Brittle Cornea Syndrome ZNF469 Mutation Carrier Phenotype and Segregation Analysis of Rare ZNF469 Variants in Familial Keratoconus

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Citation: Davidson AE, Borasio E, Liskova P, et al. Brittle cornea syndrome 1 (BCS1) is a rare recessive condition characterized by extreme thinning of the cornea and sclera, caused by mutations in ZNF469. Keratoconus is a relatively common disease characterized by progressive thinning and ectasia of the cornea. The etiology of keratoconus is complex and not yet understood, but rare ZNF469 variants have recently been associated with disease. We investigated the phenotype of BCS1 carriers with known pathogenic ZNF469 mutations, and recruited families in which aggregation of keratoconus was observed to establish if rare variants in ZNF469 segregated with disease.

METHODS. Patients and family members were recruited and underwent comprehensive anterior segment examination, including corneal topography. Blood samples were donated and genomic DNA was extracted. The coding sequence and splice sites of ZNF469 were PCR amplified and Sanger sequenced.

RESULTS. Four carriers of three BCS1-associated ZNF469 loss-of-function mutations (p.Glu1392Ter, p.Gln1930Argfs*6, p.Gln1930fs*133) were examined and none had keratoconus. One carrier had partially penetrant features of BCS1, including joint hypermobility. ZNF469 sequencing in 11 keratoconus families identified 9 rare (minor allele frequency [MAF] ≤ 0.025) variants predicted to be potentially damaging. However, in each instance the rare variant(s) identified, including two previously reported as potentially keratoconus-associated, did not segregate with the disease.

CONCLUSIONS. The presence of heterozygous loss-of-function alleles in the ZNF469 gene did not cause keratoconus in the individuals examined. None of the rare nonsynonymous ZNF469 variants identified in the familial cohort conferred a high risk of keratoconus; therefore, genetic variants contributing to disease pathogenesis in these 11 families remain to be identified.

Keywords: keratoconus, ZNF469, brittle cornea syndrome, familial keratoconus

Keratoconus (OMIM #148300) is characterized by progressive corneal thinning and ectasia, leading to irregular astigmatism, impairment of visual function, and reduced quality of life.1 It is a relatively common condition, affecting approximately 1 individual per 1000,2 although it is markedly more frequent in some ethnic groups. It typically presents in the teens or early 20s with progression until the fourth decade. Keratoconus is the single most common reason for corneal transplantation in the developed world, but the etiology and pathophysiology of this complex disease is not understood.3 Although the majority of patients with keratoconus have sporadic disease, familial clustering and a high concordance among monozygotic twins imply that genetic factors are likely to contribute to disease pathogenesis.4,5 Identification of the underlying genetic risk factors that contribute to the development of keratoconus presents an opportunity to understand the underlying disease processes. Different approaches have been used to identify risk loci and genes for keratoconus, including linkage analysis and candidate gene screening.3 Genome-wide association studies (GWAS) have identified common variants associated with keratoconus,6–9 but the actual causal variants tagged by GWAS hits and the associated underlying molecular mechanisms remain unknown. As for nearly all complex traits, GWAS associated variants explain only a limited fraction of the disease heritability. Additional factors, including rare variants with stronger effect and not tagged by GWAS SNPs, could plausibly account for some of the unexplained heritability. While the lack of robust linkage data suggests that highly penetrant variants are rare in keratoconus families, variants that increase disease
risk by approximately 4-fold are likely to generate inconsistent evidence for linkage and, therefore, may have been missed.\textsuperscript{10,11} There also has been much interest in defining risk loci associated with the highly heritable quantitative trait central corneal thickness (CCT),\textsuperscript{1,3} because reduced CCT is a risk factor for primary open-angle glaucoma and myopia, and axial myopia and progressive corneal thinning are features of keratoconus.\textsuperscript{9,13} Single nucleotide polymorphisms (SNPs) located in an intergenic region upstream of ZNF469 have been associated with CCT by numerous GWAS in different populations.\textsuperscript{9,14–18} Importantly, a recent genome-wide meta-analysis for CCT identified the major (A) allele of rs9938149 (allele frequency = 0.75; 1000Genomes phase I genotyping data), which is located approximately 162 kb upstream of ZNF469, to be associated significantly with increased CCT and keratoconus.\textsuperscript{9} This association with keratoconus was validated in an independent case control study.\textsuperscript{19} This finding is somewhat counterintuitive, as one might expect that an allele associated with keratoconus would more likely be associated with reduced, instead of increased, CCT.\textsuperscript{9}

The association of a SNP upstream of ZNF469 with risk of keratoconus is of potential biologically relevance, since biallelic nonsynonymous mutations in ZNF469 cause autosomal recessive Brittle Cornea Syndrome (BCS) type 1.\textsuperscript{20} BCS1 (OMIM #229200) is a connective tissue disorder characterized by extreme thinning and fragility of the cornea, joint hypermobility and hyperelasticity of the skin, often leading to corneal rupture after minor trauma.\textsuperscript{20} Brittle Cornea Syndrome type 2 (OMIM #614170) is caused by biallelic mutations in PRDM5. Ocular and systemic phenotypes have been observed in some heterozygote PRDM5 carriers, who have blue sclera and small joint hypermobility as well as mildly reduced CCT (range, 480–505 μm, \(n = 6\)).\textsuperscript{21} Interestingly, one of these six individuals also had keratoconus.\textsuperscript{21} It has not yet been determined if heterozygous carriers of BCS1-associated ZNF469 mutations display a carrier phenotype, such as reduced CCT or keratoconus, although blue sclera was documented previously in one of three molecularly confirmed carriers.\textsuperscript{22}

Given the association of rs9938149 (A) with keratoconus, and that biallelic mutations in ZNF469 cause BCS1, it is possible that rare coding and/or splice site variants in ZNF469 could contribute to the development of keratoconus.\textsuperscript{23–25} A recent study of ZNF469 in a cohort of unrelated Europeans with keratoconus reported an enrichment of potentially pathogenic rare (minor allele frequency [MAF] < 0.001) ZNF469 alleles in 12.5% cases (\(n = 112\)),\textsuperscript{26} while another study reported an occurrence of rare (MAF < 0.01) ZNF469 missense variants at a frequency of 23% in a Polynesian and white New Zealand keratoconus cohort (\(n = 43\)).\textsuperscript{27}

To evaluate this hypothesis further we recruited a cohort of patients in which there was familial clustering of keratoconus, indicating that genetic variants of relatively large or moderate effect contributed to disease. We determined if any rare variants in ZNF469 were present, and if these alleles segregated with familial disease. In addition, we thoroughly evaluated, for the first time to our knowledge, the corneal phenotype of carriers of BCS1 ZNF469 pathogenic mutations.

METHODS

Patient Recruitment and Diagnostic Criteria

Local research ethics committees approved the study and all investigations were conducted in accordance with the principles of the Declaration of Helsinki. Patients and family members were ascertained in Moorfields Eye Hospital, London, UK; Moorfields Eye Hospital, Dubai, United Arab Emirates; and King Faisal Specialist Hospital, Saudi Arabia. In total, 11 families were recruited, comprised of 39 affected patients, 15 unaffected individuals <40 years, 5 unaffected individuals >40 years, and 7 individuals diagnosed as keratoconus suspects (see below). After informed consent was obtained, venous blood samples were donated and genomic DNA was extracted from peripheral blood lymphocytes.

All individuals had an anterior segment examination that included corneal topography using Pentacam (Oculus Optikgeräte GmbH, Wetzlar, Germany) version 1.20r36 (Saudi) or version 1.20r02 (UK). A diagnosis of keratoconus was confirmed if there were clinical features on slit-lamp examination of either eye of regional corneal thinning, ectasia, or other signs of keratoconus (e.g., Fleischer ring, Vogt striae)\textsuperscript{28} with confirmation by corneal topography. Previous keratoplasty for keratoconus was also considered to be confirmation of disease. For individuals without clinical signs of keratoconus, three specialists experienced in the interpretation of topography who were masked to the patients’ identity reviewed the scans. If there were topographic abnormalities in either eye, they were assigned a diagnosis of keratoconus suspect, or unaffected if the topography was normal.\textsuperscript{29} Because the onset of keratoconus can be delayed, individuals were only assigned a diagnosis of unaffected if they were over the age of 40 years.

Four parents of children with BCS1, previously determined to be heterozygous carriers of BCS1-associated mutations,\textsuperscript{22,30} had anterior segment examinations including corneal topography (Pentacam). They were also questioned regarding systemic clinical features previously identified in BCS1 and BCS2, including poor healing/abnormal scarring, soft skin/easy bruising, prior treatment for dysplasia of the hip, femoral epiphyseal changes, scoliosis, small joint hypermobility, fractures, myalgia, abnormal gait, deafness, and hypercompliant tibial bowing.\textsuperscript{21}

PCR and Sanger Sequencing

The coding region and the predicted splice site junctions of ZNF469 were amplified using a Go-Taq Long PCR master mix (Promega, Southampton, UK) in 8 overlapping fragments ranging from 2 to 3 kb. Amplimers were subsequently bidirectionally Sanger sequenced using a series of internal primers (primer sequences are available on request). The ZNF469 cDNA is numbered according to the reference sequence NM_001127464.1 (which includes a potentially coding 84 bp long intronic sequence) and +1 represents the A of the translation start codon.

In Silico Analysis of ZNF469 Variants, Control Datasets, and Filtering Strategies

The allelic frequencies of all variants identified by direct sequencing were cross referenced with four independent control databases: (1) The NHLBI Exome Sequencing Project (ESP) dataset (release Version v0.0.25, accessed August 2014), (2) 1000 Genomes (1KG) dataset (release version 14), (3) whole exome sequencing (WES) data for 521 individuals of Saudi Arabian decent with a variety of Mendelian conditions (SA WES), and (4) 1100 individuals of varying ethnicity analyzed by WES at UCL (UCL WES).

Sequence data were filtered using two alternative approaches. In filter strategy 1, the following variants were removed: all synonymous variants, all variants with a MAF > 0.001 in the 1KG dataset, all variants with a MAF > 0.001 in ESP dataset, and all variants predicted to be tolerated by SIFT. In filter strategy 2, the following variants were removed: all synonymous variants, all variants with a MAF > 0.025 in the 1KG dataset, all variants with a MAF > 0.025 in ESP dataset, and all variants with a MAF > 0.025 in our control WES datasets.

RESULTS

BCS1 Mutation Carriers

The parents of a BSC1 patient, previously determined to be compound heterozygous for disease-associated ZNF469 alleles c.5788delC;[5788dupC (p.[Gln1930Argfs*6]; p.[Gln1930fs*133]),30 were clinically examined. The mother and father were heterozygous carriers of c.5788delC and c.5788dupC, respectively.30 Ophthalmic examination showed they were emmetropic, and slit-lamp examination of the cornea and anterior segment was normal. Corneal topography showed that the mother, age 39 years, (BCS1 carrier 1 in Fig. 1) had CCT measurements within the normal range of 567 μm (right eye) and 582 μm (left eye), with borderline lack of concordance between the point of maximum curvature and the point of maximum corneal elevation (1.01 mm right eye; 1.08 mm left eye, normal < 1.0 mm) with no evidence of keratoconus. The father, age 43 years, (BCS1 carrier 2 in Fig. 1) had CCT measurement of 510 μm right eye and 503 μm left eye. Topography of the right eye showed an asymmetric bow-tie pattern of astigmatism, with concordance between the anterior and posterior maximum elevation, and a 0.84-mm displacement of the corneal apex from the location of the thinnest point. On the left side, although there was a symmetric bow-tie pattern of astigmatism, there was a lack of concordance between the point of maximum curvature of the anterior surface compared to the point of maximum elevation, with a displacement of the corneal apex from the thinnest location of 1.20 mm. BCS1 carrier 3 (p.[Glu1392X], column 5) appeared normal, while BCS1 carrier 4 (p.[Glu1392X], column 6) had a symmetric bow-tie pattern of corneal astigmatisms, but was otherwise normal. The topography of normal individuals are shown for comparison (columns 4 and 7). The apex of the cornea of BSC1 carrier 3 and control B are centered, but the elevation maps are within normal limits. All images were acquired using Pentacam (Oculus Optikgeräte GmbH). Data for columns 1 to 4 were analyzed using software version 1.20r2. Data for columns 5 to 7 were analyzed using software version 1.20r36.
A familial keratoconus cohort

Given that biallelic mutations in ZNF469 are associated with BCS1,20 that common variants close to ZNF469 are associated with reduced CCT9,14–18 and keratoconus risk9,19 and the recent observation that rare alleles are enriched in keratoconus cases,26,27 we wanted to determine if rare ZNF469 variants were present and associated with increased risk in a familial keratoconus cohort. We reasoned that identification and recruitment of families in which occurrence of keratoconus cannot readily be accounted for by chance, would offer an opportunity to identify rare alleles of moderate or relatively large effect compared to a consecutive series of unrelated individuals. We, therefore, assembled a cohort of 11 families, each comprising two or more first-degree relatives affected with keratoconus, and screened the coding sequence and splice sites of ZNF469 by direct sequencing.

Of the 11 families (Figs. 2, 3), six families of Middle Eastern origin had evidence of consanguinity and presented with an inheritance pattern consistent with risk alleles conferring an apparent autosomal recessive mode of inheritance (Families 1–6, Fig. 2). For the remaining families, of mixed ethnicities,
affected individuals were identified in more than one generation (with the exception of Family 9), consistent with a suggestive autosomal dominant (Families 7–11, Fig. 3) or potentially pseudodominant (Family 10) inheritance pattern for predisposing alleles. In total, the cohort included 39 keratoconus patients, 15 unaffected relatives under 40 years of age, 5 unaffected relatives over 40 years of age, and 7 individuals assigned a diagnosis of keratoconus suspect. All \textit{ZNF469} sequence variants identified are listed in Supplementary Table S1.

Initially, with the aim of identifying any rare and potentially deleterious variants in the keratoconus familial cohort, we applied a stringent filtering strategy (Filter strategy 1) similar to that applied by Lechner et al.,\textsuperscript{26} filtering out the following variants: (1) all synonymous variants, (2) all variants with a MAF > 0.001 in the 1KG dataset, (3) all variants with a MAF > 0.001 in the ESP dataset, and (4) all variants predicted to be tolerated by SIFT. Using this stringent filtering strategy, we identified 5 rare \textit{ZNF469} variants predicted to be potentially deleterious using SIFT (Table 1). However, 3 of these 5 variants (c.4337C>T, p.[Ala1446Val], c.2035G>A, p.[Glu679Lys], and c.9011_9025del, p.[Leu3004_Thr3008del]) were present with a MAF > 0.001 in our in-house control data sets (UCL WES, SA WES) and, therefore, failed to meet the set criteria (Table 1).

The in-frame deletion, p.(Leu3004_Thr3008del), has been described previously to be potentially pathogenic.\textsuperscript{26} Interestingly, we observed this variant in the heterozygous state in one individual affected with keratoconus (IV:2 in family 5; Fig. 2), but was absent in his affected brother (IV:1 in family 5, Fig. 2). Furthermore, the variant was inherited from his unaffected mother who is over 40 years of age (III:2 in family 5, Fig. 2). We also observed the same variant in two unaffected members of Family 9, one female over 40 years (II:4) and her unaffected son (III:3) under 40 years of age (Fig. 3). These data demonstrated that this allele is a polymorphism that, in isolation, does not confer substantial risk of keratoconus. The p.(Glu679Lys) variant with a UCL and SA WES MAF of 0.0049 and 0.0030, respectively (Table 1), is present in the heterozygous state in two affected siblings in Family 1 (Fig. 2). Unfortunately, other familial DNA samples were not available, but at least one parent is expected to carry this allele, and neither has keratoconus. Similarly, the p.(Ala1446Val) variant with a UCL and SA WES MAF of 0.0122 and 0.0236, respectively (Table 1), was homozygous in an affected individual (II:2), and two unaffected individuals (II:3, II:4) in Family 3 (Fig. 2).
**TABLE 1.** ZNF469 sequence variants identified using stringent filtering criteria

<table>
<thead>
<tr>
<th>Nucleotide Change</th>
<th>Protein Change</th>
<th>dbSNP</th>
<th>ESP (MAF &gt; 0.001)</th>
<th>1KG WES</th>
<th>UCL WES</th>
<th>SA WES</th>
<th>Affected</th>
</tr>
</thead>
<tbody>
<tr>
<td>c.664G&gt;T</td>
<td>p.(Ala1446Val)</td>
<td>rs199897247</td>
<td>Damaging (0.03)</td>
<td>NI</td>
<td>NI</td>
<td>0.0122</td>
<td>0.0236</td>
</tr>
<tr>
<td>c.2035G&gt;A</td>
<td>p.(Arg1875His)</td>
<td>NA</td>
<td>Damaging (0)</td>
<td>NI</td>
<td>NI</td>
<td>0.0004</td>
<td>NI</td>
</tr>
<tr>
<td>c.4337C&gt;T</td>
<td>p.(Ser1446Val)</td>
<td>NA</td>
<td>Damaging (0.03)</td>
<td>NI</td>
<td>NI</td>
<td>0.0036</td>
<td>0.0104</td>
</tr>
<tr>
<td>c.9011_9025del</td>
<td>p.(Leu3004_Thr3008del)</td>
<td>NA</td>
<td>NA</td>
<td>NI</td>
<td>NI</td>
<td>0.0068</td>
<td>0.0089</td>
</tr>
</tbody>
</table>

Based on a filtering strategy applied previously, all ZNF469 variants identified in the familial keratoconus cohort were stringently filtered to remove: (1) all synonymous variants, (2) all variants with a MAF < 0.001 in the 1KG dataset, (3) all variants with a MAF > 0.025 in the ESP dataset, and (4) all variants predicted to be tolerated by SIFT. The 5 variants listed were identified. In silico analysis of rare variants identified is presented. SIFT results are reported as tolerant if the tolerance index is < 0.05.

- **MAF in control datasets:**
  - **ESP:** denotes variants in the NHLBI ESP Release Version: v.0.0.25. (Feb. 7, 2014); 1000 Genomes (1KG).
  - **1000 Genomes (1KG):** donates variants in the release version 14 (October 2013); SA WES, refers to 521 Saudi Arabian whole exome sequenced (WES) controls; UCL WES, refers to 1100 WES individuals of varying ethnicity; NA, not applicable; NI, not identified.

**Discussion**

BCS1 is an autosomal recessive condition characterized by extreme corneal fragility, often leading to corneal rupture after
minor trauma, in addition to other features, such as joint hyperextensibility. Given the rarity of this condition, the occurrence of phenotypes in parents of affected individuals, who are heterozygous ZNF469 mutation carriers, has not been previously fully assed to our knowledge. We examined four carriers of three different presumed loss-of-function heterozygous BCS1 pathogenic mutations; p.(Glu1392X), p.(Gln1930Argfs*6), and p.(Gln1930fs*133). The carriers had an age range of 39 to 43 years and none had keratoconus. One carrier of the c.5788dupC, p.(Gln1930fs*135) allele, age 43 years (BCS1 carrier 2 in Fig. 1), who was keratoconus suspect in one eye, had small joint hypermobility and recurrent ankle dislocation suggestive of partial penetrance of BCS. A keratoconus suspect is an individual with minor changes in corneal shape that are features of keratoconus, which are detectable only with corneal topography, and without clinical disease. These changes may be nonprogressive and, because of the age of this individual, it is highly likely that they will not progress. BCS1 carrier 3 (Fig. 1) also was noted to suffer from back and bilateral lower foot pain, which may or may not represent partially penetrant features of BCS. This investigation of BCS1 carriers leads us to conclude that loss-of-function ZNF469 alleles in the heterozygous state (i.e., presumed haploinsufficiency of ZNF469) do not cause keratoconus, but carriers of recessive mutations could present with partially penetrant features of BCS.

We observed 9 rare (MAF ≤ 0.025) coding ZNF469 variants in our familial keratoconus cohort that were predicted to be potentially damaging by one or more bioinformatic tools (Table 2). Interestingly, two of these variants have been previously reported to be pathogenic mutations causing keratoconus in the heterozygous state; p.(Leu3004_Thr3008del) and p.(Ala566Val). However, we observed the p.(Leu3004_Thr3008del) variant (UCL WES cohort MAF = 0.0068; SA WES cohort MAF = 0.0089) in the heterozygous state in 3 unaffected individuals in our familial cohort, and inheritance of the p.(Ala566Val) variant (UCL WES cohort MAF = 0.0119; SA WES cohort MAF = 0.0020) was not consistent with a high risk of developing keratoconus in two families. Therefore, these variants are most likely polymorphic alleles that, in isolation, do not confer a substantial risk of keratoconus.

Known ZNF469 loss-of-function mutations in the heterozygous state, as observed in the carriers of BCS1 mutations, are not sufficient to cause keratoconus. Therefore, ZNF469 variants that could predispose an individual to a high risk of developing keratoconus, when in the heterozygous state, must have a gain-of-function or a dominant-negative effect on the normal gene/protein function. However, segregation analysis revealed that none of the remaining nonsynonymous variants was consistent with such an allele having a dominant mechanism of disease, even allowing for reduced penetrance. Importantly, through interrogation of our in-house WES dataset for 1100 individuals of mixed ethnicity (UCL WES), by applying the same stringent filtering criteria (Filtering strategy 1) we applied initially to our keratoconus family data, we identified 4 heterozygous presumed loss-of-function variants (stop or frameshift) and 224 nonsynonymous variants in the ZNF469 gene, illustrating the allelic variability of this large gene.

Our data suggested that the allelic variation of ZNF469 in the general population is somewhat under-represented in currently available public databases (EVS and 1000 Genomes), perhaps due to poor coverage of the gene using previous generation WES capture techniques. Our extensive UCL WES internal control dataset (n = 1100), in combination with the familial cohort we have studied likely contributed to the differences observed between our study and others.26,27
Our understanding of the functional role of ZNF469 in the cornea, or other tissues, and the mechanisms that mediate the development and maintenance of the structural integrity of the cornea is limited. The ZNF469 gene is a relatively polymorphic gene that is poorly conserved among lower mammals and vertebrates.\(^{20}\) It is thought to function as a nuclear transcription factor regulating extracellular matrix protein expression in the cornea, or as an extranuclear regulatory molecule involved in the synthesis and/or organization of extracellular matrix proteins.\(^{20,21,30}\) The functional impact of rare ZNF469 variants can be established only once more is known about the role of ZNF469 in the human cornea.

In the absence of a functional assay, and given the relatively polymorphic and poor evolutionary conservation of ZNF469, performing segregation analysis in familial cohorts to evaluate whether a variant confers a substantial risk of disease is extremely valuable. The lack of segregation of any of the rare variants identified in our study suggests that they do not, in isolation, confer a substantial risk of disease. However, rare ZNF469 variants may still contribute to mutational load or the genetic architecture of keratoconus.

The keratoconus families described here, some with many affected individuals (e.g., Family 8 with 6 affected individuals in 2 generations) offer an opportunity to use next generation sequencing (NGS) approaches to identify the genetic factors that are involved in the risk of developing keratoconus. Given the consanguineous nature of families 1 to 6, and the potential autosomal recessive inheritance patterns of keratoconus observed, autozygosity mapping in combination with NGS may prove to be a powerful way to determine keratoconus-associated variants in these families.

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