Brittle Cornea Syndrome *ZNF469* mutation carrier phenotype and segregation analysis of rare *ZNF469* variants in familial Keratoconus

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Abstract

Purpose: Brittle cornea syndrome 1 (BCS1) is a rare recessive condition characterised by extreme thinning of the cornea and sclera, caused by mutations in *ZNF469*. Keratoconus is a relatively common disease characterised by progressive thinning and ectasia of the cornea. The aetiology 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 (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 non-synonymous *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.

INTRODUCTION

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 1,000 [2] although it is markedly more frequent in some ethnic groups. It typically presents in the teens or early twenties with progression until the fourth decade. Keratoconus is the single most common reason for corneal transplantation in the developed world, but the aetiology 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 or robust linkage data suggests that highly penetrant variants are rare in keracotonus families, variants that increase disease risk by about four fold are likely to generate inconsistent evidence for linkage and may therefore have been missed [10, 11].

There has also been much interest in defining risk loci associated with the highly heritable quantitative trait central corneal thickness (CCT) [12], 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 [9, 13]. SNPs located in an intergenic region upstream of *ZNF469* have been associated with CCT by numerous GWAS in different populations [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 significantly associated with increased CCT and keratoconus [9]. This association with keratoconus was validated in an independent case control study [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 [9].

The association of a SNP upstream of *ZNF469* with risk of keratoconus is of potential biologically relevance, since bi-allelic non-synonymous mutations in *ZNF469* cause autosomal recessive Brittle Cornea Syndrome (BCS) type 1 [20]. BCS1 (OMIM #229200) is a connective tissue disorder characterised by extreme thinning and fragility of the cornea, joint hypermobility and hyperelasticity of the skin, often leading to corneal rupture after minor trauma [20]. BCS type 2 (OMIM #614170) is caused by bi-allelic 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) [21]. Interestingly one of these six individuals also had keratoconus [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 previously documented in one of three molecularly confirmed carriers [22].

Given the association of rs9938149 (A) with keratoconus, and that bi-allelic 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 [23-25]. A recent study of *ZNF469* in a

cohort of unrelated Europeans with keratoconus reported an enrichment of potentially pathogenic rare (MAF<0.001) *ZNF469* alleles in 12.5% cases (n=112) [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) [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, 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, 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) [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 [29]. Because the onset of keratoconus can be delayed, individuals were only assigned a diagnosis of unaffected in they were over the age of 40 years.

Four parents of children with BCS1, previously determined to be heterozygous carriers of BCS1-associated mutations [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 [21].

PCR and Sanger sequencing

The coding region and the predicted splice site junctions of *ZNF469* was amplified using a Go-Taq Long PCR master mix (Promega, Southampton, UK) in 8 overlapping fragments ranging from 2-3 kb. Amplimers were subsequently bi-directionally Sanger sequenced using a series of internal primers (primer sequences are available on request). *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) NHLBI Exome Sequencing Project (ESP) dataset (release Version v.0.0.25, 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); (4) 1,100 individuals of varying ethnicity analyzed by WES at UCL (UCL WES).

The predicted biological effect of rare non-synonymous variants identified in *ZNF469* were scored for likely pathogenicity utilizing SIFT (http://sift.jcvi.org/), PolyPhen2 (http://genetics.bwh.harvard.edu/pph2/), and Blosum62 (http://www.ncbi.nlm.nih.gov/Class/FieldGuide/BLOSUM62.txt).

Sequence data was filtered using two alternative approaches. Filter strategy 1- the following variants were removed: (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 ESP dataset, (4) all variants predicted

to be tolerated by SIFT. Filter strategy 2 - the following variants were removed: all synonymous variants, (2) all variants with a MAF >0.025 in the 1KG dataset, (3) all variants with a MAF >0.025 in ESP dataset, and (4) 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 found to be 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 Figure 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 Figure 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 and the point of maximum corneal elevation, with a 1.20 mm displacement of the corneal apex from the location of the thinnest point. The CCT measurements of the father are in the range reported for mild keratoconus (CCT= 503± 34.15 μm)[31], and, in combination with the mild abnormalities in contour, are consistent with keratoconus suspect in the left eye. The father also had small joint hypermobility (double jointed fingers and wrist) and his sports activity was limited due to recurrent ankle dislocation; no additional systemic features were noted in either parent. In combination, these data suggest that in the father the c.5788dupC, p.(Gln1930fs*133) *ZNF469* allele is partially penetrant.

Similarly, the heterozygous carrier parents of two affected children homozygous for the BCS1-associated *ZNF469* allele, c.4174G>T p.(Glu1392X) were examined [22]. The father was emmetropic, the mother had mild stable astigmatism. They both presented with a normal slit lamp examination of the cornea and anterior segment. Corneal topography of the father (BCS1 carrier 3 in Figure 1), age 40 years, was normal with CCT measurements of 510 µm (right eye) and 503 µm (left eye). Corneal topography of the mother (BCS1 carrier 4 in Figure 1), age 42 years, was normal with a symmetric bow-tie pattern of corneal astigmatism and CCT measurements of 543 µm (right eye) and 541 µm (left eye). There were no topographic features of keratoconus in either parent. The father had complained of lower back pain for the last 7 years. He also had bilateral lower foot pain that lasted for an hour before his going to sleep. There were no additional systemic features associated with BCS1 reported in either parent. In both sets of parents the variation in the direction of CCT measurement compared to the adult population (mean 535± 31 µm) [32] was inconsistent.

Familial keratoconus cohort

Given that bi-allelic mutations in *ZNF469* are associated with BCS1 [20], that common variants close to *ZNF469* are associated with reduced CCT [9, 14-18] and keratoconus risk [9, 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 eleven 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 eleven families (Figure 2 and 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, Figure 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, Figure 3) or potentially pseudo-dominant (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 *ZNF469* sequence variants identified are listed in 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 and colleagues [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 ESP dataset, (4) all variants predicted to be tolerated by SIFT. Using this stringent filtering strategy we identified 5 rare *ZNF469* variants predicted to be potentially deleterious using SIFT (Table 1). However, 3/5 of these 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 previously described to be potentially pathogenic [26]. Interestingly, we observed this variant in the heterozygous state in one individual affected with keratoconus (IV:2 in family 5; Figure 2) but was absent in his affected brother (IV:1 in family 5, Figure 2). Furthermore, the variant was inherited from his unaffected mother who is over 40 years of age (III:2 in family 5, Figure 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 (Figure 3). These data demonstrate 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 (Figure 2). Unfortunately, other familial DNA samples were not available, but at least one parent is expected to carry this allele, and neither have keratoconus. Similarly, the p.(Ala1446Val) variant with a UCL and SA WES MAF of 0.0122 and 0.0236, respectively (Table 1), was found to be homozygous in an affected individual (II:2), and two unaffected individuals (II:3, II:4) in Family 3 (Figure 2).

Only 2 variants survived this filtering strategy, c.664G>C, p.(Gly222Arg) and c.5624G>A, p.(Arg1875His), identified in Families 3 and 8, respectively (Figure 2 and 3). The p.(Gly222Arg) variant was identified in the heterozygous state in only one of two affected male siblings in Family 3, and although at least one parent probably carries this allele, neither were affected, so this variant did not segregate with keratoconus in this family. The p.(Arg1875His) variant was identified in several affected members of Family 8 in either the heterozygous state (II:5, II:6 and III:4) or homozygous state (II:11). It was also present in the heterozygous state in two family members who are keratoconus suspect; III:3 (age 38 years) and III:7 (age 24 years), and an unaffected individual III:6 (age 27 years) in the homozygous state. The possibility that all three of these individuals will develop keratoconus cannot currently be fully excluded, as disease onset can be delayed. It is also interesting to note that an affected family member (II:10) was wild-type for this allele, however it is possible that this individual may be a phenocopy.

For comparison purposes the assembled UCL WES dataset, comprising WES data for 1,100 individuals of mixed ethnicity with full coverage of the *ZNF469* gene (used as a control cohort for filtering purposes, as described above) was also filtered according to Filter Strategy 1. Interestingly, we identified 4 heterozygous presumed loss-of-function variants (stop or frameshift) and 224 non-synonymous variants in *ZNF469*.

Given that keratoconus is a relatively common condition [2], we next assessed the genetic load of *ZNF469* alleles (Table S1) with a more relaxed filtering criteria, such that one or more

potentially deleterious variants of moderate effect, present at low frequency in the normal population, would not be excluded. For this analysis we filtered out the following variants: (1) all synonymous variants, (2) all variants with a MAF >0.025 in the 1KG dataset, (3) all variants with a MAF >0.025 in ESP dataset, and (4) all variants with a MAF >0.025 in our control WES datasets. This alternative filtering strategy highlighted a further 4 variants of potential interest [(c.1697C>T p.(Ala566Val); c.2803G>A, p.(Glu935Lys); c.6956C>T, p.(Ala2319Val); c.10277G>A, p.(Arg3426Gln)] in families 1, 3, and 10 (Table 2; Figures 2 and 3).

The p.(Ala566Val) variant was identified in one of the affected siblings in Family 1 (V:5, Figure 2), but both parents are unaffected. This p.(Ala566Val) variant was also identified in two unaffected members of Family 3 under 40 years of age (II:3 and II:4) and one of their affected siblings (II:1), the parents, at least one of which is expected to carry the allele, are also unaffected in this family. Interestingly, the variant has previously been identified in 3 unrelated sporadic keratoconus patients of Caucasian (n=2) and Indian (n=1) origin [27]. The segregation data present here for Families 1 and 3 suggest this allele, in isolation, does not confer substantial risk of disease. Two rare heterozygous variants were identified in Family 10, c.6956C>T, p.(Ala2319Val) and c.10277G>A, p.(Arg3426Gln) (Table 2). All affected individuals (II:1, III:2, III:4) share the p.(Arg3426Gln) variant, as does unaffected individual III:3 (32 years of age). Both the affected father (II:1) and the unaffected mother (II:2), who are related, are heterozygous for the c.6956C>T, p.(Ala2319Val) variant, and this allele was not inherited by their affected children (III:2 and III:4). The p.(Glu935Lys) variant was identified in Family 1 in only one of two affected siblings with unaffected parents (Figure 2). In Families 2, 4, 6, 7 and 11 no rare variants were identified that passed either of the filtering criteria applied (Figure 2 and 3).

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 previously been fully assessed. Here, 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 39-43 years and none had keratoconus. One carrier of the c.5788dupC, p.(Gln1930fs*133) allele, age 43 yrs (BCS1 carrier 2 in Figure1), who was keratoconus suspect in one eye, also 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 only detectable with corneal topography, and without clinical disease. These changes may be non-progressive and because of the age of this individual, it is highly likely that they will not progress. BCS1 carrier 3 (Figure 1) was also 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) [26, 27]. 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. These variants are therefore 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 non-synonymous variants were consistent with such an allele having a dominant mechanism of disease, even allowing for reduced penetrance.

Importantly, interrogation of our in-house WES dataset for 1,100 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 non-synonymous variants in the *ZNF469* gene, illustrating the allelic variability of this large gene.

Our data suggest 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=1,100), in combination with the familial cohort we have studied likely contributes 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. *ZNF469* is a relatively polymorphic gene that is poorly conserved amongst 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 extra-nuclear regulatory molecule involved in the synthesis and/or organization of extracellular matrix proteins [20, 21, 30]. The functional impact of rare *ZNF469* variants can only be established 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 utilise 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 1-6, 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.

FIGURE LEGENDS

Figure 1. Computer generated analysis of Scheimpflug images of, heterozygous carriers of pathogenic BCS1 *ZNF469* alleles compared to a keratoconus patient. For each individual the axial/sagittal curvature of the anterior corneal surface (row 1), the regional corneal thickness (row 2), the anterior corneal surface elevation compared to the mean anterior elevation (row 3), and posterior corneal surface elevation compared to the mean posterior elevation (row 4) of the left eye are presented. In the keratoconus patient there is marked thinning and steepening of the curvature of the anterior and posterior surfaces of the central cornea. BCS1 carrier 1 (p.[Gln1930Argfs*6]) has nasal displacement of the point of maximum curvature of the anterior corneal surface but no other abnormality. In BCS1 carrier 2 (p.[Gln1930fs*133]) there is a symmetric bow-tie pattern of astigmatism on the anterior curvature, but with 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 and 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 decentered but the elevation maps are within normal limits. All images were acquired using Pentacam (Oculus, Germany). Data for columns 1-4 were analyzed using software version 1.20r02. Data for columns 5-7 were analyzed using a software version 1.20r36.

Figure 2. Segregation of rare *ZNF469* coding variants in consanguineous keratoconus families with affected individuals in one generation

Pedigrees of Families 1-6 of Middle Eastern origin are shown. A diagnosis of keratoconus was given if there were clinical features on slit lamp examination in either eye of regional corneal thinning, ectasia, or other signs of keratoconus, with confirmation by corneal

topography. Documented keratoplasty for keratoconus was also considered diagnostic. Individuals were assigned a diagnosis of keratoconus suspect, denoted as 'S', on the basis of presence of topographic abnormalities in either eye, or unaffected if the topography was normal. All unaffected individuals under the age of 40 years are denoted with a '?' as onset of keratoconus can be delayed. Segregation of rare *ZNF469* alleles presented in Tables 1 and 2 are shown. Het = heterozygous state; Homo = homozygous state; WT= wild-type.

Figure 3. Segregation of rare *ZNF469* coding variants in keratoconus families with affected individuals in more than one generation and non-consanguineous families

Pedigrees of Families 7-11 are shown. A diagnosis of keratoconus was given if there were clinical features on slit lamp examination in either eye of regional corneal thinning, ectasia, or other signs of keratoconus, with confirmation by corneal topography. Documented keratoplasty for keratoconus was also considered diagnostic. Individuals were assigned a diagnosis of keratoconus suspect, denoted as 'S', on the basis of presence of topographic abnormalities in either eye, or unaffected if the topography was normal. All unaffected individuals under the age of 40 years are denoted with a '?' as onset of keratoconus can be delayed. Segregation of rare *ZNF469* alleles presented in Tables 1 and 2 are shown. Het = heterozygous state; Homo = homozygous state; WT= wild-type.

FUNDING

The research was supported by the National Institute for Health Research Biomedical Research Centre at Moorfields Eye Hospital and UCL Institute of Ophthalmology, Moorfields Special Trustees, Moorfields Eye Charity, The Lanvern Foundation and Rosetrees Trust.

ACKNOWEDGEMENTS

We thank Dr. Ahmed Shamia for his help with analyzing the allele frequency in the Saudi cohort and we thank all the families for participating in this research. We also thank Soctt Hau for his interpretation of the corneal topography data.

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