the proband and the shared diffuse cornea steepening and thinning observed in the proband and her daughter led us to suspect an underlying hereditary connective tissue disorder. We excluded the diagnosis of Ehler-Danlos syndrome type VI (EDSVIB) since neither the proband nor her daughter manifested extraocular features of a connective tissue disorder. Based on the corneal phenotype and clinical course, we suspected an ocular only phenotype of brittle cornea syndrome (BCS).[32, 33] Therefore, we screened the coding regions of the BCS-associated genes, PR domain-containing protein 5 (*PRDM5)* and zinc finger transcriptional factor protein 469 (*ZNF469*), in the proband and her daughter.[24, 25]

Screening of *PRDM5* in the proband did not reveal any rare or novel variants (Table 2). Screening of *ZNF469* in the proband identified three heterozygous, rare, non-synonymous single nucleotide variants (SNVs) (c.2035G>A, c.10244G>C, and c.11119A>G), all with a MAF ≤ 0.01 (Table 2). Screening of *ZNF469* in the proband's affected daughter and the unaffected biological father of the daughter identified the same three variants in the heterozygous state in the daughter but not in the daughter's father (Table 2). Three common *ZNF469* single nucleotide polymorphism (SNPs) that were present in the heterozygous state in proband's daughter (c.7072G>C, c.8009T>A, c.10888G>C) were present in the homozygous state in her father but absent in the proband, indicating that they were on the opposite allele from the three rare SNVs (c.2035G>A, c.10244G>C, and c.11119A>G) and the three rare SNVs were on the same allele transmitted from the proband to her daughter (Table 2). The presence of all three potentially pathogenic rare SNVs on the same allele effectively excludes the diagnosis of autosomal recessive brittle cornea syndrome.

As the proband is of Ashkenazi Jewish decent, we further determined the MAFs of the three rare *ZNF469* SNVs within the Ashkenazi Jewish population (Supplemental Table 1). In the gnomAD database, the MAFs of c.2035G>A, c.10244G>C, and c.11119A>G in the Ashkenazi Jewish population were 0.0320, 0.0167, and 0.0265 respectively, significantly higher than the global MAFs (0.001, 0.001 and 0.0003) and failed to meet the selected MAF for rare variants (MAF ≤ 0.01) (Supplemental Table 1).

To predict the potential impact of the three missense variants on ZNF469 protein function, *in silico* analysis of the resulting amino acid substitutions was performed (Supplemental Table 2). Located within the first proline-rich domain of the ZNF469 protein (Supplemental Fig. 1), c.2035G>A (p.(Glu679Lys)) leads to the replacement of the conserved acidic glutamic acid (Consurf conservation score 7) with a basic lysine and is predicted to be "Damaging" to ZNF469 protein function by SIFT. The p.(Gly3415Ala) change secondary to c.10244G>C

variant is located in the last of 7 Zinc-finger binding domains and is predicted to be "Possibly Damaging" by PolyPhen-2. None of the programs predicted the polar to nonpolar amino acid substitution p.(Ser3707Gly) resulting from c.11119A>G to be damaging.

DISCUSSION

We here report a case of repetitive spontaneous corneal perforations after CXL in a patient with atypical keratoconus to increase the awareness of this potential complication among corneal specialists. Careful review of the patient and her affected daughter's clinical and imaging records revealed several atypical features for keratoconus that might be associated with progressive corneal thinning following CXL: stable refraction during early adulthood, in contrast to the typical progression of keratoconus; topographic evidence of progressive corneal steepening noted in the sixth decade of life, resulting in a late diagnosis of keratoconus; and diffuse stromal thinning, rather than being localized to the area of corneal ectasia. In addition, progression of steepening was observed after CXL, in contrast to the reports of minimal corneal topographical changes after CXL in multiple clinical trials.[34]

Genetic analysis of the proband and the daughter revealed three potentially pathogenic heterozygous *ZNF469* SNVs *in cis*, indicative of a potential underlying genetic predisposition to progressive corneal thinning and perforation after CXL. Encoding a protein that is involved in corneal extracellular matrix development and maintenance[25], *ZNF496* is reported as the gene most strongly associated with central corneal thickness (CCT) in multiple genome-wide association studies (GWAS) that include various ethnic populations.[35–37] While homozygous frameshift or truncating *ZNF469* mutations are associated with BCS,[4, 24, 33] heterozygous missense mutations are associated with a keratoconic phenotype, as seen in the proband and her daughter.[35, 38, 39] Therefore, given the association of *ZNF496* with decreased central corneal thickness, BCS and keratoconus, it is possible that one, two or all of the three heterozygous *ZNF469* SNVs identified in the proband and her daughter are causative of the atypical keratoconic presentation in both and corneal perforation following CXL in the proband. Evidence for the pathogenicity of the three identified SNVs include: each is associated with a MAF < 0.01; *in silico* analysis predicted c.2035G>A to be "Damaging" and c.10244G>C to be "Possibly Damaging"; c.2035G>A was identified in the heterozygous state in two affected siblings with keratoconus;[38] a variant involving the same nucleotide as c.10244G>C SNV (c.10244G>T) was identified in a family and two sporadic cases with keratoconus; and c.11119A>G was submitted by Illumina Clinical Services Laboratory to NCBI with a clinical diagnosis of "corneal fragility keratoglobus, blue sclerae and joint hypermobility (BCS1)".[40] However, evidence against the pathogenicity of the three identified SNVs includes: the fact that the MAF of each in the Ashkenazi Jewish population was significantly higher than the global MAFs; the c.2035G>A was associated with a MAF ≥ 0.001 in ethnically-matched controls[38]; and the c.10244G>T variant was associated with a MAF of 0.1304 in ethnically-matched controls.[40]

Keratoconus has been reported to occur in a higher percentage of the Jewish population than in other populations,[41] although the prevalence in the Ashkenazi Jewish population has not been reported. If this prevalence were known, it would facilitate our judgement in assigning significance to the three identified SNVs. While we observed a positive association between

keratoconus prevalence and the MAFs of each of the three *ZNF469* variants in specific ethnic groups, including Jewish, Middle Eastern and South Asian populations,[42–45] the MAFs were above the threshold for rare variants (MAF ≤ 0.01) (supplemental table 1), decreasing the likely pathogenicity of these variants in regards to keratoconus. However, if the identified SNVs are in fact associated with keratoconus, the higher MAFs of the three variants in these ethnic groups would explain the observed multiple-fold higher prevalence of keratoconus in these ethnic groups.[41, 45]

Low frequency SNVs with MAFs between 0.01 – 0.05 can be pathogenic in the setting of a complex disease such as keratoconus and a quantitative trait such as CCT. There is an inverse relationship between a variant's "regression effect size" (the strength of the relationship between pathogenic variant and disease's quantitative trait phenotype on a numeric scale) and its frequency in the population.[46] Trait inheritability is contributed by common variants (MAF > 0.05) of very weak effect and low frequency (MAF 0.01 – 0.05) and rare variants (MAF < 0.01) of small to modest effect, or a combination of both.[47] Isolated populations are particularly helpful in the identification of low-frequency disease-causing variant(s) affecting complex traits, because the founding event and genetic drift can cause an increase in allele frequency that can significantly increase the power to detect an association. The prevalence of keratoconus in Jewish (2.2%, CI 1.2 – 3.3% in Israeli Jewish[41]) and Maori/Polynesian[45, 48] (26.9%, CI 21.0 – 33.7% of Maori/Polynesian teenagers are topographically keratoconus suspect[45]) populations is multiple-fold higher than in non-isolated populations (e.g. 0.054% in Minnesota, USA[49]). These two populations would be very helpful in deciphering the role of *ZNF469* SNVs in keratoconus if one could document keratoconus phenotype as a quantitative trait, so statistical regression models could be applied to estimate the effect size of candidate SNVs on keratoconus phenotype.

In summary, we report a patient with a delayed onset of corneal ectasia diagnosed as keratoconus who developed spontaneous bilateral corneal perforation after CXL and was subsequently found to have thee potentially pathogenic heterozygous *ZNF496* missense variants on the same allele, all of which have been previously implicated in the pathogenesis of corneal ectasia.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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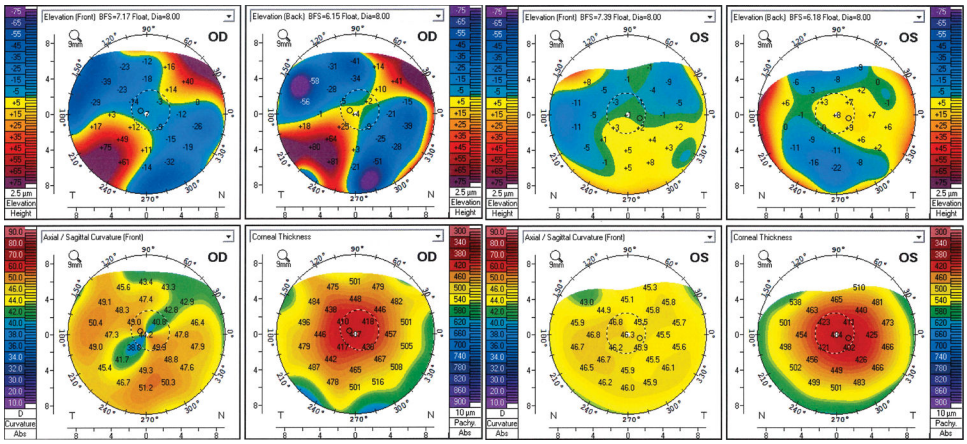

Figure 1:

Corneal tomographic imaging of the proband diagnosed with late-onset keratoconus demonstrating significant inferotemporal steepening and high oblique astigmatism in the right eye with diffuse stromal thinning and centrally located minimum thickness of approximately 400 μm in each eye.

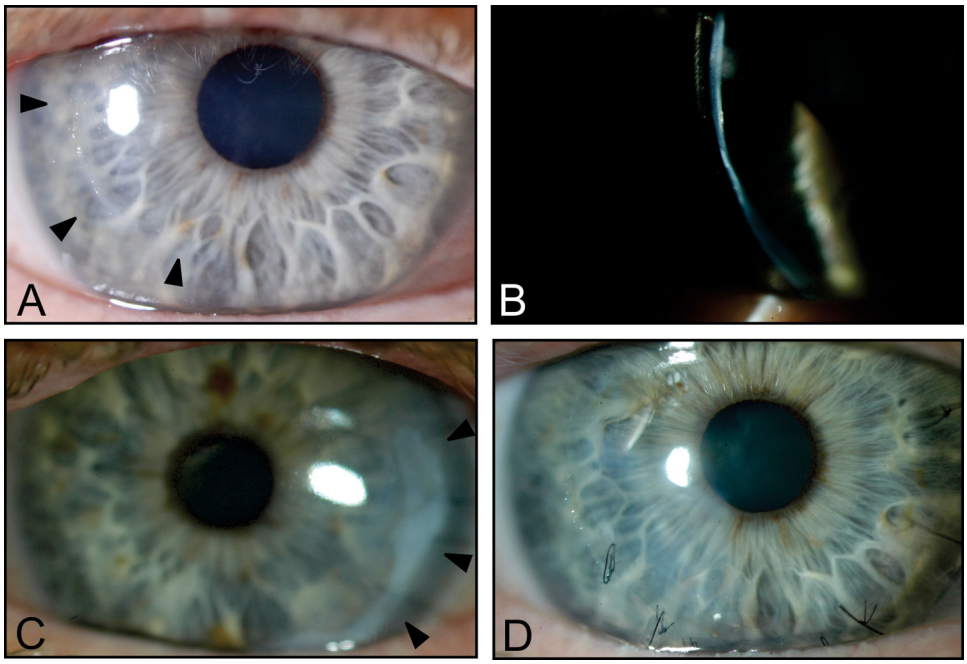

Figure 2:

Slit-lamp photos of the proband's right cornea demonstrating the location of prior temporal paracentral perforations (arrowheads) (A) and diffuse thinning (B). Images obtained 4 months after epi-off CXL. Slit-lamp photos of the proband's left cornea demonstrating the location of prior temporal paracentral spontaneous perforation that developed 8 days after epi-on CXL (arrowheads) (C). Three months after the third spontaneous perforation in the right cornea, which occurred 3 years after CXL, sutures are present in the location of the corneal perforation (D).

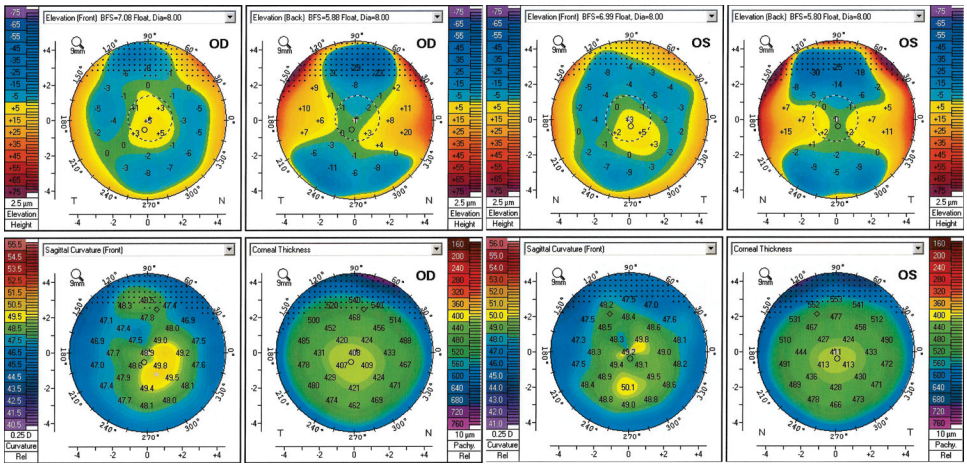

Figure 3:

Corneal tomographic imaging of the proband's daughter demonstrating mild inferior paracentral steepening with low astigmatism as well as diffuse thinning with centrally located minimum thicknesses of approximately 400 μm in both eyes.

Table 1.

Corneal thickness profiles of proband and her daughter compared to reported keractoconic cohort.

Corneal thickness (μm)	**Proband (OD / OS)**	**Proband's daughter (OD / OS)**	**Keractoconus cohort[30, 31] (mean ± SD)**
Central	404 / 399	405 / 410	428.0 ± 72.0
Paracentral (4 mm)	452 / 447	454 / 467	536.5 ± 48.3
Peripheral (8 mm)	540 / -	605 / -	695.6 ± 56.4

"-" indicates corneal thickness cannot be obtained.

Table 2.

Identified variants in proband, affected daughter and daughter's unaffected father

		Proband (Affected Mother)	Affected Daughter	Unaffected Biological Father of the Daughter	dbSNPs #	TOPMed MAF	Consequence Type
ZNF469 (NM_001127464.2)	exon1	**het.c.2035G>A**	**het.c.2035G>A**	**no variant**	**rs551591362**	**0.001**	**missense**
	exon2	het.c.3432T>C	het.c.3432T>C,	-	rs111557381	0.063	synonymous
		no variant	*het.c.7072G>C*	*homo.c.7072G>C*	rs12598474	0.229	synonymous
		no variant	*het.c.8009T>A*	*homo.c.8009T>A*	rs3812956	0.308	missense
		homo.c.8520C>T	het.c.8520C>T	-	rs3812953	0.432	synonymous
		het.c.10244G>C	**het.c.10244G>C**	**no variant**	**rs140056980**	**0.001**	**missense**
		no variant	*het.c.10888G>C*	*homo.c.10888G>C*	rs1105066	0.460	missense
		het.c.11119A>G	**het.c.11119A>G**	**no variant**	**rs536054902**	**0.0003**	**missense**
PRDM5 (NM_018699.3)	exon6	no variant	het.c.681A>G	-	rs343192	0.279	synonymous
	intron8	(intron)het.c.946–60G>C	(intron)het.c.946–60G>C	(intron)het.c.946–60G>C	rs4833677	0.225	intron variant
		(intron)het.c.946–41G>C	(intron)het.c.946–41G>C	(intron)het.c.946–41G>C	rs4833676	0.225	intron variant
	intron9	(intron)het.c.1030+35C>A	(intron)het.c.1030+35C>A	no variant	rs1511310	0.151	intron variant
		(intron)het.c.1030+45A>G	(intron)het.c.1030+45A>G	(intron)het.c.1030+45A>G	rs1511309	0.278	intron variant
	exon10	het.c.1066T>A	het.c.1066T>A	-	rs140634372	0.018	missense
	exon11	het.c.1234T>C	het.c.1234T>C	-	rs12499000	0.170	synonymous

Identified rare variants (TOPMed < 0.01) shared between proband and the daughter are **bolded**. Common SNPs seen only in daughter and father are *underlined and italicized*. The cells labelled "-" indicate regions not screened.