Genetic variation on chromosome 11 is associated with keratoconus

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Key points

Question Which genetic loci associate with keratoconus?

Findings In this case-control genome-wide association study, with three independent replication cohorts, a locus containing multiple variants across six protein-coding genes on chromosome 11 was associated with keratoconus. Several of these genes are likely involved in apoptotic pathways.

Meaning This study of patients with keratoconus demonstrates a potential role of genes involved in apoptotic pathways.

Abstract

Importance Keratoconus is a condition in which the cornea progressively thins and protrudes in a conical shape, severely affecting refraction and vision. It is a major indication for corneal transplantation. To discover new genetic loci associated with keratoconus and to better understand the causative mechanism of this disease, we performed a genome-wide association study (GWAS) on patients with keratoconus.

Objective To identify keratoconus genetic susceptibility regions in the human genome.

Design The discovery cohort was genotyped using the Illumina HumanCoreExome single nucleotide polymorphism (SNP) array. Following quality control and data cleaning, genotypes were imputed against the 1000 Genomes Project reference panel (Phase III, version 5) and association analysis was completed using PLINK. SNPs with $P < 1 \times 10^{-6}$ were assessed for replication in three additional cohorts.

Setting Eye clinics in Australia, the USA, and Northern Ireland.

Participants The discovery cohort comprised 522 Australian keratoconus cases and 655 Australian controls. Controls were drawn from the Blue Mountains Eye Study and normal individuals previously recruited as controls for a study of glaucoma. Replication cohorts were a previous keratoconus GWAS dataset from the USA (222 cases, 2927 controls); 331 cases and 229 controls from Australia and Northern Ireland (AUS+NI), and an Australian case–control cohort (VIC, 265 cases, 702 controls).

Main Outcomes and Measures Associations between keratoconus and 6,252,612 genetic variants were estimated using logistic regression, were adjusting for ancestry using the first three principal components.

Results Two novel loci reached genome-wide significance (defined as $P < 5 \times 10^{-8}$), with $P = 7.46 \times 10^{-9}$ at rs61876744 in *PNPLA2* on chromosome 11 and $P = 6.35 \times 10^{-12}$ at rs138380 2.2kb upstream of *CSNK1E* on chromosome 22. One additional locus was identified with $P < 10^{-6}$, in *MAML2* on chromosome 11. The novel locus in *PNPLA2* reached genome-wide significance in meta-analysis of all four cohorts ($P = 2.45 \times 10^{-8}$).

Conclusions and Relevance In this relatively large keratoconus GWAS, we identified a genome-wide significant locus for keratoconus in the region of *PNPLA2* on chromosome 11.

Introduction

Keratoconus is characterized by progressive thinning of the cornea, the clear tissue at the front of the eye. Asymmetrical bulging and conical protrusion of the cornea leads to extreme refractive error (myopia and irregular astigmatism) causing severe visual impairment.[1] Keratoconus is relatively common, with a reported prevalence of around 55 per 100,000 in white populations[1] and up to 229 per 100,000 in Asian populations.[2] Due to recent advances in diagnostic imaging the true incidence and prevalence of keratoconus may be 5 to 10 times higher than previously reported.[3] The etiology of keratoconus is poorly understood. Links have been made to eye rubbing and atopy, but no direct causative connection has been established.[4] Various biochemical pathways may be involved, including oxidative stress, apoptosis, and disruption to extracellular matrix turnover.[5]

Although many cases of keratoconus present as sporadic, there is a well-recognised genetic component to the disease. The estimated prevalence in relatives of keratoconus patients is 3.34% (95% CI: 3.22–3.46%), which is 15–67 times higher than in the general population.[6] In addition, more than 20 syndromes are associated with keratoconus, including Down syndrome, Leber congenital amaurosis, and several connective tissue disorders.[7] Linkage studies have identified at least 16 loci for keratoconus;[8,9] however, the causative genes and variants have remained elusive. Common variants in the *DOCK9*[10] and *LOX*[11] genes have been implicated as well as rare mutations in *MIR184,*[12,13] although these loci have not been broadly replicated. Genome-wide association studies (GWAS) have implicated several loci. Variation in the promoter region of the *HGF* gene[14,15] and upstream of *RAB3GAP1*[16,17] have both been associated in multiple independent studies. Further, a GWAS for central corneal thickness (CCT) identified loci that are also associated with keratoconus, including *RXRA–COL5A1, FOXO1* and *FNDC3B,*[18] and more recently a suggestive association at *DCN*.[19]

We present findings from a GWAS of 522 patients with keratoconus, a relatively large sample for this complex disease. We show association and independent replication at the *PNPLA2* region with keratoconus.

Methods

Study design

We report a study of 4 independent Caucasian cohorts of patients with keratoconus. The first case–control cohort used for the discovery phase comprised 522 keratoconus patients and 655 controls. All single nucleotide polymorphisms (SNPs) with $P < 1 \times 10^{-6}$ were looked up in imputed genotypes from a previously published GWAS study of 222 keratoconus patients and 3324 controls from the USA. In addition, 27 SNPs were genotyped in an independent replication cohort of 331 cases and 229 controls from Australia and Northern Ireland (AUS+NI), and in an additional 265 cases and 702 controls from Victoria, Australia (VIC). The demographics for each cohort are given in Table 1.

Discovery cohort

The protocol was approved by the Southern Adelaide Clinical Human Research Ethics Committee (HREC) and the HREC of the Royal Victorian Eye and Ear Hospital and the Health and Medical HREC of the University of Tasmania. All participants gave written informed consent and the study conformed to the tenets of the Declaration of Helsinki. Participants with keratoconus were ascertained through the eye clinic of Flinders Medical Centre, Adelaide; optometry and ophthalmology clinics in Adelaide and Melbourne; or 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.

The diagnosis of keratoconus was based on both clinical examination and videokeratography pattern analysis as described previously.[20] Clinical examination included slit-lamp biomicroscopy, cycloplegic retinoscopy, and fundus evaluation. Slit-lamp biomicroscopy was used to identify stromal corneal thinning, Vogts' striae, or a Fleischer ring. Retinoscopy examination was performed on 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, Salt Lake City, UT, USA). Patients were classified as having keratoconus if they had at least one clinical sign of keratoconus and a confirmatory videokeratography.[21] A history of penetrating keratoplasty performed because of keratoconus was also sufficient for inclusion as a case. Patients with syndromic forms

of keratoconus were excluded, and if multiple individuals from the same family presented, only one was included.

Controls were obtained from the Australian cohort previously described in a GWAS for age-related macular degeneration (AMD) from the International AMD Genomics Consortium[22] and have been described in detail previously. For the current analysis, data from 676 Australian unaffected controls (including 465 from the Blue Mountains Eye Study[23] and 211 normal individuals previously recruited as controls for a study of glaucoma[24]) were combined as controls for keratoconus. Related individuals and those who did not pass all sample QC for the AMD GWAS were excluded.

DNA for cases and controls was extracted from whole blood using the QiaAMP DNA Maxi kit (Qiagen, Hilden, Germany).

Genotyping and data quality control

Cases were genotyped for 551,839 variants using the HumanCoreExome array (HumanCoreExome-24v1-1_A, Illumina, San Diego, CA, USA). For the controls, genotypes of 569,645 variants were generated with a customized Illumina HumanCoreExome array ("HumanCoreExome_Goncalo_15038949_A") as described previously.[22]

Quality control was carried out according to the protocol described by Anderson et al[25], modified as follows. Reverse and ambiguous strand SNPs were detected using snpflip (https://github.com/biocore-ntnu/snpflip, accessed March 24, 2017) and flipped or excluded. Only SNPs common to both arrays were included. Individuals with discordant sex information, missing genotype rate >0.05, or heterozygosity more than 3 standard deviations from the mean were excluded. Related individuals were detected by calculating pairwise identity by descent (IBD), and the individual with the lower genotyping rate in any pair with IBD >0.185 was removed. Ancestry outliers were identified by principal component analysis (PCA) using EIGENSTRAT,[26] and removed. Markers were excluded if they had missing genotype rate >3%, significantly different missing data rates between cases and controls, minor allele frequency (MAF) <0.01, or deviated significantly ($P < 10^{-5}$) from Hardy–Weinberg equilibrium. Following all exclusions, there were 522 cases and 655 controls genotyped for 264,115 common platform SNPs.

Genomic imputation and association analysis

We phased autosomal genotype data using Eagle (version 2.3.5)[27] and then imputed genotypes on the basis of the EUR subset of the 1000 Genomes Project reference panel (Phase III, version 5)[28] using Minimac3 (version 2.0.1).[29] We excluded indels, SNPs within 5bp of an indel, rare variants (MAF <0.01), and variants with poor imputation quality ($R^2 < 0.8$). This filtering yielded a total of 6,252,612 quality-controlled variants, including 250,964 genotyped variants. Association analysis was performed on most-likely genotypes under a logistic regression model using PLINK (version 1.90)[30] using the first three principal components as covariates. A P value less than 5×10^{-8} was considered significant.

Replication and meta-analysis

The USA cohort has been previously described.[18] Briefly, clinically affected Caucasian keratoconus cases (n = 240) were enrolled into the GWAS[31] as a part of the longitudinal videokeratography and genetic study at the Cornea Genetic Eye Institute.[6] After removing samples with poor genotyping quality, 222 samples were included in the analysis. Caucasian controls (n = 3324) were obtained from the Cardiovascular Health Study (CHS), a population-based cohort study of risk factors for cardiovascular disease and stroke in adults 65 years of age or older, recruited at 4 field centres.[32,33] CHS was approved by the Institutional Review Board at each recruitment site, and subjects provided informed consent for the use of their genetic information. African-American CHS participants were excluded from analysis due to insufficient number of ethnically-matched cases. Participants did not have an eye examination to exclude keratoconus. The samples included in the analysis were from self-reported Caucasians. Outliers detected by PCA were excluded, and the analysis was adjusted for the top 3 principal components.

IMPUTE version 2.3.0 was used to perform imputation of the genotyping data from 370K BeadChip arrays (Illumina) in keratoconus patients and CHS Caucasian controls using 1000 Genomes Phase I data as the reference panel. All SNPs with $P < 10^{-6}$ identified in the discovery analysis were looked up and extracted, with the exception of 4 SNPs at the *CSNK1E* locus that were not imputed in the USA study.

SNPs with $P < 10^{-6}$ in the discovery cohort were selected for genotyping in additional replication cohorts using the MassARRAY® System (Agena Bioscience, San Diego, CA, USA) by the Australian Genome Research Facility. SNPs were chosen from each locus that were compatible with the assay design, with

preference given to SNPs with the smallest *P* value in the discovery cohort. Twenty-seven SNPs were genotyped in 186 additional cases recruited under the same protocol as the discovery cohort as well as in 145 patients from Northern Ireland, described previously,[14] for a total of 331 cases. Cases were compared to 229 unaffected examined controls, consisting of 84 individuals from the Blue Mountains Eye Study not included in the discovery cohort and 145 older individuals recruited from nursing homes in the Launceston area of Tasmania, Australia. All controls underwent a thorough ocular examination and keratoconus was excluded. These SNPs were also genotyped in a replication cohort from Melbourne, Victoria, consisting of 265 cases and 702 examined controls described previously.[15]

Association was assessed in each cohort individually using logistic regression. Meta-analysis of results from discovery and replication cohorts was performed using METAL.[34]

Functional annotation of associated variants

The lead SNP at the novel locus on chromosome 11, rs61876744, was queried in HaploReg V4.1 (https://pubs.broadinstitute.org/mammals/haploreg/haploreg.php; accessed 28 May 2019),[35] including data from the Genotype–Tissue Expression (GTex) pilot analysis.[36] Genes in the associated region were assessed for ocular tissue expression using the Ocular Tissue Database (https://genome.uiowa.edu/otdb/)[37] and for differential expression between corneas from keratoconus and myopia patients in a previously published study.[38]

Results

Genome-wide association testing of the discovery cohort

Genome-wide association analysis was conducted in the discovery cohort (Figure 1, eFigure 1 in the Supplement). The genomic inflation factor was $\lambda_{1000} = 1.023$ and all included samples were of European ancestry (eFigure 2 in the Supplement). Two novel loci reached genome-wide significance (defined as $P < 5 \times 10^{-8}$), with $P = 7.46 \times 10^{-9}$ at rs61876744 in the *PNPLA2* gene on chromosome 11 and $P = 6.35 \times 10^{-12}$ at rs138380 2.2kb upstream of the *CSNK1E* gene on chromosome 22. One additional locus was identified with $P < 10^{-6}$, in the *MAML2* gene on chromosome 11 (rs10831500, $P = 3.91 \times 10^{-7}$). Association results for all

SNPs with $P < 10^{-6}$ are shown in eTable 1 in the Supplement. Locus-specific plots for all 3 loci are shown in eFigure 3 in the Supplement.

The locus on chromosome 11 showing genome-wide significance included 25 SNPs with $P < 10^{-6}$ spanning 6 protein-coding genes (*CEND1*, *SLC25A22*, *PANO1*, *PIDD1*, *RPLP2* and *PNPLA2*) and multiple RNA genes.

Because previously-reported keratoconus risk loci were initially identified in GWAS for CCT, we assessed each of the loci reaching suggestive significance in the current analysis in our previously reported meta-analysis for CCT. As shown in Table 2, only the *MAML2* locus shows nominal association with CCT. We also looked up the lead SNPs from our CCT meta-analysis in the results from our discovery cohort (eTable 2 in the Supplement). SNPs at *FNDC3B*, *MPDZ*, and *SMAD3* show nominal association in our analysis.

Association testing of the replication cohorts

All SNPs with $P < 10^{-6}$ identified in the discovery cohort were analysed in the previously generated GWAS data for the USA cohort, and 27 SNPs compatible with a single assay design were genotyped in the AUS+NI and VIC replication cohorts. (Table 3 and eTable 3 in the Supplement).

Multiple SNPs in both novel loci at *PNPLA2* and *MAML2* showed association with $p < 0.05$ in the USA cohort. None of the SNPs in the *CSNK1E* locus reached significance, although all showed the same direction of association as in the discovery cohort. None of the 3 loci reached significance in either the AUS+NI or the VIC replication cohort, but most SNPs showed the same direction of association as the discovery cohort. MAFs for cases and controls in each cohort are given in eTable 3 in the Supplement.

Meta-analysis

Meta-analysis of the combined data from the discovery and all 3 replication cohorts found 12 SNPs at the novel *PNPLA2* locus to be associated with keratoconus at genome-wide significance ($P < 5 \times 10^{-8}$). The *MAML2* locus on chromosome 11 showed suggestive association with $P = 3.83 \times 10^{-6}$ at rs10831500. The *CSNK1E* locus on chromosome 22 reached $P = 3.18 \times 10^{-4}$ at rs138378. For all 3 loci, there were other SNPs with smaller *P* values on meta-analysis, but these did not include data for all replication cohorts (eTable 3 in the Supplement).

Functional annotations of novel associated loci

At the significant locus on chromosome 11, the lead SNP rs61876744 is located in the second intron of the *PNPLA2* gene (NM_020376.3). The associated region extends for around 40kbp encompassing multiple transcripts. The *PNPLA2* gene is highly expressed in all eye tissues assessed in the ocular tissue database, including cornea, as are other protein-coding genes at this locus (eTable 4 in the Supplement). A recent study compared gene expression in corneal epithelium from keratoconus and myopia patients,[38] and found that *PNPLA2* and *PIDD1* were differentially expressed with a false discovery rate (FDR) < 0.05 (eTable 4 in the Supplement) while *RPLP2* and *CSNK1E* are significant at FDR < 0.1.

HaploReg identified 7 SNPs in strong linkage disequilibrium with the lead SNP (rs61876744), and all report an eQTL for an antisense RNA transcript *AP006621* (multiple transcripts 1–8) in multiple tissues assessed in GTex (eTable 5 in the Supplement) where the more common allele, C, is associated with increased transcript levels (eFigure 4 in the Supplement). This RNA gene is not represented in the ocular tissue database. A similar trend is seen in GTex for the *PIDD1* gene in sun exposed skin (eFigure 4 in the Supplement).

Discussion

This study has identified a candidate locus for keratoconus on chromosome 11 that shows replication in the USA data and consistent direction of association in the other cohorts. The lead SNP is located in an intron of *PNPLA2*. This gene encodes Patatin-like phospholipase domain-containing protein 2, which catalyzes the initial step in triglyceride hydrolysis. The relevance of this pathway to keratoconus is not obvious, but it is well known that the closest gene to an association signal is not necessarily the causative gene. At least 4 other protein coding genes at this locus are also expressed in the cornea, and RNA coding genes are also annotated in the region. There is a strong eQTL signal of the lead SNP rs61876744 for an antisense RNA gene, *AP006621.8* located on the opposite strand to the protein coding *PNPLA2* gene. The antisense RNA *AP006621* transcripts may have a role in regulating *PNPLA2* or other genes at this locus and elsewhere. The minor allele at rs61876744, T, is associated with reduced risk of keratoconus and with reduced expression of *AP006621* in many tissues. This suggests that overexpression of *AP006621* may destabilise corneal structures. Oxidative stress and apoptosis have been suggested as part of the pathogenesis of keratoconus[5] and sun (or UV light) exposure is known to trigger oxidative stress and DNA damage pathways.[39] Several

genes at this locus likely play a role in apoptotic pathways, including *PPID* (p53-induced death domain protein 1) and *PANO1* (proapoptotic nucleolar protein 1).

The chromosome 11 locus overlaps with a previously reported (although not genome-wide significant) association signal for Fuchs Endothelial Corneal Dystrophy (FECD).[40] The lead SNP in the FECD GWAS is rs12223324 in the *PDDC1* gene, upstream of *PNPLA2.* This SNP does not reach significance in our keratoconus GWAS, ($P = 2.02 \times 10^{-5}$). It is not known how this locus might lead to FECD, but the overlap of genetic association with keratoconus is intriguing, given both diseases affect the cornea. Although rare, there are reports in the literature of patients with both FECD and keratoconus.[41,42] The participants in the current study do not have FECD and thus this disease does not account for the association observed here.

Although we observed the strongest association in the discovery cohort at the *CSNK1E* locus, this result was not replicated. The signal appears to be driven by a single genotyped SNP that has influenced the imputation of a surrounding LD block (see eTable 1; eFigure 3C in the Supplement). The signal at the *MAML2* locus is supported by the USA replication cohort. Further replication of these loci is required before any firm conclusions can be drawn.

All control cohorts used in this study were older than the case cohorts, thus it will be important to assess these loci for age-related effects in future studies. Batch effects are a potential problem in an analysis where cases and controls are genotyped separately; however, the low inflation factor seen in our analysis reassures us that batch effects are unlikely to be having a major impact.

Previous GWAS for keratoconus (using a subset of cases involved in the current study) reported *RAB3GAP1*[31] and a region upstream of the *HGF*,[14] although neither study reached genome-wide significance. The lead SNPs at these loci reach *P* values of 0.066 and 0.038 respectively in the current discovery cohort, suggesting these loci are not major contributors in this better powered study. Previously reported genome-wide significant keratoconus associated loci (*RXRA–COL5A1, FOXO1* and *FNDC3B)* are also associated with CCT. The current findings suggest that mechanisms other than susceptibility to a thinner cornea may also be at play in the genetic risk of keratoconus.

In summary, we have identified a locus for keratoconus on chromosome 11. The lead SNP is in an intron of the *PNPLA2* gene and is an eQTL for a long non-coding RNA *AP006621.8*. We also suggest loci near

MAML2 and *CSNK1E* that require further replication. It is very likely that additional risk loci exist for keratoconus, and larger studies will be needed to identify them.

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Conflict of Interest:

None of the authors has any conflicts of interest to disclose.

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Figure 1: Manhattan plot of association results in the discovery cohort. Results of logistic regression with the first three PCs as covariates ($-\log_{10}$ *p*-values) are plotted for each chromosome. The red and blue lines represent the genome-wide significance threshold of $p = 5 \times 10^{-8}$ and our threshold for follow-up of $p = 10^{-6}$, respectively.

Table 1: Demographics of the Australian discovery cohort and the three replication cohorts.

Cohort	Cases			Controls		
	N	% male	Mean age [SD]	N	% male	Mean age [SD]
Discovery: Australia	522	56	45 [15.2]	655	47	65 [10.6]
Replication: USA	222	55	44 [13.3]	2927	39	72 [5.4]
Replication: AUS+NI	331	61	41 [15.9]	229	37	75 [11.5]
Replication: VIC	265	60	35 [14.9]	702	38	52 [15.2]

Table 2: Lead SNPs at all three loci with $p < 1 \times 10^{-6}$ in discovery cohort.

Locus[a]	Chr	Lead SNP	Base pair[b]	A1/2	F_A[c]	F_U[c]	OR[d] [95% CI]	*P*	*P* (CCT)[e]
PNPLA2	11	rs61876744	820754	T/C	0.341	0.447	0.59 [0.49–0.71]	7.46×10^{-9}	0.553
MAML2	11	rs10831500	95982642	G/T	0.422	0.330	1.59 [1.33–1.91]	3.91×10^{-7}	0.014
CSNK1E	22	rs138380	38796629	G/A	0.384	0.524	0.49 [0.40–0.60]	6.35×10^{-12}	0.790

[a]Locus assigned to the RefSeq protein-coding gene within or near the association signal interval.

[b]Genomic positions are based on hg19.

[c]F_A, minor allele frequency in cases; F_U, minor allele frequency in controls.

[d]Odds ratios with respect to A1.

[e]*P*-values in our previously reported analysis for CCT.[19]

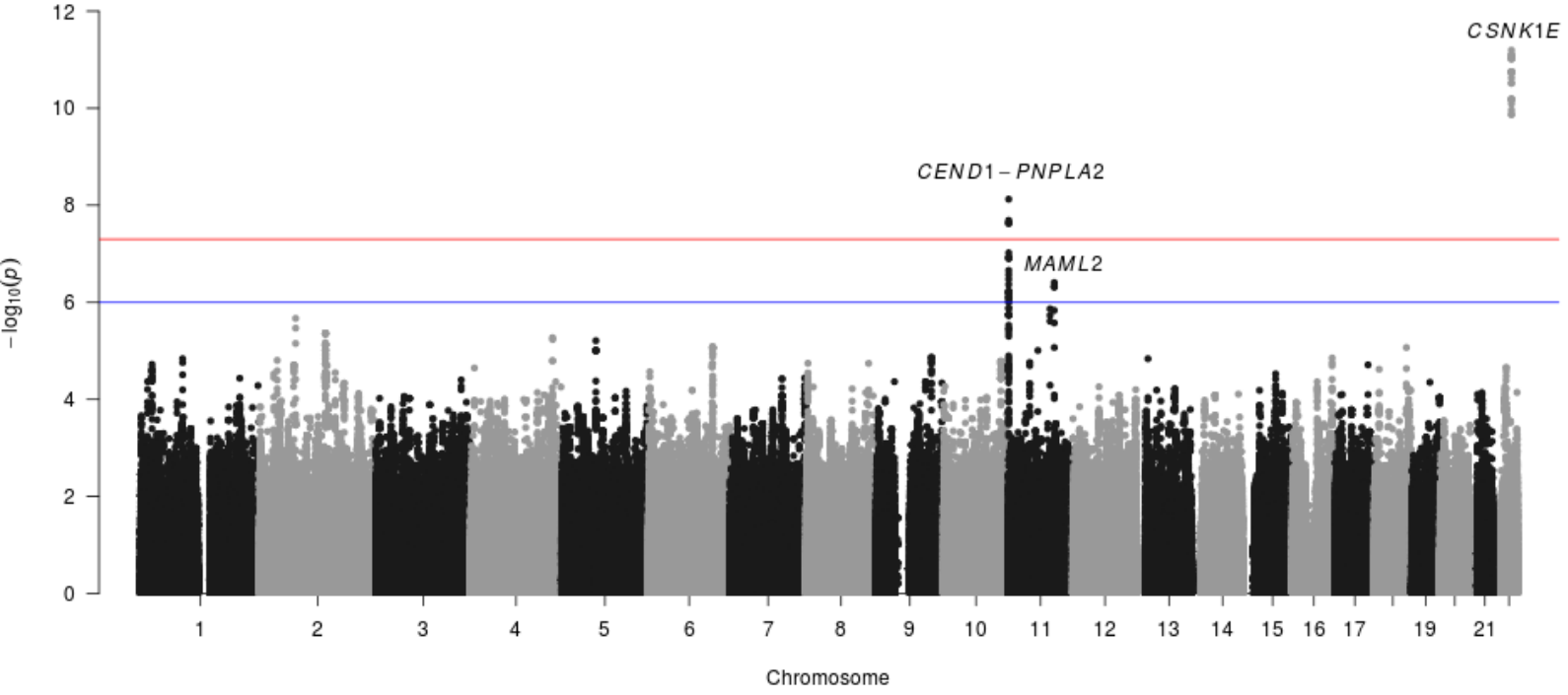
Table 3: Replication and meta-analysis of association results at lead SNPs.

					Discovery		Replication:USA		Replication: AUS+NI		Replication: VIC		Meta-analysis	
Locus[a]	**Chr**	**Lead SNP**[b]	**Base pair**[c]	**A1/2**	**OR [95% CI]**	***P***	**OR**	***P***	**OR [95% CI]**	***P***	**OR [95% CI]**	***P***	***P***	**direction**
PNPLA2	11	rs61876744	820754	T/C	0.59 [0.49–0.71]	7.46×10^{-9}	0.77 [0.63–0.95]	8.88×10^{-4}	0.84 [0.65–1.08]	0.179	0.97 [0.79–1.19]	0.763	2.45×10^{-8}	−−−−
MAML2	11	rs10831500	95982642	G/T	1.59 [1.33–1.91]	3.91×10^{-7}	1.35 [1.11–1.65]	5.69×10^{-3}	1.09 [0.85–1.40]	0.515	1.03 [0.84–1.26]	0.792	3.83×10^{-6}	++++
CSNK1E	22	rs138378	38796159	A/G	0.50 [0.41–0.61]	1.77×10^{-11}	0.95 [0.78–1.16]	0.790	0.97 [0.76–1.24]	0.818	0.93 [0.76–1.13]	0.448	3.18×10^{-4}	−−−−

[a]Locus assigned to the RefSeq protein-coding gene within or near the association signal interval.

[b]The lead SNP at *CSNK1E* (rs138380) was not successfully genotyped in the AUS+NI and VIC cohorts, and a proxy (rs138378, R^2=0.938) was used for replication instead.

[c]Genomic positions are based on hg19.


CSNK1E
CEND1 – PNPLA2
MAML2
12
10
8
6
4
2
0
– log10(p)
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
19
21
Chromosome