Replication and Meta-Analysis of Candidate Loci Identified Variation at *RAB3GAP1* Associated With Keratoconus

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Purpose. Keratoconus is a common complex corneal ectasia that can lead to severe visual impairment. Although a genetic component is well recognized, the genetic risk factors for keratoconus are yet to be fully elucidated. A recent genome-wide association study (GWAS) by Li et al. identified 15 potentially associated single nucleotide polymorphisms (SNPs). Here, we aimed to replicate these associations, and conduct a meta-analysis of the current and previous studies.

Methods. We genotyped the 15 reported associated SNPs in 524 Australian Caucasian cases with keratoconus and 2761 controls. Association analysis was conducted in PLINK. A meta-analysis of this study with the adjusted *P* values of the previously published GWAS was conducted using the method of Fisher to combine *P* values.

Results. Our Australian cohort showed association ($P < 0.003$) at SNPs near *RAB3GAP1, KCND3*, *IMMPL2,* and in a gene desert on chromosome 13q33.3, providing evidence of replication of the published results. The meta-analysis showed SNP rs4954218 near *RAB3GAP1* gene was associated significantly with keratoconus, with $P = 9.26 \times 10^{-9}$ passing the genome-wide significance level.

Conclusions. Although the mechanism of disease association is yet to be determined, SNP rs4954218 is associated consistently with keratoconus and likely tags a functional variant that contributes to disease susceptibility.

Keywords: keratoconus, cornea, genetics, genome-wide association study, meta-analysis

Keratoconus is a bilateral noninflammatory corneal ectasia characterized by progressive thinning of the cornea. It leads to asymmetric bulging of the cornea, altering refractive power, and causing irregular astigmatism and severe visual impairment.[1–3] The disease progresses through the prime earning and child-rearing years, consequently resulting in high costs for health care system and the economy.[4,5] It is estimated that the prevalence of keratoconus varies from 50 to 230 individuals per 100,000 in the general population, dependent on ethnicity and definition of disease, with a reported annual incidence of 2 per 100,000.[3] Keratoconus is the most common indication for corneal transplantation in Australia.[6]

Keratoconus is a multifactorial disease, likely caused by the interaction of multiple disease susceptibility genes and environmental factors, such as contact lens wear, chronic eye rubbing, and atopy (allergy).[7,8] In recent years, several susceptibility genes have been reported by using family and case-control studies, and have provided strong evidence of a genetic component.[9]

Recently, a genome-wide association study (GWAS) for keratoconus was conducted in a United States-based Caucasian population.[10] The study identified 15 keratoconus-associated single nucleotide polymorphisms (SNPs) from 13 loci using multiple case-control and family-based cohorts. The meta-analysis of all three cohorts in the study revealed a SNP rs4954218, located near the *RAB3GAP1* gene, which was highly associated with keratoconus (adjusted $P = 1.6 \times 10^{-7}$), although not at genome-wide significance ($P < 5 \times 10^{-8}$).

RAB3GAP1 encodes the catalytic subunit of the heterodimeric enzyme RAB3GAP (RAB3GTPase-activating protein), a key regulator of the RAB3 cycle, which controls calcium-mediated exocytosis of neurotransmitters and hormones.[11] Mutations in *RAB3GAP1* cause Warburg Micro syndrome (OMIM 600118) and Martsolf syndrome (OMIM 212720), phenotypically overlapping autosomal recessive conditions characterized by ocular and neurodevelopmental manifestations.[12,13]

In our study, we evaluated the 15 GWAS-derived putative keratoconus susceptibility SNPs to investigate their association with keratoconus and provide further evidence for their association, if it exists. We genotyped an independent cohort of Australian Caucasian keratoconus cases and controls. Meta-analysis of this study with the US study then was applied to combine current replication data with previous GWAS results.

Methods

Patient Recruitment

The protocol was approved by the Southern Adelaide Clinical Human Research Ethics Committee. All participants gave

informed consent, and the study conformed to the tenets of the Declaration of Helsinki. Participants with keratoconus (n = 524) were ascertained through the eye clinic of Flinders Medical Centre, Adelaide, South Australia, Australia, through optometry clinics in Adelaide and Melbourne, or through an Australia-wide invitation to members of Keratoconus Australia, a community-based support group for patients. Clinical data were obtained from the participants' eye care practitioner. Unaffected controls were participants of the Blue Mountains Eye Study, a population-based study of older Australians aged 50+ years living in two postcode areas of the Blue Mountains, west of Sydney, Australia. The cohort has been previously described.[14]

Definition of Keratoconus

The diagnosis of keratoconus was based on clinical examination and videokeratography pattern analysis using the instrumentation available in each contributing practice and has been described previously.[15] Clinical examination included slit-lamp biomicroscopy, cycloplegic retinoscopy, and fundus evaluations. Slit-lamp biomicroscopy was used to identify stromal corneal thinning, Vogt's striae, or a Fleischer ring. Retinoscopy examination was performed with a fully dilated pupil to determine the presence or absence of retro-illumination signs of keratoconus, such as the oil droplet sign and scissoring of the red reflex. Videokeratography evaluation was performed on each eye using the Orbscan (Orbtek; Bausch & Lomb, Rochester, NY). Patients were classified as having keratoconus if they had at least one clinical sign of keratoconus and a confirmatory videokeratography.[16] A history of penetrating keratoplasty performed because of keratoconus also was sufficient for inclusion as a case. All control participants also had a detailed eye examination, including slit-lamp biomicroscopy to exclude severe keratoconus. Forme fruste keratoconus was not excluded specifically in controls and, thus, a small number of controls may have been misclassified.

Genotyping

The 15 previously reported SNPs[10] were genotyped in cases using iPLEX Gold chemistry (Sequenom, Inc., San Diego, CA) on an Autoflex MassArray mass spectrometer (Sequenom, Inc.). The controls were previously typed on the HumanHap610 Genome-wide SNP array (Illumina, Inc., San Diego, CA) and the genotype data for the 15 SNPs of interest were extracted.

Analysis

Genotype data from Australian cases and controls were checked for presentation on the same strand, flipped if necessary and merged using PLINK.[17] SNPs were assessed for deviation from Hardy Weinberg equilibrium and association analysis under a χ^2 test for allelic association was conducted. Logistic regression under an additive genetic model in PLINK was used to adjust for the effects of sex. To correct for multiple testing of 15 SNPs, a Bonferroni correction was applied. A P value of less than 0.003 was considered statistically significant for the replication analysis.

A meta-analysis was conducted for SNPs where the direction of association was consistent between this study and the previously reported GWAS.[10] P values were combined using the method of Fisher as no standard error or allele frequency information was available for the published cohorts. The P values utilized from the published study were the combined analysis of the discovery and both replication cohorts, adjusted for principal components. For four SNPs, replication data were not available from the US cohort and, thus, the P value from the discovery cohort alone was used for the current meta-analysis. Published P values were combined with the sex-adjusted P value from the current Australian study. Genome-wide significance was set at a P value of $<5 \times 10^{-8}$.

Results

The Australian cohort consisted of 524 patients with keratoconus and 2761 examined controls. The cases had a mean age of 43.2 ± 15.4 years, whereas the controls were significantly older with a mean age of 69.8 ± 10.1 ($P < 0.001$), reflecting the choice of the older control cohort to exclude patients yet to have keratoconus. The sex distribution was similar between cases and controls (45% female cases compared to 43% in controls, $P = 0.34$). All SNPs met the requirements of Hardy-Weinberg equilibrium ($P > 0.003$, accounting for 15 SNPs tested).

In total, 5 of the 15 SNPs assessed showed significant replication in the Australian sample ($P < 0.003$, Table 1). The most significant SNP was rs4839200 near *KCND3*, with a P value of 1.40×10^{-4} and odds ratio (OR, 95% confidence interval [CI]) of 1.43 (1.19–1.72). The strongest SNP from the US GWAS, rs4954218 near *RAB3GAP1*, also was significantly associated in this sample with $P = 3.5 \times 10^{-4}$. Four additional SNPs, at two loci, also reached the threshold for significance. These were rs757219 and rs214884 near *IMMPL2*, and rs1328083 and rs1328089 on chromosome 13q33.3 (Table 1). Following an adjustment for the effects of sex, the same 6 SNPs remained significant (Table 1).

Meta-analysis was performed to combine the sex-adjusted results with the association results reported in the previous US-based study[10] (Table 2). The published study reported P values for the discovery cohort and two replication cohorts, as well as a meta-analysis of all 3 cohorts. The meta-analysis P values were used in the current meta-analysis of both countries. In the Australian sample, two SNPs, (rs3749350 and rs1428642) had effect directions inconsistent with that published in the US study[10] and, thus, were excluded from the meta-analysis as nonreplications. Of the remaining 13 SNPs, 7 showed improved significance on meta-analysis, while 6 showed reduced significance. Following meta-analysis, SNP rs4954218 near *RAB3GAP1* was the most significantly associated with keratoconus ($P = 9.26 \times 10^{-9}$) and was the only SNP to reach genome-wide significance. The top ranked SNP from the Australian study, rs4839200 in *KCND3*, approached genome-wide significance ($P = 1.61 \times 10^{-7}$) and was associated more significantly than in the US study alone. The two SNPs near *IMMPL2* (rs757219 and rs214884) showed increased significance under meta-analysis. The two 13q33.3 SNPs (rs1328083 and rs1328089) and rs6430585 near *UBXD2* also show a trend toward association and an improvement of significance when both studies are combined.

Discussion

This replication and meta-analysis study showed that genetic variation upstream of the *RAB3GAP1* gene is highly likely to be a contributor to the genetic risk of keratoconus development. The previously published GWAS study and our replication study support its association, and together provided evidence at genome-wide significance for the association of SNP rs4954218 with keratoconus. We also provided supportive evidence for SNPs near *KCND3*, *IMMPL2*, and in a gene desert on chromosome 13q33.3, although even in meta-analysis, these SNPs do not reach genome-wide significance. Although these regions were highly ranked in the GWAS discovery cohort, the

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Table 1. Association Results in Australian Sample and SNPs Reported Associated With Keratoconus in a US Cohort

SNP	Gene/Locus	Alleles		MAF		*P* Value	OR (95% CI)	*P* Adj
		1	2	Cases	Controls			
rs4839200*	*KCND3*	A	G	0.165	0.121	1.40×10^{-4}	1.43 (1.19–1.72)	2.11×10^{-5}
rs12407427	*KIF26B*	T	C	0.128	0.125	0.820	1.02 (0.82–1.25)	0.563
rs4954218*	*RAB3GAP1*	G	T	0.251	0.306	3.50×10^{-4}	0.76 (0.65–0.88)	0.003
rs6430585	*UBXD2*	A	C	0.201	0.171	0.021	1.22 (1.03–1.44)	0.023
rs3749350	*LRRN1*	T	G	0.120	0.133	0.250	0.89 (0.72–1.09)	0.115
rs6442925	*BHLHB2*	T	C	0.168	0.163	0.670	1.04 (0.87–1.24)	0.830
rs6792542	3q26.2	C	A	0.250	0.250	1.000	1.00 (0.85–1.17)	0.637
rs2659546	*PPP3CA*	A	G	0.056	0.052	0.630	1.08 (0.80–1.44)	0.852
rs757219*	*IMMPL2*	C	T	0.174	0.136	0.001	1.34 (1.12–1.60)	7.21×10^{-5}
rs214884*	*IMMPL2*	G	A	0.110	0.078	0.001	1.45 (1.17–1.81)	1.36×10^{-4}
rs1978238	12p13.3	C	A	0.369	0.366	0.880	1.01 (0.88–1.16)	0.708
rs1328083*	13q33.3	G	T	0.204	0.159	3.50×10^{-4}	1.36 (1.15–1.61)	9.03×10^{-4}
rs1328089*	13q33.3	C	T	0.297	0.251	0.002	1.26 (1.09–1.46)	0.003
rs8111998	19p12	T	C	0.069	0.054	0.054	1.30 (1.00–1.71)	0.191
rs1428642	*BIRC8*	A	G	0.483	0.479	0.780	1.02 (0.89–1.16)	0.641

Allele 1 is the minor allele. MAF, minor allele frequency; *P* Adj, *P* value adjusted for sex.
* Significantly associated SNPs.

SNP near *KCND3* (rs4839200) and one of the 13q33.3 SNPs rs1328089 were not able to be assessed in the US replication cohorts, and, thus, to our knowledge our report provided the first attempt at replicating these findings in a comparable population. While the reported association at *IMMPL2* (rs214884 and rs757219) was not consistent among all three US cohorts, our Australian cohort provided additional evidence that this locus is likely to contribute to keratoconus risk.

Nine of the 15 SNPs were not associated to any degree in the Australian cohort, including 2 regions ranked highly by the US GWAS discovery cohort (rs6442925 near *BHLB2* and rs1428642 near *BIRC8*), suggesting that the effects observed at these SNPs in the US GWAS may be false positives. This is consistent with the results reported in the two US replication cohorts, which also failed to find association at these variants.

The published GWAS utilized, in total, 672 cases and 4003 unaffected controls, including a family study. The current Australian study included 524 cases and 2761 controls, and, thus, meta-analysis of these two studies almost doubles the size of the study and, thus, provides sufficient power to observe the association of rs4954218 at genome-wide significance.

The Australian cases and controls were typed independently of each other using different technologies. This could lead to a false association caused by different genotyping error rates between the two platforms. However, of the 15 SNPs typed, there was no consistent or systematic bias in allele frequencies observed between cases and controls, although this does not rule out bias at an individual SNP. When directly comparing the observed OR, the effect sizes are smaller or equivalent in the Australian cohort than the first published finding, and 13 of 15 SNPs are in the same direction, as would be expected for a replication study.

Through this meta-analysis, SNP rs4954218 has been confirmed as a keratoconus risk locus. It demonstrates association in all 4 cohorts tested to date, although an additional replication is required formally to complement the

Table 2. Results of Meta-Analysis of Previously Reported Association Results From the US-Based Study and the Australian Study

SNP	Gene	Minor Allele	OR US Study	*P* Value US Study	Meta *P* Value
rs4839200*	*KCND3*	A	1.79	3.90×10^{-4}	1.61×10^{-7}
rs12407427*	*KIF26B*	T	1.79	4.10×10^{-5}	2.69×10^{-4}
rs4954218†	*RAB3GAP1*	G	0.62	1.60×10^{-7}	9.26×10^{-9}
rs6430585	*UBXD2*	A	1.39	0.002	4.96×10^{-4}
rs3749350	LRRN1	T	1.39	1.40×10^{-4}	N/A
rs6442925	*BHLHB2*	T	1.56	9.60×10^{-5}	9.60×10^{-4}
rs6792542	*3q26.2*	C	1.31	0.003	0.014
rs2659546	*PPP3CA*	A	1.63	0.021	0.090
rs757219	*IMMPL2*	C	1.36	0.006	6.77×10^{-6}
rs214884	*IMMPL2*	G	1.44	0.007	1.42×10^{-5}
rs1978238*	*12p13.3*	C	0.64	3.40×10^{-6}	3.35×10^{-5}
rs1328083	*13q33.3*	G	1.31	0.0110	1.24×10^{-4}
rs1328089*	*13q33.3*	C	1.61	8.8×10^{-4}	3.47×10^{-5}
rs8111998	*19p12*	T	1.71	4.90×10^{-4}	9.60×10^{-4}
rs1428642	*BIRC8*	A	0.84	0.014	N/A

The previously reported OR and *P* values from the US study also are given. N/A, meta-analysis not conducted due to opposite direction of effect at these SNPs.
* Meta-analysis includes *P* value from the US discovery cohort and Australian sample only. US replication cohort data not available for these SNPs.
† Genome-wide significant results.

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genome-wide significant result obtained here. This SNP is located 6.4 kb upstream of the *RAB3GAP1* gene. While the SNP itself is not located in any known regulatory element, as reported by the ENCODE project and others available on the University of California, Santa Cruz (UCSC) genome browser (available in the public domain at http://genome.ucsc.edu, accessed November 30 2012), it is in close proximity to the promoter of this gene. The SNP sits in the middle of a 130 kb block of linkage disequilibrium (LD) in Caucasians (HapMap; available in the public domain at www.hapmap.org), extending from SNP rs4953945 to rs6749119. It is in strong LD ($D' = 1.0$) with the majority of common SNPs in this block. A notable feature in this LD block is an H3K27Ac mark identified in 7 ENCODE Project cell lines, overlapping with exons 1 and 2 of the *RAB3GAP1* gene. This type of acetylation mark typically is found near active regulatory elements. The associated SNP rs4954218 may be tagging variation associated with this, or other, regulatory elements in the linkage disequilibrium block, although further genetic and functional investigation is required to confirm this, and elucidate the role of the SNP in corneal biology.

In conclusion, our replication study and meta-analysis has reported a genome-wide significant association of SNP rs4954218 upstream of *RAB3GAP1* with keratoconus and provided additional support for the association of SNPs near *KCND3*, *IMMPL2* and in a gene desert on chromosome 13q33.3. It appears that the genetics of this complex disease also are complex, with multiple genes of moderate effect size contributing to keratoconus risk. The genetics of keratoconus have long remained elusive, but well powered GWAS and meta-analysis studies now are able to begin to dissect the genetic contribution to this complex disease.

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