Genetic Association of *COL5A1* Variants in Keratoconus Patients Suggests a Complex Connection between Corneal Thinning and Keratoconus

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PURPOSE. Single nucleotide polymorphisms (SNPs) located near or within the *COL5A1* gene, at 9q34.2-q34.3 chromosomal region have been reported in association with central corneal thickness (CCT). Using family linkage analysis, we identified a keratoconus susceptibility locus at 9q34. These findings led us to perform an association study between *COL5A1* variation and keratoconus susceptibility.

METHODS. A Caucasian case–control cohort of 222 keratoconus patients and 3324 controls was selected as the discovery panel. An independent case–control panel of 304 cases and 518 controls and a family panel of 186 subjects were replicated for genotyping and association. Forty-four SNPs (21 for discovery and 23 for fine-mapping) spanning 300 kilobases in and around *COL5A1* were genotyped and tested for genetic association. Logistic regression models implemented in PLINK were used to test for association in case controls. Generalized estimating equation models accounting for familial correlations implemented in genome-wide interaction analyses with family data were used for association testing in families.

RESULTS. Two CCT associated SNPs (rs1536482 and rs7044529 near and within *COL5A1*) were identified in the keratoconus discovery cohort (P values of 6.5×10^{-3} and 7.4×10^{-3}). SNP rs1536482 was replicated in the second case–control sample ($P = 0.02$), and SNP rs7044529 was replicated in a keratoconus family panel ($P = 0.03$). Meta P values of rs1536482 and rs7044529 in the keratoconus cohorts were 1.5×10^{-4} (odds ratio [OR] = 1.30) and 2.9×10^{-3} (OR = 1.39). After Bonferroni correction, the association of SNP rs1536482 remained significant ($P = 6.5 \times 10^{-3}$).

CONCLUSIONS. SNPs in the *COL5A1* region, which regulate normal variation in CCT, may play a role in the thinning associated with keratoconus.

Keywords: keratoconus, association, *COL5A1*

K eratoconus (KC, MIM #148300) is a noninflammatory corneal dystrophy characterized by progressive corneal thinning resulting in moderate to marked visual impairment.[1] The prevalence of keratoconus has been estimated between approximately 1 in 1200[2] to 1 in 2000[3] in the Caucasian population. Although spectacles or contact lenses may be used for vision correction in early and moderate disease, corneal transplantation is often required for visual rehabilitation in severe cases, making it one the most common indications for corneal transplantation.[4]

One of the hallmarks of keratoconus is marked corneal thinning. There is an average difference of 75 μm in central corneal thickness (CCT) in keratoconus patients when compared with normal controls.[5–8] Extreme corneal thinning is also a dramatic clinical feature for rare congenital connective tissue disorders with gross corneal abnormalities, such as Brittle cornea syndrome and several types of osteogenesis imperfecta.[9,10] Yet mildly reduced CCT is associated with more common eye conditions, including keratoconus[11,12] and primary open-angle glaucoma.[13] Variation in CCT is one of the most highly heritable human traits.[14,15] Several recently performed genome-wide association studies (GWAS) identified a number of genomic loci associated with differences in CCT and revealed differences in genetic determinants of CCT between ethnic groups.[16–18] Since the corneal stroma is composed of collagen fibrils, it is not surprising that a number of genomic loci associated with CCT contain genes that code for various forms of collagen, such as *COL1A1* and *COL1A2*,[19] *COL8A2*,[17] and *COL5A1*.[16–18] Interestingly, *COL5A1* is located under a keratoconus linkage peak at 9q34 we identified through a prior linkage analysis study, using sib-pair families, with the maximum logarithm of odds of 2.7.[20]

In the present study, we selected SNPs located within or near *COL5A1*, including those associated with CCT, for genotyping and testing for association with keratoconus in a Caucasian population. We identified genetic evidence of an association between SNPs within or near *COL5A1* and keratoconus, possibly independent of the association with CCT, supporting a potential involvement of *COL5A1* in both familial and sporadic keratoconus.

MATERIALS AND METHODS

Study Subjects

Clinically affected keratoconus patients were recruited at three major sites: the Cornea Genetic Eye Institute ($n = 551$) at Cedars-Sinai Medical Center, Los Angeles, CA; the Jules Stein Eye Institute ($n = 26$) at UCLA, Los Angeles, CA; and the University Hospitals Eye Institute, Case Western Reserve University, Cleveland, OH ($n = 46$). Institutional Review Board (IRB) approval was obtained at all clinic sites. Written informed consent was obtained from all subjects after explanation of the nature and possible consequences of the study. The study was conducted in concordance with the provisions of the Declaration of Helsinki.

Discovery Cohort. Clinically affected Caucasian individuals with keratoconus ($n = 240$) were enrolled into the GWAS as a part of the longitudinal videokeratography and genetic study at the Cornea Genetic Eye Institute at Cedars-Sinai Medical Center in Los Angeles.[21] After removing samples with poor quality of genotyping, 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 four field centers.[22,23] In all, 5201 predominantly Caucasian individuals were recruited in 1989 to 1990 from random samples of Medicare eligibility lists, followed by an additional 687 African-Americans recruited in 1992–1993 (total $n = 5888$). All these patients underwent ocular evaluations. CHS was approved by the IRB 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 an insufficient number of ethnically matched cases.

Replication Cohort. An independent group of keratoconus cases and controls was recruited as a part of the collaborative replication study, consisting of 304 keratoconus cases: 232 independent keratoconus cases recruited at the Cornea Genetic Eye Institute, 26 cases recruited at the Jules Stein Eye Institute, and 46 cases recruited at University Hospitals Eye Institute; 518 normal controls were also recruited at Cedars-Sinai Medical Center in Los Angeles.

Keratoconus Families. Family members of keratoconus cases diagnosed at the Cornea Genetic Eye Institute were recruited to perform family-based studies. A total of 186 individuals from 41 Caucasian families consisting of 79 keratoconus patients and 107 unaffected family members were obtained for this study. This panel was ascertained through a clinically affected keratoconus proband as a part of the longitudinal videokeratography and genetic study of keratoconus at the Cedars-Sinai Medical Center in Los Angeles, CA.[20]

Clinical Diagnosis

The diagnosis of keratoconus was based on clinical examination and confirmed with videokeratography. All family members underwent clinical examinations including slit-lamp biomicroscopy, cycloplegic retinoscopy, and fundus evaluations. The slit-lamp biomicroscope was used to identify corneal stromal thinning, Vogt striae, or a Fleischer ring. The retinoscopy examination was performed with a fully dilated pupil (20 minutes after phenylephrine 2.5% and cyclopentolate 1% drops had been instilled in the eye) 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 also performed on each eye using the Topographic Modeling System (TMS-4). Subjects were assigned as having keratoconus if they had at least one clinical sign of keratoconus and a confirmatory videokeratography map with an asymmetric bowtie with skewed radial axis above and below the horizontal meridian (AB/SRAX) pattern.[24]

CCT Measurements

CCT was measured using a Fourier domain optical coherence tomography (OCT) device (Optovue, Inc., Fremont, CA). The OCT system operated at a 1.3-µm wavelength with a scan rate of 2000 axial scans per second. A pachymetry scan pattern (8 radials, 128 axial scans each; 10-mm diameter) centered at the corneal vertex was used to map the corneal thickness. The pachymetry map was divided into zones by octants and annular rings. Five pachymetric parameters were calculated from the region inside the 5-mm diameter: minimum, minimum–median, inferior–superior (I-S), inferotemporal–superonasal (IT-SN), and the vertical location of the thinnest cornea. The 1-percentile value of the normal group was used to define the diagnostic cutoff. This method has been shown to be highly reproducible and as accurate as ultrasonic pachymetry and scanning slit tomography for CCT assessment.[8,25]

DNA Extraction

DNA was extracted from Epstein–Barr virus transformed lymphoblastoid cell lines established from peripheral whole blood of each study participant (NucleoSpin Tissue kit; Macherey-Nagel Inc., Bethlehem, PA). The salt extraction protocol was used to extract DNA from buffy coats whenever cell lines were not available.

Genotyping

Discovery. All genotyping was performed at the genotyping laboratory of the General Clinical Research Center and the Medical Genetics Institute at Cedars-Sinai Medical Center. Discovery genotyping was performed using a whole genome genotyping beadchip (Illumina Human CNV370 Quad; Illumina, Inc., San Diego, CA), following the manufacturer's protocol (Illumina Inc.).[26,27] The details of GWAS genotyping and quality control were performed as previously described.[28] In all, 21 SNPs located within and near *COL5A1* were genotyped and analyzed in the discovery cohort.

Replication. Using a commercial software package (Tagger [http://www.broad.mit.edu/mpg/tagger] as implemented in Haploview[29,30]; Broad Institute of MIT and Harvard University, Cambridge, MA) and data from the CEPH population in the International HapMap project, release 2,[31,32] we designed a panel of 44 SNPs spanning 300 kilobases in and around *COL5A1* (including 21 SNPs genotyped in the discovery cohort and 23 fine-mapping SNPs in the replication cohort) in *COL5A1* and genotyped them using custom beadchip technology[33] (iSelect Infinium; Illumina Inc.) in both case–control and family samples. The average genotyping rate for samples genotyped on the iSelect platform and passing quality control was 99.98%. Genotyping concordance among 20 pairs of blind duplicates was 100%.

One SNP (rs7044529, genotyped in the discovery cohort) failed for genotyping in the iSelect panel and was regenotyped using predesigned genotyping assay (*Taq*Man; Applied Biosystems/Life Technologies, Carlsbad, CA) with allelic discrimination on a real-time PCR system (ABI 7900; Applied Biosystems/Life Technologies).

Imputation

Since 23 fine-mapping SNPs were genotyped in replication cohorts but not in the original discovery cohort, multilevel imputation software (IMPUTE version 2.1.0; SAS Institute, Cary, NC)[34] was used to perform imputation for the Caucasian participants in the discovery cohort on chromosome 9 (HapMap Phase I and II, CEU as the reference panel, release #24; National Center for Biotechnology Information Build 36 [dbSNP 126], Bethesda, MD).

Statistical Analyses

Discovery and Replication Study. Under the additive genetic model, we examined the association between keratoconus and each SNP individually, adjusted for potential covariates, using the logistic regression model. Odds ratios (ORs) and their SE values associated with each additional minor allele for each SNP were calculated. Association tests by the logistic regression model for case–control samples were performed with the use of the PLINK program (v1.07) (http://pngu.mgh.harvard.edu/purcell/plink/).[35] Principal component analysis (PCA) for population stratification was performed in the discovery GWAS panel using EIGENSTRAT.[36] Sex and three significant principal components variables identified from PCA were used as covariates. Because of the large age difference between patients and controls in the discovery panel, age was not used as a covariate. We also adjusted for the sample collection site in the replication case–control samples. Association tests controlling for familial correlations by a generalized estimating equation (GEE) model were performed in families using the GWAF package.[37]

Association Testing After Conditioning on Significant SNPs. Additional association tests in the discovery cohort were performed after adjustment for each of the top two significant SNPs, and both of them together by logistic regression.

Meta-Analysis. Meta-analyses of 44 SNPs obtained from the three cohorts were calculated using an inverse-variance weighting by PLINK.[35] We also calculated and present the adjusted *P* values based on Bonferroni correction for the number of SNPs tested. Given that a number of 44 SNPs tested were in linkage disequilibrium (LD), that is a rather conservative approach. Regional association plots were generated by LocusZoom[38] (http://cgs.sph.umich.edu/locuszoom/).

Analysis of CCT in Patients Carrying Different Alleles of *COL5A1* SNPs. To identify whether the association

between identified *COL5A1* SNPs and keratoconus was independent of CCT, we tested the difference in CCT among individuals carrying different genotypes of SNPs rs1536482 and rs7044529 using a regression model in a subset of 197 keratoconus patients (all cases from the replication case–control cohort).

RESULTS

In this study, we have studied three independent cohorts of patients with keratoconus. Table 1 summarizes the characteristics of study cohorts.

SNPs in *COL5A1* Gene Region Are Associated With Keratoconus in the Discovery and Replication Cohorts

Twenty-one SNPs located within and near *COL5A1* were genotyped (Illumina Human CNV370 Quad beadchip) in the keratoconus GWAS discovery phase.[28] An additional 23 fine-mapping SNPs were further genotyped in replication cohorts. To have genotyping data for all 44 SNPs analyzed in both the discovery and replication cohorts, 23 fine-mapping SNPs were also imputed in the discovery cohort. Four of the 44 SNPs (rs3118516: imputed; rs1536482: genotyped; rs3118520: imputed; and rs7044529: genotyped) were found to be associated with keratoconus in the discovery cohort with nominal $P \leq 0.03$ (Table 2). The same two SNPs, rs1536482 and rs7044529, have previously been associated with CCT in three independent GWAS[16-18] and in large-scale meta-analysis of CCT.[39] Results of the association testing for SNPs rs1536482 and rs7044529 in the discovery cohort were previously published in a collaborative study of keratoconus risk of the CCT-associated loci.[39]

Three of these four SNPs (rs3118516, rs1536482, and rs3118520, clustered in one LD block) also demonstrated significant association with keratoconus in the replication cohort ($P = 0.02, 0.02,$ and $0.009,$ respectively). However, although the three SNPs demonstrated the same direction of association in the familial cohort, they did not attain individual significance in that cohort. Only one of the four SNPs that were genotyped (rs7044529) demonstrated significant association in the familial cohort (although all four SNPs had an effect in the same direction). Although there were differences in age and sex distribution between cases and controls, the *P* values remained similar before and after adjustments for age, sex, and other covariates (data not shown).

Meta-analysis combing the three cohorts identified rs1536482 as having the greatest association (meta *P* value $= 1.5 \times 10^{-4}$; Bonferroni corrected $P = 6.5 \times 10^{-3},$ representing the first LD block), followed by rs7044529 (meta *P* value $= 2.9 \times 10^{-3}$; Bonferroni corrected $P = 0.12$) (Table 2, Figs. 1A, 1B).

TABLE 1. Characteristics of the Study Samples

	Discovery			Replication, Case–Control			Replication, Family		
Variable	Case, *n* = 222	Control, *n* = 3324	*P*	Case, *n* = 304	Control, *n* = 518	*P*	Case, *n* = 79	Control, *n* = 107	*P*
Age (SD)*	44 (13)	72 (5)	<0.01	43 (16)	45 (14)	0.02	41 (15)	55 (18)	<0.01
Male, %	55	39	<0.01	68	52	<0.01	55	50	0.58
CCT (SD)*	—	—	—	466 (49)†	—	—	—	—	—

* Mean and SD.
† 197 patients had CCT measurements.

TABLE 2. Association Testing of SNPs Located in the *COL5A1* Gene Associated in GWAS Discovery Cohort*

| SNP	Gene Location	Chromosomal Position	MA	Discovery				Replication, Case–Control				Replication, Family				Meta-Analysis		
				F_A	F_U	OR	P	F_A	F_U	OR	P	F_A	F_U	OR	P	OR	P	P_A†
rs3118516	5′ to *COL5A1*	136,579,612	A	0.39	0.28	1.37	0.01	0.42	0.36	1.29	0.02	0.36	0.31	1.26	0.23	1.31	3.6E-4	0.02
rs1536482	5′ to *COL5A1*	136,580,348	A	0.41	0.33	1.32	6.5E-3	0.42	0.36	1.28	0.02	0.37	0.31	1.27	0.20	1.30	1.5E-4	6.5E-3
rs3118520	5′ to *COL5A1*	136,581,415	G	0.47	0.37	1.31	0.03	0.48	0.41	1.31	0.009	0.44	0.37	1.33	0.11	1.31	2.0E-4	8.6E-3
rs7044529	*COL5A1*	136,707,872	T	0.17	0.14	1.44	7.4E-3	0.16	0.15	1.02	0.92	0.22	0.12	1.96	0.03	1.39	2.9E-3	0.12

* P value < 0.05 in replication case–control and family cohorts and by meta-analysis.
† Bonferroni-corrected P values.
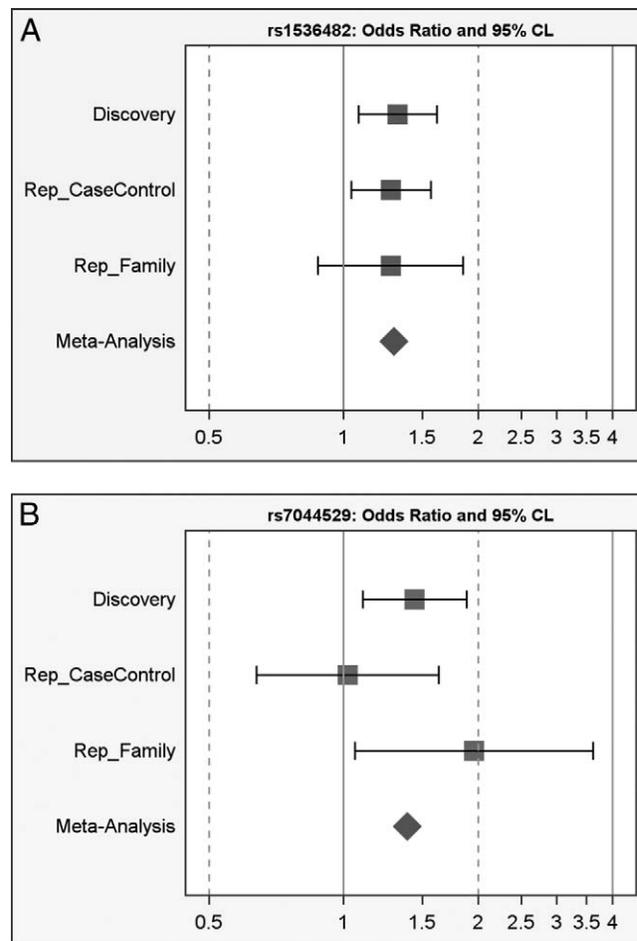
FIGURE 1. Forest plots for top SNPs. (**A**) rs1536482-A: OR and 95% CI; (**B**) rs7044529-T: OR and 95% CI.

Variation Across *COL5A1* Gene Region and Association With Keratoconus

Two independent signals were identified in the region of *COL5A1* as evident from the regional plot of meta P values (Fig. 2). Three SNPs, rs3118516, rs1536482, and rs3118520, are clustered in one LD block, located in the intergenic region between *RXRA* and *COL5A1*. The other tentative association signal was observed at rs7044529 located in another LD block, in an intron of *COL5A1* and located approximately 126 kilobases away from rs1536482. To determine whether these signals represent independent associations, we used genotyping data of the GWAS discovery cohort to perform conditional testing for genetic association after adjusting for the top SNPs using logistic regression. After adjustment for rs7044529, a P value of 8×10^{-3} was calculated for rs1536482. Similarly, after adjustment for rs1536482, a P value of 7×10^{-3} was calculated for rs7044529. After adjusting for both of these SNPs, no other nominally significant associations ($P < 0.05$) were identified. These results indicate two possibly independent association signals for this region.

Analysis of CCT Measurements in Keratoconus Patients With Different Alleles of Associated SNPs

In a subset of keratoconus patients ($n = 197$) collected for this study, we found the mean CCT measurements to be 466 ± 49 μm (as measured by OCT). These values are much lower than
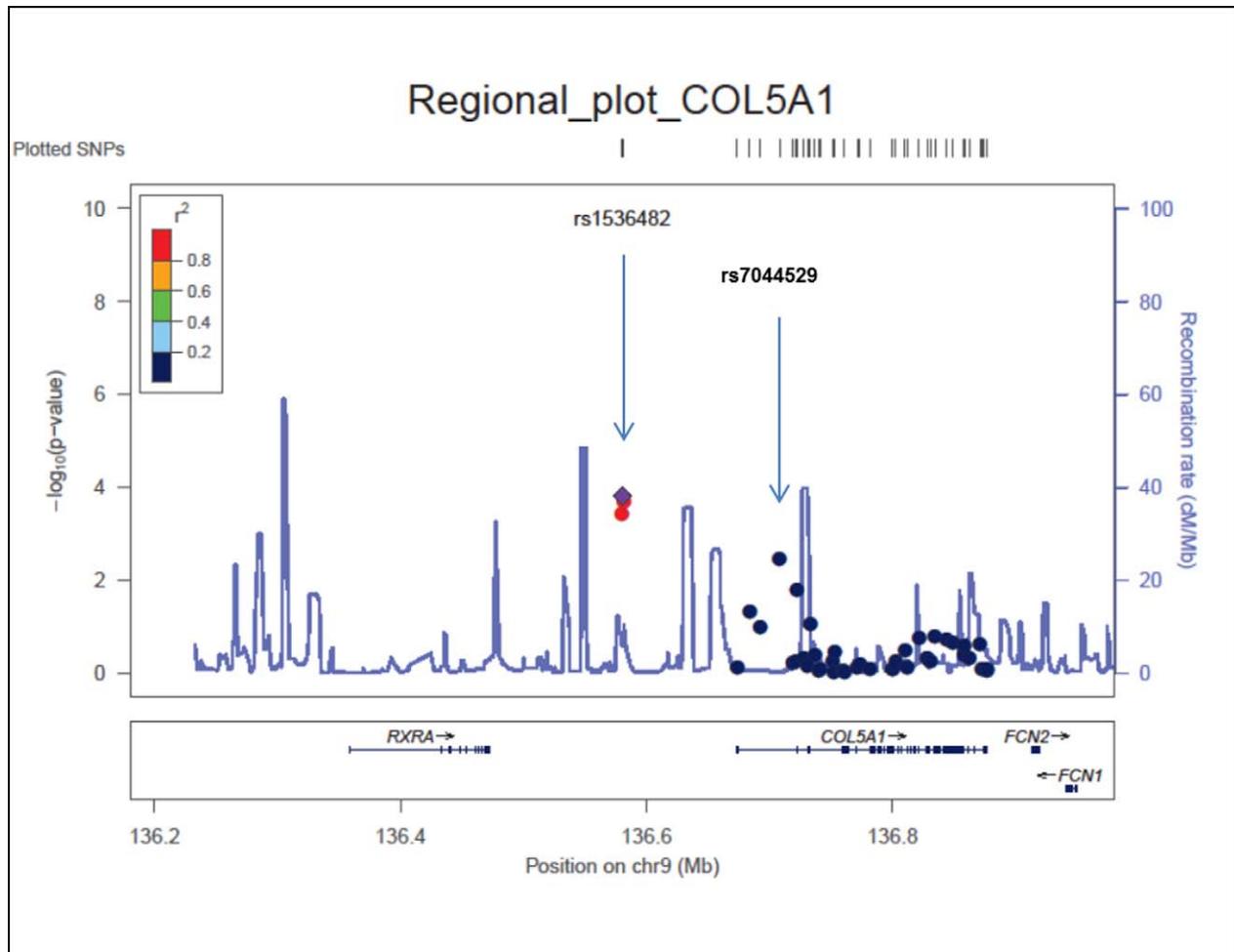
Figure 2. Regional plot of *COL5A1* region.

normal values reported for CCT measurements in the literature. This raises the question whether the *COL5A1* association with keratoconus is an independent finding or is due to association with corneal thinning in general. Although formal correction for CCT was not performed because these measurements were not available for samples in discovery cohort and also not for the control samples in the replication cohorts, we addressed this issue by assessing CCT values in 197 keratoconus patients from the replication cohort carrying different combination of alleles in the two SNPs that demonstrated the greatest association with keratoconus (rs1536482 and rs7044529). The distribution of CCT between three genotypes of rs1536482 was: GG, 470 ± 49 μm ($n = 67$); GA, 465 ± 52 μm ($n = 93$); and AA, 465 ± 42 μm ($n = 35$) μm. For rs7044529, the distribution of CCT was: CC, 470 ± 49 μm ($n = 149$); CT, 453 ± 54 μm ($n = 41$); and TT, 465 ± 37 μm ($n = 7$). Although the difference in CCT between the three genotypes was not statistically significant for rs1536482 and rs7044529 ($P > 0.05$), the effect size of the risk allele was −3 and −10 μm, respectively, which was similar with GWAS in the general population.[16–18] In addition, in keeping with data reported in the literature,[40] there was no correlation between CCT and age ($r^2 = 0.002$, $P = 0.55$). No difference of CCT between males and females was identified either ($P = 0.09$). These results suggest that the association between keratoconus and this gene may be independent of CCT.

Examples of Relationship Between Low CCT and Keratoconus

Because CCT measurements are available to us for only a moderate sample size of keratoconus patients, we had limited power to address whether the genetic effect with CCT and keratoconus is independent; however, we have observed many first-degree relatives of patients with keratoconus with abnormally low CCT values without any topographic abnormalities or progression over time. An illustrative example of such a case is an 18-year-old male referred to rule out keratoconus, prior to refractive surgery, who had two cousins with keratoconus on his mother's side. He had no videokeratography signs of keratoconus, but an abnormally low CCT (see Figs. 3A, 3B). His mother also had a low CCT without signs of keratoconus on topography (see Figs. 4A, 4B).

Discussion

An association between SNPs rs1536482 and rs7044529 located near and within *COL5A1* and CCT has been documented in both Caucasian and Asian populations,[16,17] with the risk alleles of these SNPs associated with reduced CCT. Based on data from HapMap 3 Release 2 data, SNP rs3118520 is in strong linkage disequilibrium with SNP rs1536482 with pairwise r^2 of 0.8 (SNAP; http://www.broadinstitute.org/mpg/snap/ldsearchpw.php). However,

A

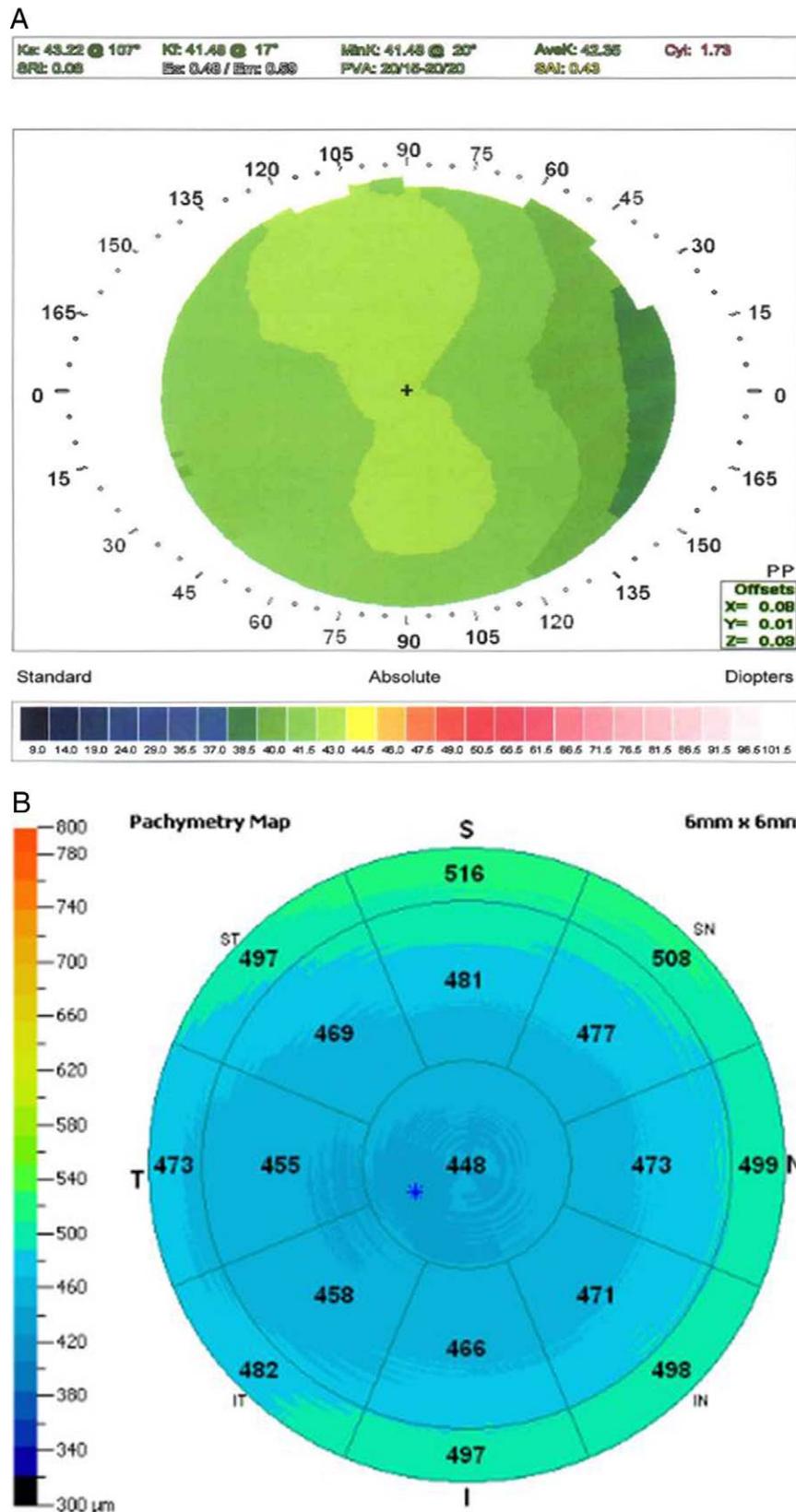| Ka: 43.22 @ 107° | Kf: 41.48 @ 17° | MinK: 41.48 @ 20° | AveK: 42.35 | Cyl: 1.73 |
| SRI: 0.08 | Ex: 0.48 / Err: 0.89 | PVA: 20/15-20/20 | SAI: 0.43 | |

B

FIGURE 3. **(A)** Videokeratography right of an 18-year-old male with a family history of keratoconus (two cousins on maternal side). Topography illustrates with the rule astigmatism and no evidence of "forme frusta" keratoconus. **(B)** OCT pachymetry of the same eye demonstrating low CCT measurements with diffuse corneal thinning and no evidences of focal ectasia.
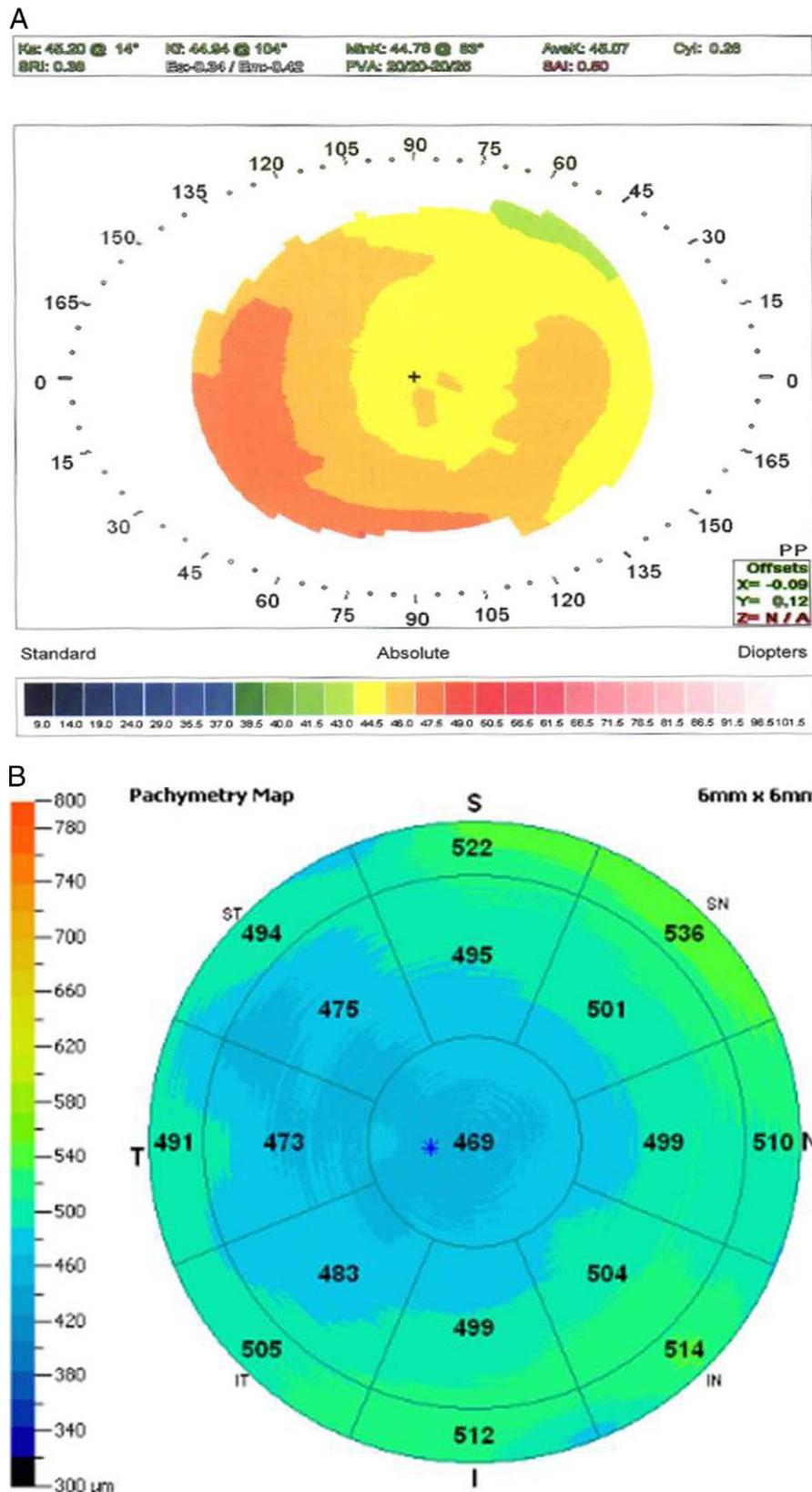
FIGURE 4. (A) Videokeratography of the right eye of the mother of the 18-year-old male described in Figure 3A. Topography demonstrates mild contact lens warpage with no evidence of "forme frusta" keratoconus. (B) OCT pachymetry of this eye demonstrating low CCT measurements with diffuse corneal thinning and no evidence of focal ectasia.

based on the same data set, r^2 between both rs3118520/rs1536482 and rs7044529 is <0.1, confirming the existence of two independent signals in this region. Correspondingly, we observed two independent association peaks in the region of *COL5A1*, although only SNP rs1536482 remained significant after stringent Bonferroni correction was applied. However, the Bonferroni correction approach with the number of SNPs test is conservative, given that the number of LD blocks in this region is much less than that of SNPs (Fig. 2). This could indicate that the second signal associated with rs7044529 (although not significant after Bonferroni correction), identical to the one repeatedly identified in normal individuals with thinner corneas, is related to the underlying corneal thinning that characterizes keratoconus.

COL5A1 encodes an alpha chain for type V collagen, one of the low-abundance fibrillar collagens. It is a constituent of type I collagen-rich fibrils in many connective tissues including the cornea[41] and appears to regulate the diameter of heterotypic fibers composed of both type I and type V collagen.[42] Several studies have been performed to compare composition and distribution of different collagen types in normal and keratoconus corneas with conflicting results.[43-46] Preliminary studies of corneas in our laboratory indicate a strong presence of *COL5A1* transcripts. We are following this up with more detailed expression studies of this gene, comparing keratoconus corneal postkeratoplasty buttons to normal.

In addition to our molecular findings, there is clinical evidence to support findings suggested by our genetic studies: that the variation in CCT may be an independent genetic risk factor that predisposes individuals to developing keratoconus. Corneal thinning associated with keratoconus more often than not commences paracentrally and inferotemporally and may be progressive in nature.[1,47] This phenomenon frequently occurs in clinically normal family members of patients with keratoconus with mild topographic abnormalities and in the clinically normal fellow eyes of patients with unilateral keratoconus as first shown by our group[48,49] and further confirmed by the work of Colin et al.[11] and Salabert et al.[50]

Phenotypically subclinical CCT is different from that associated with keratoconus. It is central in position, nonprogressive in nature, and it occurs in the absence of clinical or topographic features of keratoconus.[14] In a retrospective review of 171 patients who developed ectasia after LASIK surgery (an adverse outcome indicating a high probability of underlying undiagnosed keratoconus), Randleman et al.[51] found that decreased CCT measurements constituted a major risk factor predisposing LASIK patients to developing ectasia.

One of the limitations of our study is the unmatched design of cases and controls, especially the large difference in age between cases and controls in the discovery panel. We could not adjust for age as the covariate in the association tests. However, keratoconus typically commences in the early teens and progresses to the mid-30s, when it typically arrests. Therefore, using an elderly population as controls, the misclassification probability of disease due to age is small, although age-related genes could potentially be detected as false positives. For replication cohorts, we did comparisons of association tests before and after adjustments for age and sex, and did not identify significant age and/or sex effect.

In summary, both our genetic findings and clinical evidence suggest that the variants in CCT-associated gene *COL5A1* both contribute to normal variation in central corneal thickness and are associated with clinical corneal thinning in keratoconus. However, the lack of association between *COL5A1* variants and CCT in keratoconus patients raises the possibility that abnormal thinning in keratoconus is dependent on the effects of other genetic polymorphisms in other keratoconus-associated genes. This hypothesis is being tested in extended families with keratoconus in our clinic and laboratories.

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