Cornea

Evaluating the Association Between Keratoconus and the Corneal Thickness Genes in an Independent Australian Population

Srujana Sahebjada,[1,2] Maria Schache,[1] Andrea J. Richardson,[1] Grant Snibson,[1,3] Stuart MacGregor,[4] Mark Daniell,[1] and Paul N. Baird[1]

[1]Ocular Genetics Unit, Centre for Eye Research Australia, East Melbourne, Australia
[2]The University of Melbourne, Melbourne, Australia
[3]Corneal Unit, The Royal Victorian Eye and Ear Hospital, East Melbourne, Australia
[4]Statistical Genetics Lab, Queensland Institute of Medical Research Berghofer Medical Research Institute, Royal Brisbane Hospital, Brisbane, Australia

Purpose. A recent genome-wide association study (GWAS) identified six loci associated with central corneal thickness that also conferred associated risk of keratoconus (KC). We aimed to assess whether genetic associations existed for these loci with KC or corneal curvature in an independent cohort of European ancestry.

Methods. In total, 157 patients with KC were recruited from public and private clinics in Melbourne, Australia, and 673 individuals without KC were identified through the Genes in Myopia study from Australia. The following six single-nucleotide polymorphisms (SNPs) that showed a statistically significant association with KC in a recent GWAS study were selected for genotyping in our cohort: rs4894535 (*FNDC3B*), rs1324183 (*MPDZ-NF1B*), rs1536482 (*RXRA-COL5A1*), rs7044529 (*COL5A*), rs2721051 (*FOXO1*), and rs9938149 (*BANP-ZNF469*). The SNPs were assessed for their association with KC or corneal curvature using logistic or linear regression methods, with age and sex included as covariates. Bonferroni corrections were applied to account for multiple testing.

Results. Genotyping data were available for five of the SNPs. Statistically significant associations with KC were found for the SNPs rs1324183 ($P = 0.001$; odds ratio [OR], 1.68) and rs9938149 ($P = 0.010$; OR, 1.47). Meta-analysis of previous studies yielded genome-wide significant evidence of an association for rs1324183, firmly establishing it as a KC risk variant. None of the SNPs were significantly associated with corneal curvature.

Conclusions. The SNPs rs1324183 in the *MPDZ-NF1B* gene and rs9938149 (between *BANP* and *ZNF4659*) were associated with KC in this independent cohort, but their association was via a non–corneal curvature route.

Keywords: keratoconus, central corneal thickness, candidate genes

Keratoconus (KC) is a common corneal condition in individuals of European descent, with a reported incidence of approximately 1 case per 2000 and a prevalence of 54.5 per 100,000 in the general population, although this is likely to be an underestimate.[1] It is the major reason for corneal transplantation, accounting for some 31% of corneal grafts in Australia.[2] Typically, the condition has its onset in the teenage years and is characterized by a progressive corneal thinning that results in corneal protrusion, irregular astigmatism, and decreased vision.[3] Keratoconus is a heterogeneous disorder believed to be caused by both genetic and environmental factors.[4] It has been reported to occur through several routes, including eye rubbing,[4] allergy,[5] connective tissue dysfunction,[6] and contact lens wear,[7] as well as in those with a family history.[8] Studies have also indicated a genetic involvement in KC, including a twin study,[9] a familial aggregation study,[10] and formal genetic analyses.[11]

Within the general population, central corneal thickness (CCT) is a normally distributed quantitative trait, with evidence from twin and familial studies[12–14] indicating that

it is highly heritable. Numerous genetic loci from genome-wide association study (GWAS) data have been reported to be associated with CCT in Caucasians and Asians, including *AKAP13*,[15,16] *COL5A1*,[16,17] *RXRA-COL5A1*,[16–18] *COL8A2*,[17] *FAM53B*,[19] *FOXO1*,[19] *IBTK*,[15] *LRRK1*,[15] and *ZNF469*.[16–19] Recently Gao et al.[20] performed a GWAS of CCT among 1768 Latinos and reported single-nucleotide polymorphisms (SNPs) associated with CCT in the *LOC100506532* gene. Also, Lu et al.[21] recently performed a GWAS of CCT and identified 27 associated loci. Of these 27 loci, the following six genes were also associated with KC: *FNDC3B* (rs4894535), *MPDZ-NF1B* (rs1324183), *RXRA-COL5A1* (rs1536482), *COL5A1* (rs7044529), *FOXO1* (rs2721051), and *BANP-ZNF469* (rs9938149).

Given the paucity of replication studies confirming genetic associations for KC, we undertook a replication study of the six significantly associated SNPs identified by Lu et al.[21] We investigated their involvement in an independent Caucasian cohort with KC and assessed their association with corneal curvature.

METHODS

Participants

All patients for this study were recruited from public clinics at The Royal Victorian Eye and Ear Hospital, private rooms, optometry clinics, or the consenting general public with KC. A patient information sheet, consent form, privacy statement, and patient rights documentation were provided to all individuals participating in the study. Participants without KC were recruited through the Genes in Myopia (GEM) study from Australia, in which a similar testing protocol was used with the administration of a general questionnaire, comprehensive eye examination, and blood collection. The methods of the GEM study[22] have been published elsewhere.

Protocol

Patients with KC were required to complete a study questionnaire, undergo a clinical examination, and provide complete details of their family history of disease. Blood or saliva samples were also collected. DNA was extracted from the blood or saliva sample as previously described.[23] The study protocol was approved by The Royal Victorian Eye and Ear Hospital Human Research and Ethics Committee (project 10/954H). Written informed consent was obtained from each participant, and all protocols followed the tenets of the Declaration of Helsinki.

Inclusion and Exclusion Criteria

Individuals with KC of European background seen at clinics and private practices were invited to participate in the study. Keratoconus was diagnosed on the basis of the presence of one or more of the following three characteristics: (1) an irregular cornea, as determined by distortion of keratometric mires or computerized videokeratography; (2) scissoring of the retinoscopic reflex; and (3) at least one biomicroscopic sign, including Vogt's striae, Fleicher's ring, or corneal thinning, and scarring typical of KC. Participants also had to demonstrate one or more of the following three changes in the topographic map: (4) focal steepening of areas greater than 47 diopters (D) located in the cone protrusion zone surrounded by concentric decreasing power zones; (5) angling of the hemimeridians exceeding 20 or 30° in the bow tie pattern; and (6) inferosuperior asymmetry greater than 1.4 D within the midperipheral cornea. Individuals with non-KC ocular disease in both eyes such as corneal degenerations and dystrophies, macular disease, and optic nerve disease (e.g., optic neuritis and optic atrophy) and other disease-associated KC were excluded from the study.

Recruited from the GEM study were individuals who had some form of refractive error (±6 D and astigmatism < 4.00 D) and were mainly myopic. Excluded from the statistical analysis were individuals with known or subsequently identified ocular disorders that may lead to changes in refractive error such as amblyopia (Snellen difference between the eyes > 2 lines), strabismus, visually significant lens opacification, glaucoma, or any other corneal abnormality. Individuals with connective tissue disease such as Marfan's syndrome or Stickler's syndrome were also excluded from the study. The latter conditions were identified by the individual's medical history, obtained via a general questionnaire.

SNP Selection and Genotyping

The SNPs chosen for this project were rs1324183, rs1536482, rs2721051, rs4894535, rs7044529, and rs9938149 based on the study by Lu et al.[21] A total of 10 ng genomic DNA was amplified using PCR. A mass extend reaction was initiated by adding a Hotstart DNA polymerase (Bioline USA, Inc., Taunton, MA) and a common primer that allowed a 1–base pair primer extension of either allele at the polymorphic site using the designed primers (Integrated DNA Technologies, Inc., Coralville, IA). Deoxynucleotides incorporated at the polymorphic site were terminated with the incorporation of a dideoxynucleotide, and excess nucleotides were removed through the use of shrimp alkaline phosphatase. Using this approach, two allele-specific products of different mass were generated. Samples were conditioned using SPECTROCLEAN (Spectron Gas Control Systems GmbH, Frankfurt am Main, Germany) resin to remove excess salt that might interfere with matrix-assisted laser desorption ionization–time-of-flight (MALDI-TOF) analysis. Each sample was transferred from a 384-well microtiter plate and spotted onto the pad of a 384 SpectroCHIP II microarray (Sequenom, San Diego, CA). The microarray was placed into the MALDI-TOF, and genotypes were simultaneously called in real time with MassArray Spectro Typer software version 4.0 (Sequenom). Genotyping was performed on the MassArray platform (Sequenom) at the Murdoch Childrens Research Institute, Melbourne, Australia. Accuracy of the machine is checked annually and is calibrated to 98% mass spectrometer assays call rate by a field service engineer from Sequenom. Quality control was done by positive and negative internal duplicates in which the duplicates were similar to each other.

Statistical Analysis

All statistical analyses were performed using PLINK version 1.04 (available in the public domain at http://pngu.mgh.harvard.edu/~purcell/plink/). Keratoconus associations were analyzed using a logistic regression model, and corneal curvature was analyzed using a linear regression model. For all analyses, age and sex were used as covariates. Corrections for multiple testing were undertaken using Bonferroni methods. All SNPs were assessed for deviations from Hardy-Weinberg equilibrium with $P < 0.01$. Only SNPs passing the Hardy-Weinberg equilibrium test were used for statistical analysis. Power calculations were performed using Quanto version 1.2.4 (available in the public domain at http://hydra.usc.edu/gxe) for allele frequencies ranging from 0.1 to 0.38 and $\alpha = 0.05$.

In Silico Analysis

Detection of evolutionary conservation across species, enhancer elements, microRNA (miRNA) sequences, and transcription factor binding sites was undertaken using a 0.5-kilobase (kb) genomic sequence upstream and downstream of each of the SNPs. Evolutionary conservation analysis was undertaken using the ECR Browser (available in the public domain at http://ecrbrowser.dcode.org),[24] enhancer element detection using the VISTA Enhancer Browser (available in the public domain at http://enhancer.lbl.gov org),[25] miRNA detection using miRBase (avialable in the public domain at http://www.mirbase.org),[26–29] and transcription factor binding site prediction analysis using Match version 1.0 (BIOBASE Biological Databases, Beverly, MA).

RESULTS

Clinical Data

Recruited into the study were 830 individuals (354 male [43%] and 476 female [57%]), with a mean (SD) cohort age of 49.85 (16.30) years. Of these, 157 had KC (93 male [59%] and 64

TABLE 1. Characteristics of the Study Cohort

Characteristic	KC	Non-KC	P Value
Male sex, %	59	39	<0.05
Age, mean (SD), y	37.81 (15.65)	52.67 (15.15)	<0.05
Spherical equivalent, mean (SD), D	−4.54 (−4.71)	−1.96 (−3.77)	<0.05
Axial length, mean (SD), mm	24.32 (1.55)	24.64 (1.71)	0.77
Corneal curvature, mean (SD), D	49.28 (8.19)	42.69 (2.54)	<0.05
Anterior chamber depth, mean (SD), mm	3.52 (0.57)	3.49 (0.41)	0.97

female [41%]; mean [SD] age, 37.81 [15.65] years), and 673 did not have KC (260 male [39%] and 413 female [61%]; mean [SD] age, 52.67 [15.15] years). There was a very high correlation between measures for the right eyes and left eyes ($r^2 > 0.99$); therefore, only data from the right eyes were used in the analyses. The KC eyes were significantly steeper and more myopic compared with the non-KC eyes ($P < 0.05$). There was no significant difference in axial length ($P = 0.77$) or anterior chamber depth ($P = 0.97$) between the KC eyes and the non-KC eyes. Characteristics of each group are summarized in Table 1.

Association Study

Six SNPs (rs1324183, rs1536482, rs2721051, rs4894535, rs7044529, and rs9938149) were genotyped based on the study by Lu et al.[21] All SNPs were in Hardy-Weinberg equilibrium, and the cutoff for SNP genotyping was set at 95%. The SNP rs4894535 was excluded from further analysis because the genotyping rate fell below quality control measures at 89%. The SNP genotyping call rates for each of the other SNPs were 96.1% for rs1324183, 95.2% for rs1536482, 99.3% for rs2721051, 96.7% for rs7044529, and 95.7% for rs9938149. Genetic association studies were performed for the remaining five SNPs between KC eyes and non-KC eyes. A Bonferroni-corrected P value of 0.05 divided by 5 (= 0.01) was applied as appropriate for dependent SNPs because of the multiple comparisons made in the study. The risk allele A of SNP rs1324183 (odds ratio [OR], 1.68; 95% confidence interval [CI], 1.22–2.30; $P = 0.001$) and the risk allele A of SNP rs9938149 (OR, 1.47; 95% CI, 1.10–1.96; $P = 0.010$) were significantly associated with KC eyes compared with non-KC eyes (Table 2). Quantitative analysis of the five SNPs for corneal curvature using linear regression methods did not show any statistically significant associations for any of the SNPs analyzed (Supplementary Table S1).

In Silico Analysis

The two significantly associated SNPs are located on chromosome 9p23 (rs1324183) and on chromosome 16q11 (rs9938149). Both SNPs are intergenic with the nearest gene to rs1324183, being *MPDZ* at 437 kb downstream, and the nearest gene to rs9938149, being *ZNF469* at 162 kb downstream.

An analysis of evolutionary conservation 0.5 kb upstream and downstream of rs1324183 indicated complete conservation (100%) between the human DNA sequence and that of the dog, mouse, rhesus macaque, cow, rat, and chimpanzee. There was no evolutionary conservation between the human DNA sequence and sequences from any other species analyzed (i.e., fugu, chicken, opossum, frog, zebra fish, and *Tetraodon*). A search for putative enhancer binding sites detected none. A search for known miRNA sequences in the region around rs1324183 detected 102 known miRNA elements. A search for transcription factor binding sites detected four, including sequences for BR-C Z4, Evi-1, BR-C Z1, and COMP1.

An analysis of the evolutionary conservation 0.5 kb upstream and downstream of rs9938149 indicated conservation between the human DNA sequence and that of the chimpanzee (100%) and the rhesus macaque (91%). Moderate to low evolutionary conservation was observed between the human DNA sequence and sequences from the mouse (61%), cow (28%), opossum (28%), and dog (27%). A search for putative enhancer binding sites found none. A search for known miRNA sequences in the region around rs9938149 detected 42 known miRNA elements. A search for transcription factor binding sites detected two, including sequences for Pax-4 and MCM1.

Because of the intergenic location of each SNP, the biological mechanisms of rs1324183 and rs9938149 are unclear at this stage. It is known how miRNA sequences in each region might be associated with KC.

DISCUSSION

This study was undertaken to assess replication of recent GWAS hits from a study by Lu et al.[21] that suggested an association of six loci with KC. However, in the present study no significant association was evident for four of the SNPs reported by Lu et al. Meta-analysis of previously published studies yielded genome-wide significant evidence of an association for rs1324183 (Supplementary Fig. S1), firmly establishing it as a KC risk variant. Most important, we replicated the surprising effect of the direction of the A allele at rs9938149 reported by Lu et al. as being associated with

TABLE 2. Logistic Regression Analysis of KC in the Present Study

SNP	Risk Allele Name	Non-KC Frequency	KC Frequency	OR (95% CI)	P Value
rs1324183	**A**	**0.19**	**0.29**	**1.68 (1.22–2.30)**	**0.001**
rs1536482	A	0.29	0.33	1.15 (0.84–1.57)	0.387
rs2721051	A	0.10	0.15	1.45 (0.99–2.13)	0.057
rs7044529	T	0.16	0.17	1.11 (0.76–1.60)	0.596
rs9938149	**A**	**0.62**	**0.72**	**1.47 (1.10–1.96)**	**0.010**

Boldface indicates significantly associated with KC. P value for statistical significance is 0.05 divided by 5 (= 0.01).

increased risk of KC. In their article, they indicated that the *A* allele was associated with increased CCT, which is perhaps counterintuitive because it is expected that this allele would be associated with corneal thinning, as found in KC. Lu et al. suggested that the finding of a different effect direction relative to that proposed by epidemiological prediction may either reflect a false-positive association or a genuine pleiotropic action of a gene on diseases. Our data suggest that this is not a false-positive association but acts through another, as yet unknown, pleiotropic role.

Our results suggested that these two SNPs confer only a modest increase in the risk (OR of 1.68 for rs1324183 and OR of 1.47 for rs9938149) of KC. When considered together, the six SNPs identified as being significantly associated with KC in the study by Lu et al.[21] explain approximately 2% of the variance of CCT.

Progressive corneal thinning of the cornea is a well-known feature of the pathophysiology of KC.[30] We (Sahebjada S, Chan E, Daniell M, Baird PN, unpublished data, 2013) and Emre et al.[31] have shown that progressive corneal thinning is an indicator of the progression of KC. Because KC is a disease characterized by extreme corneal curvature, we also assessed the association of the five genotyped SNPs with this quantitative trait. The estimates of the effect of an allele on corneal curvature at any of the tested SNPs were small (β [SE] range, -0.5 to 0.5 [0.3 to 0.4]). Such small effects would explain less than 1% of the variance in corneal curvature. Based on a power calculation, our study has greater than 80% power to detect variants explaining 1% of trait variance. Therefore, the results of the present study suggest that the SNPs rs1324183 and rs9938149 are associated with risk of KC, and the effect most likely acts via a non–corneal curvature route.

Our sample size is smaller than that in the study by Lu et al.,[21] and this is reflected in the 95% CIs in Supplementary Figure S1. Although our study has limited power to show significant effects for the six loci, all loci have 95% CIs that overlap between each data set, with some loci significant ($P < 0.05$) in our study alone. Based on a power calculation, our study has 43% to 80% power to detect ORs in the range of 1.25 to 1.60 found by Lu et al.

Given that we present a well-defined clinical phenotype and cohort of homogeneous ethnicity, why is it that our results differ from those of the equally robust study by Lu et al.[21]? We compared findings from the present study with those by Lu et al. using a series of forest plots (Supplementary Fig. S1). The significantly replicated SNP rs1324183 was positively associated with the risk allele in our cohort (OR, 1.68) and in the same direction but of greater magnitude compared with a South Australian (SA) cohort and the US cohort used by Lu et al. and the meta-analysis (each with an OR of 1.33). The second significantly replicated SNP rs9938149 was positively associated with the risk allele in our cohort (OR, 1.47) and in the same direction but of greater magnitude compared with the SA cohort (OR, 1.19) and the meta-analysis (OR, 1.25) but was similar to that of the US cohort (OR, 1.52) (Supplementary Fig. S1). The other three genotyped SNPs showed effects in the same direction as previous data but with 95% CIs that included both 1.00 and the ORs in previous investigations.

A recent study by Li et al.[32] assessed an association between the *COL5A1* variant (21 SNPs located within and near the gene) and KC. A Caucasian case-control cohort of 222 patients with KC and 3324 control subjects was selected as the discovery panel. An independent case-control panel of 304 cases and 518 controls and a family panel of 186 individuals were used for replication of genotyping and association. Twenty-one SNPs located within and near *COL5A1* were genotyped, of which SNPs rs1536482 and rs7044529 were common to our study and the study by Lu et al.[21] Li et al.[32] showed that the SNPs rs1536482 ($P = 6.5 \times 10^{-3}$) and rs7044529 ($P = 7.4 \times 10^{-3}$) were significantly associated with KC in the discovery cohort. The SNP rs1536482 was replicated in the second case-control sample ($P = 0.02$), and the SNP rs7044529 was replicated in a KC family panel ($P = 0.03$). Meta-analysis significance levels were $P = 1.5 \times 10^{-4}$ (OR, 1.30) for rs1536482 and $P = 2.9 \times 10^{-3}$ (OR, 1.39) for rs7044529 in the KC cohorts. After Bonferroni correction, only the association of SNP rs1536482 remained significant ($P = 6.5 \times 10^{-3}$) (Supplementary Fig. S2). For the SNPs rs4894535 (in the *FNDC3B* gene) and rs2721051 (in the *FOXO1* gene), an association was detected in both the SA and US cohorts in the study by Lu et al.[21] In our study, SNP rs4894535 was not included in the analysis because of its low genotyping call rate. The SNP rs2721051 was estimated to have an OR similar to that previously reported by Lu et al., but in our study it was only borderline significant ($P = 0.057$).

We found no association of SNPs rs1536483 and rs7044529, previously associated with KC in a GWAS,[21] in the present independent cohort. However, we were able to replicate an association of rs1324183 and rs9938149 with KC in our cohort. The SNP rs2721051 demonstrated an effect size similar to that in previous investigations but did not achieve significance based on our data alone. Our results also suggest that there is unlikely to be a strong association between the five KC SNPs and corneal curvature. Therefore, the SNPs thus far identified and replicated are of moderate effect size. In addition, these SNPs explain only a small percentage of the variance of KC, so the genetic influences on KC are likely complex and many.

References

1. Kennedy RH, Bourne WM, Dyer JA. A 48-year clinical and epidemiologic study of keratoconus. *Am J Ophthalmol*. 1986; 101:267–273.

2. Williams KA, Lowe MT, Keane MC, Jones VJ, Loh RSK, Coster DJ. The Australian Corneal Graft Registry 2012 Report. 2012. Available at: http://hdl.handle.net/2328/25860. Accessed November 19, 2013.

3. Rabinowitz YS. The genetics of keratoconus. *Ophthalmol Clin North Am*. 2003;16:607–620. vii.

4. Karseras AG, Ruben M. Aetiology of keratoconus. *Br J Ophthalmol*. 1976;60:522–525.

5. Bawazeer AM, Hodge WG, Lorimer B. Atopy and keratoconus: a multivariate analysis. *Br J Ophthalmol*. 2000;84:834–836.

Investigative Ophthalmology & Visual Science

6. Beardsley TL, Foulks GN. An association of keratoconus and mitral valve prolapse. *Ophthalmology*. 1982;89:35–37.

7. Hartstein J. Keratoconus that developed in patients wearing corneal contact lenses: report of four cases. *Arch Ophthalmol*. 1968;80:345–346.

8. Falls HF, Allen AW. Dominantly inherited keratoconus. *J Genet Hum*. 1969;17:317–324.

9. McMahon TT, Shin JA, Newlin A, Edrington TB, Sugar J, Zadnik K. Discordance for keratoconus in two pairs of monozygotic twins. *Cornea*. 1999;18:444–451.

10. Owens H, Gamble G. A profile of keratoconus in New Zealand. *Cornea*. 2003;22:122–125.

11. Wang Y, Rabinowitz YS, Rotter JI, Yang H. Genetic epidemiological study of keratoconus: evidence for major gene determination. *Am J Med Genet*. 2000;93:403–409.

12. Toh T, Liew SH, MacKinnon JR, et al. Central corneal thickness is highly heritable: the twin eye studies. *Invest Ophthalmol Vis Sci*. 2005;46:3718–3722.

13. Landers JA, Hewitt AW, Dimasi DP, et al. Heritability of central corneal thickness in nuclear families. *Invest Ophthalmol Vis Sci*. 2009;50:4087–4090.

14. Zheng Y, Ge J, Huang G, et al. Heritability of central corneal thickness in Chinese: the Guangzhou Twin Eye Study. *Invest Ophthalmol Vis Sci*. 2008;49:4303–4307.

15. Cornes BK, Khor CC, Nongpiur ME, et al. Identification of four novel variants that influence central corneal thickness in multi-ethnic Asian populations. *Hum Mol Genet*. 2012;21:437–445.

16. Vitart V, Bencic G, Hayward C, et al. New loci associated with central cornea thickness include *COL5A1*, *AKAP13* and *AVGR8*. *Hum Mol Genet*. 2010;19:4304–4311.

17. Vithana EN, Aung T, Khor CC, et al. Collagen-related genes influence the glaucoma risk factor, central corneal thickness. *Hum Mol Genet*. 2011;20:649–658.

18. Hoehn R, Zeller T, Verhoeven VJ, et al. Population-based meta-analysis in Caucasians confirms association with *COL5A1* and *ZNF469* but not *COL8A2* with central corneal thickness. *Hum Genet*. 2012;131:1783–1793.

19. Lu Y, Dimasi DP, Hysi PG, et al. Common genetic variants near the brittle cornea syndrome locus *ZNF469* influence the blinding disease risk factor central corneal thickness. *PLoS Genet*. 2010;6:e1000947. Available at: http://www.ncbi.nlm.

nih.gov/pmc/articles/PMC2869325/. Accessed November 20, 2013.

20. Gao X, Gauderman WJ, Liu Y, et al. A genome-wide association study of central corneal thickness in Latinos. *Invest Ophthalmol Vis Sci*. 2013;54:2435–2443.

21. Lu Y, Vitart V, Burdon KP, et al. Genome-wide association analyses identify multiple loci associated with central corneal thickness and keratoconus. *Nat Genet*. 2013;45:155–163.

22. Baird PN, Schache M, Dirani M. The Genes in Myopia (GEM) study in understanding the aetiology of refractive errors. *Prog Retin Eye Res*. 2010;29:520–542.

23. Richardson AJ, Narendran N, Guymer RH, Vu H, Baird PN. Blood storage at 4°C: factors involved in DNA yield and quality. *J Lab Clin Med*. 2006;147:290–294.

24. Ovcharenko I, Nobrega MA, Loots GG, Stubbs L. ECR Browser: a tool for visualizing and accessing data from comparisons of multiple vertebrate genomes. *Nucleic Acids Res*. 2004;32:W280–W286.

25. Visel A, Minovitsky S, Dubchak I, Pennacchio LA. VISTA Enhancer Browser: a database of tissue-specific human enhancers. *Nucleic Acids Res*. 2007;35:D88–D92.

26. Kozomara A. Griffiths-Jones S. miRBase: integrating microRNA annotation and deep-sequencing data. *Nucleic Acids Res*. 2011;39:D152–D157.

27. Griffiths-Jones S, Grocock RJ, van Dongen S, Bateman A. Enright AJ. miRBase: microRNA sequences, targets and gene nomenclature. *Nucleic Acids Res*. 2006;34:D140–D144.

28. Griffiths-Jones S, Saini HK, van Dongen S. Enright AJ. miRBase: tools for microRNA genomics. *Nucleic Acids Res*. 2008;36:D154–D158.

29. Griffiths-Jones S. The microRNA Registry. *Nucleic Acids Res*. 2004;32:D109–D111.

30. Sherwin T, Brookes NH. Morphological changes in keratoconus: pathology or pathogenesis. *Clin Exp Ophthalmol*. 2004;32:211–217.

31. Emre S, Doganay S, Yologlu S. Evaluation of anterior segment parameters in keratoconic eyes measured with the Pentacam system. *J Cataract Refract Surg*. 2007;33:1708–1712.

32. Li X, Bykhovskaya Y, Canedo AL, et al. Genetic association of *COL5A1* variants in keratoconus patients suggests a complex connection between corneal thinning and keratoconus. *Invest Ophthalmol Vis Sci*. 2013;54:2696–2704.