Association of Polymorphisms in the Hepatocyte Growth Factor Gene Promoter with Keratoconus

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Purpose. Keratoconus is a progressive disorder of the cornea that can lead to severe visual impairment or blindness. Although several genomic regions have been linked to rare familial forms of keratoconus, no genes have yet been definitively identified for common forms of the disease.

Methods. Two genome-wide association scans were undertaken in parallel. The first used pooled DNA from an Australian cohort, followed by typing of top-ranked single-nucleotide polymorphisms (SNPs) in individual DNA samples. The second was conducted in individually genotyped patients, and controls from the USA. Tag SNPs around the hepatocyte growth factor (*HGF*) gene were typed in three additional replication cohorts. Serum levels of HGF protein in normal individuals were assessed with ELISA and correlated with genotype.

Results. The only SNP observed to be associated in both the pooled discovery and primary replication cohort was rs1014091, located upstream of the *HGF* gene. The nearby SNP rs3735520 was found to be associated in the individually typed discovery cohort ($P = 6.1 \times 10^{-7}$). Genotyping of tag SNPs around *HGF* revealed association at rs3735520 and rs17501108/rs1014091 in four of the five cohorts. Meta-analysis of all five datasets together yielded suggestive *P* values for rs3735520 ($P = 9.9 \times 10^{-7}$) and rs17501108 ($P = 9.9 \times 10^{-5}$). In addition, SNP rs3735520 was found to be associated with serum HGF level in normal individuals ($P = 0.036$).

Conclusions. Taken together, these results implicate genetic variation at the *HGF* locus with keratoconus susceptibility. (*Invest Ophthalmol Vis Sci.* 2011;52:8514–8519) DOI:10.1167/iovs.11-8261

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Keratoconus is a corneal disorder with an incidence of approximately 1 in 2000 and a prevalence of 54.5 per 100,000.[1] The disorder leads to severe refractive error and irregular astigmatism. It is typically bilateral and is characterized by progressive thinning of the cornea, leading to asymmetric bulging and, in advanced cases, formation of a conical cornea.[1]

Based on the results of twin studies, reports of familial aggregation, and formal segregation analysis (reviewed in Ref. 2), keratoconus appears to have a strong genetic component; however, to date, few if any genes have been reliably identified. Approximately 6% to 23% of individuals with keratoconus report a positive family history.[2] Linkage to multiple loci has been reported through a combination of linkage studies in extended pedigrees (3p14-q13[3]; 5q14-q21[4]; 15q22-q24[5]; 1p36 and 8q24[6]; and 13q32[7]), in collections of small families and sibling pairs (2p24[8]; 16q22-q23[9]; 9q34, 5q32-q33, and 5q21.2[10]; and 14q11.2[11]), and in an identity-by-descent approach (20q12).[12] Although the causatives genes in these regions remain to be elucidated, several reports

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have been made of candidate genes associated with keratoconus. The best studied of these is *VSX1*, the visual system homeobox-1 gene, with mutations identified in a small number of keratoconus patients,[13–17] although not all studies support these findings.[18–21] A 7-bp deletion in intron 2 of the *SOD1* gene was described in two families,[22] but no mutations were reported in another study.[23] Promoter variants in *IL1B* were also nominally associated with keratoconus in a small Korean cohort.[24] Several coding polymorphisms in the *COL4A3* and *COL4A4* genes encoding the structural proteins collagens were also found to be associated with keratoconus.[25] These results are awaiting further replication.

In addition to the candidate gene approach, recent developments in genotyping technology have made feasible large-scale genetic studies with hundreds of thousands of dense markers, such as genome-wide association studies (GWASs). Understanding of genetic susceptibility to ophthalmic diseases, such as glaucoma, macular degeneration, and refractive error,[26–28] and ocular quantitative traits, such as corneal thickness,[29–31] has been greatly enhanced by recent GWASs.

We present the results of two genome-wide association studies, both of which point to the *HGF* (hepatocyte growth factor) gene as a keratoconus susceptibility locus. Two SNPs located upstream of the *HGF* gene on chromosome 7 showed repeated association with keratoconus in independent cohorts of patients, thus providing initial evidence that they may be associated with the common form of keratoconus in Caucasian patients. We also present data indicating association of the same SNPs with the serum HGF level in normal participants.

Materials and Methods

Multiple patient cohorts were recruited and examined as described below. All studies adhered to the tenets of the Declaration of Helsinki and were approved by the relevant institutions; all participants gave written informed consent. All participants (cases and controls) were Caucasian. A flow chart indicating the cohorts studied and the genotyping strategies is given in Supplementary Figure S1 (http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-8261/-/DCSupplemental).

Definition of Keratoconus

The diagnosis of keratoconus was performed by an ophthalmologist based on clinical examination and videokeratography pattern analysis. 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. A retinoscopic examination was performed with a fully dilated pupil to determine the presence or absence of retroillumination signs of keratoconus, such as the oil droplet sign and scissoring of the red reflex. Videokeratography evaluation was performed on each eye by topographic modeling (Topographic Modeling System; Computed Anatomy, New York, NY) or with corneal topographers (ARK-9000; Nidek Inc. Freemont, CA, or Orbscan; Bausch & Lomb, Madison, NJ). Patients were considered as having keratoconus if they had at least one clinical sign of the disease and by confirmatory videokeratography map with an asymmetric bowtie pattern with skewed radial axis above and below the horizontal meridian (AB/SRAX).[32] A history of penetrating keratoplasty performed because of keratoconus was also sufficient for inclusion as a case in the Australian and Northern Ireland cohorts.

Australian GWAS

Discovery Cohort. The protocol was approved by the Southern Adelaide Health Service/Flinders University Human Research Ethics Committee. Participants with keratoconus ($n = 97$) were ascertained through the eye clinic of Flinders Medical Centre. Unaffected controls ($n = 216$) were recruited from assisted living facilities (nursing homes) in Adelaide and from healthy volunteers. All controls had an ocular examination and medical history taken.

Replication Cohorts. *Rep1.* An additional 39 cases and 72 controls from Flinders Medical Centre were recruited, along with 57 patients recruited from the Ophthalmology Department of the Belfast Health and Social Care National Health System Trust. The study was approved by the Northern Ireland Research Ethics Committee.

Rep2. This cohort was recruited through an Australia-wide mailing to members of Keratoconus Australia, a community-based support group for patients. Clinical data were obtained from the participants' eye care practitioner, and patients were included in the study only if they met the recruitment criteria—in total, 186 cases. An additional more recently recruited 29 cases from Northern Ireland were also included in this cohort. Genotype data for the SNPs of interest were downloaded for 112 unrelated CEU samples (CEPH:Utah residents with ancestry from northern and western Europe) from the International HapMap Project (www.hapmap.org) to be used as controls.

Genotyping and Analysis. Genomic DNA was extracted from peripheral whole blood with a kit (QIAamp Blood Maxi Kit; Qiagen, Valencia, CA), for each Australian participant, and with another kit (Puregene; Gentra Systems, Minneapolis, MN), for Northern Ireland participants, according to the manufacturers' protocols. Equimolar DNA pools were generated in triplicate for discovery cases ($n = 97$) and controls ($n = 216$) after step-wise dilution to 75 ng/μL and accurate quantitation of all samples at each dilution stage in a dsDNA quantitation assay (PicoGreeen; Invitrogen, Carlsbad, CA) performed on a plate reader (Fluoroskan; Thermo Fisher Scientific, Waltham, MA). A total of 451 ng of DNA from each sample was added to the pool.

Genome-wide genotyping was conducted by hybridization to SNP arrays (HumanHap 1M; Illumina, San Diego, CA). The case pool was hybridized to two independent arrays, and the larger control pool to four arrays. The output of the raw red and green bead scores from the genotyping stage was available for the pooled data analysis. The data processing protocol was similar to a method described previously.[33] Before the raw scores were calculated, SNPs with more than 10% negative scores on each array were excluded, as well as the SNPs with the sum of mean red and green scores lower than 1200 across each array. This step was included to ensure that the calibration was performed on a precleaned dataset. A normalization/correction factor (corr) was calculated by forcing the mean value of the pooling allele frequency to be 0.5 over all SNPs on each stripe (the Human 1M-Duo V3 array has six stripes on a single array). The pooling allele frequency (PAF) was then estimated based on the raw red intensities and the corrected green intensities for all the SNPs [PAF = red/(red + green/corr)].

A final set of autosomal SNPs meeting the criteria (1) more than 20 probes in each pool; (2) an MAF in the HapMap CEU samples greater than 5%; (3) no large differences (>0.3) between PAF and HapMap CEU allele frequency; and (4) estimated k value between 0.333 and 3 (where k is the estimated coefficient of unequal amplification of alleles), without a significant variance difference between case and control pools (i.e., the log10 transformed P values from an F test on the ratio of case control pool variances were smaller than 6), was taken forward to a linear regression model.[34] The underlying idea was to regress the pooling allele frequency on the case–control status for each SNP and estimate the pooling error across all the SNPs (for more details, see MacGregor et al.[34,35]). The P value from comparing the test statistic in the paper by MacGregor et al. (T2 − x) to a χ^2 distribution with 1° of freedom was computed to assess the significance of allele frequency difference between the two pools.

Highly ranked SNPs from the pooled GWAS of Australian samples were prioritized for genotyping in individual DNA samples and further follow-up according to the following criteria: (1) SNP reaching a significance threshold of $P < 1 \times 10^{-7}$ or better; (2) SNP present in a cluster of at least two SNPs within 100 kb of each other, with $P < 1 \times 10^{-4}$; or (3) SNP highly ranked with $P < 1 \times 10^{-5}$ and intragenic in a biologically relevant candidate gene. Additional SNPs representing the next best ranked SNP in any of the regions pinpointed through the above criteria were also entered into the assay design process to best use the multiplexes. In addition, to be included in follow-up individual typing, the SNP

must be suitable for genotyping (Sequenom, San Diego, CA) and able to multiplex with other chosen SNPs. In total, 52 SNPs were genotyped in three multiplexes (iPlex Gold chemistry; Sequenom) and allelic detection by mass spectrometry (MassArray; Sequenom) at the Australian Genome Research Facility (Brisbane, Australia) and were considered to be validated if they reached a nominal $P = 9.0 \times 10^{-3}$ or better. This validation threshold was chosen to allow a manageable number of candidate genes for further analysis. Additional tag SNPs around the *HGF* gene as reported by Yanovitch et al.[36] were also typed in both the discovery and Rep1 replication samples in individual DNA samples. The associated *HGF* SNPs rs17501108 and rs3735520 were typed in the Rep2 replication cohort by using predesigned genotyping probes (TaqMan; Applied Biosystems, Inc. [ABI], Foster City, CA) on a real-time PCR instrument (StepOne Plus; ABI). Association analysis was undertaken in PLINK.[37]

U.S. GWAS

Discovery Cohort: US1. This discovery cohort consisted of 222 clinically affected Caucasian keratoconus cases enrolled in a GWAS as a part of the longitudinal videokeratography and genetic study at the Cornea Genetic Eye Institute (Los Angeles, CA).[38] 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. Predominantly Caucasian individuals ($n = 5201$) were recruited in 1989 to 1990 from random samples of Medicare eligibility lists, followed by an additional 687 African-Americans recruited in 1992 to 1993 (total, $n = 5888$). The 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 an insufficient number of ethnically matched cases.[39,40]

Replication Cohort: US2. An independent group of 304 keratoconus cases and 518 normal controls were recruited as a part of the collaborative effort to form a replication cohort. Three independent groups of clinically affected keratoconus patients were recruited at three major sites: The Cornea Genetic Eye Institute ($n = 232$; Cedars-Sinai Medical Center, Los Angeles, CA); The Jules Stein Eye Institute ($n = 26$; UCLA, Los Angeles, CA); and the University Hospitals Eye Institute ($n = 46$; Case Western Reserve University, Cleveland, OH). Normal controls were recruited at the Cornea Genetic Eye Institute. Institutional Review Board approval was obtained at all clinic sites.

Genotyping and Analysis. DNA was extracted from EBV-transformed lymphoblastoid cell lines established from peripheral whole blood of each study participant (NucleoSpin Tissue kit; Macherey-Nagel, Inc., Bethlehem, PA). A salt-extraction protocol was used to obtain DNA from buffy coats.[41]

Genotyping in the discovery cohort (US1) was performed at Medical Genetics Institute at Cedars Sinai Medical Center using a whole genome genotyping bead chip assay (Human CNV370 Quad; Illumina), according to the manufacturer's protocol.[42,43] The average genotyping rate for samples passing quality control was 99.77%. Approximately 320,000 SNPs were used for the analysis after SNPs with minor allele frequency of less than 0.05 and/or a call rate of less than 0.9 were excluded.

Genotyping in the replication cohort (US2) was performed with a custom bead chip assay (iSelect Infinium; Illumina).[44] Tagging and proxy SNPs were selected in a 100-kb genomic region at 7q21.1, overlapping the *HGF* gene. General criteria were an r^2 threshold of 0.8 and an MAF of 0.05 in the CEU panel, using the software Tagger as implemented in Haploview[45,46] and data from the CEPH population in the International HapMap project, release 2.[47,48] In total, a panel of 4905 SNPs was designed that were compatible with the bead chip technology, of which 4650 SNPs remained after clustering quality control, including the SNPs reported here. The average genotyping rate for samples genotyped on this platform and passing quality control was 99.98%. Genotyping concordance among 20 replicated samples was 100%.

Meta-analysis

Meta-analysis of all cohorts was conducted by using Fisher's combined-probability test. The test was applied when all cohorts showed association in the same direction, as this test combines *P* values from multiple studies. Calculations were conducted manually (Excel; Microsoft, Redmond, WA).

Measurement of Serum HGF Concentration

Serum was obtained from whole blood collected from 191 normal control participants (controls from the pooled GWAS discovery cohort). Aliquots of serum were stored frozen at −80°C and thawed immediately before analysis. Serum HGF concentration was measured with a human HGF ELISA kit (RayBiotech, Inc., Norcross GA), per the manufacturer's protocol. Each sample was analyzed in duplicate in two independent experiments. A coefficient of variation of <12% was required for inclusion in the analysis, and outliers (values >2000; $n = 4$) were removed. A total of 78 participants were included in the analysis (SPSS Statistics, ver. 17.0; SPSS, Chicago, IL). Nonparametric Kruskal-Wallis and Mann-Whitney U tests were used to assess the differences in serum HGF concentration between genotypes of SNPs rs3735520 and rs17501108.

Results

Demographics for each cohort are given in Table 1. In all cohorts, controls were significantly older than cases, to reduce misclassification error. There were also differences in the ratio of males to females; however, the direction of this bias was not consistent between cohorts (Table 1).

A genome-wide association scan of pooled DNA from Australian cases was conducted to identify candidate genes for further analysis in keratoconus. Quality-control measures were used in obtaining allele frequency estimates from the pooled DNA for 745,982 SNPs. In total, four SNPs reached genome-wide significance of $P < 5 \times 10^{-8}$ with an additional four SNPs

Table 1. Demographics of the Study Cohorts

	n		Mean Age (SD)			% Female		
	Case	Control	Case	Control	*P*	Case	Control	*P*
Discovery (pooled)	97	216	48.4 (15.5)	76.7 (8.5)	<0.001	53.0	28.9	0.002
Rep1	96	72	42.7 (15.1)	72.9 (11.3)	<0.001	38.5	9.9	<0.001
Rep2	215	112	41.2 (14.8)	71.6 (8.5)*	<0.001	39.3	50.1	0.054
Discovery (individual) US1	222	3246	43.8 (13.3)	72.3 (5.4)	<0.001	43.7	39.2	0.188
US2	304	518	42.9 (15.6)	45.4 (13.7)	0.017	31.6	47.7	<0.001
Total	933	4164						

Comparisons were made with a *t*-test for age and χ^2 test for sex.

* Age was available only for 68/112 controls in Rep2, and this cohort was not examined.

TABLE 2. Association Results for Tag SNPs in the *HGF* Gene in the Pooled and Rep1 Samples Combined

SNP	Alleles Maj/Min	MAF Controls (n = 287)	MAF Cases (n = 196)	P	OR (95% CI)
rs7799610	G/A	0.078	0.072	0.74	1.08 (0.66–1.78)
rs12536657	G/A	0.227	0.195	0.24	1.21 (0.88–1.68)
rs2286194	T/A	0.167	0.204	0.15	1.28 (0.92–1.78)
rs3735520	C/T	0.413	0.513	**0.002**	1.50 (1.15–1.94)
rs17501108	G/T	0.144	0.067	**0.0002**	2.33 (1.17–3.69)
rs1014091	G/A	0.147	0.072	**0.0004**	2.22 (1.41–3.48)

The minor allele and its frequencies in cases and controls are shown. The OR with 95% CI is expressed with respect to the risk allele. Significant P values are highlighted bold.

reaching the $P < 1 \times 10^{-7}$ threshold (Supplementary Table S1, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-8261/-/DCSupplemental). Fifty-two top ranked SNPs were typed in individual case and control DNA samples and 19 reached the validation threshold (Supplementary Table S1), representing 13 independent loci. The only SNP showing any level of replication in the small Rep1 cohort was rs1014091, located upstream of the *HGF* gene.

Simultaneously with the Australian GWAS, the US1 cohort underwent genome-wide genotyping in individual DNA samples. Suggestive association was identified with *HGF* SNPs rs1014091 (P = 0.018; in complete LD with rs17501108) and rs3735520 ($P = 6.1 \times 10^{-7}$ OR[95% CI] = 1.63 [1.35–1.98]). The latter was the fourth ranked SNP in the GWAS and, in conjunction with the data from the Australian study, was prioritized for replication.

Genetic variation in and around *HGF* was then investigated further for association. Tag SNPs in the region of the *HGF* gene (rs12536657, rs2286194, rs3735520, and rs17501108) were genotyped in the Australian samples (Discovery and Rep1) along with the SNPs from the array (rs7799610 and rs1014091). Tag SNP rs17501108 is in complete linkage disequilibrium (r^2 =1.0) with array SNP rs1014091 in the Caucasian HapMap data, although both SNPs were typed to ensure comparison with the literature and consistency of results between cohorts. Both these SNPs are associated with keratoconus (Table 2) and survive Bonferroni correction for the six SNPs analyzed in and around this gene (P = 0.0004 for rs1014091 and P = 0.0002 for rs17501108). SNP rs3735520 was also associated (P = 0.002).

The associated SNPs, rs3735520 and rs17501108 were then typed in the Australian Rep2 and US2 replication cohorts. Association of SNP rs17501108 was replicated in Rep2 (P = 0.006; Table 3). No significant association ($P < 0.05$) was observed in the US2 replication cohort; however, the odds ratios are in the same direction with those observed in the other cohorts. All results for these SNPs are summarized in Table 3. Meta-analysis of *HGF* SNPs in all cohorts suggested that SNP rs3735520 is associated with keratoconus with a genome-wide suggestive $P = 9.9 \times 10^{-7}$. A less significant $P = 9.9 \times 10^{-5}$ was calculated for SNP rs17501108/rs1014091.

To assess the effect of SNP rs3735520 on HGF levels, we obtained serum HGF concentration that met quality control standards from 78 control participants (serum was not available from the keratoconus patients). According to a nonparametric Kruskal-Wallis test, there was a significant trend for increased serum HGF concentration with each T allele of the SNP (Table 4, P = 0.036). The minor T allele is also the risk allele for keratoconus. This association was also significant under both dominant and recessive genetic models. A similar relationship was found with the risk G allele of rs17501108, although it did not reach statistical significance (data not shown). No correlation was observed between serum HGF concentration and age in this cohort (Pearson's correlation =0.082; P = 0.47).

DISCUSSION

Two independent genome-wide association studies both identified positive association of keratoconus with SNPs in the promoter and upstream region of the *HGF* gene. Positive association was also observed in another independent cohort with a combined meta-analysis $P = 9.9 \times 10^{-7}$ in five cohorts. Although neither study was able to pinpoint this gene directly, analysis of the two GWASs in tandem identified *HGF* as a prominent candidate gene for further evaluation.

The odds ratios observed in this study are in the range of 1.5 to 2.2, indicating a relatively large effect size for a common complex disease. However, there is a possibility that they are over

TABLE 3. Results of Association Testing of HGF SNPs in Multiple Cohorts

	rs3735520				rs17501108/rs1014091*			
	MAF				MAF			
Cohort	Controls	Cases	P	OR (95% CI)	Controls	Cases	P	OR (95% CI)
Pooled+Rep1	0.41	0.51	**0.002**	1.50 (1.15–1.94)	0.14	0.07	**0.0002**	2.33 (1.17–3.69)
Rep2	0.43	0.45	0.664	1.08 (0.76–1.51)	0.17	0.10	**0.006**	1.93 (1.20–3.10)
US1	0.45	0.57	$\mathbf{6.1 \times 10^{-7}}$	1.63 (1.35–1.98)	0.11	0.07	**0.018**	1.56 (1.07–2.24)
US2	0.46	0.47	0.655	1.05 (0.86–1.28)	0.11	0.10	0.658	1.08 (0.77–1.50)

The minor allele frequency in cases and controls are shown. OR (95% CI) is also shown, expressed with respect to the risk allele defined in the pooled samples.

* As rs17501108 and rs1014091 are in very strong LD (r^2 = 0.95 in Australian samples), the results for rs17501108 are presented for Australian samples and rs1014091 for U.S. samples, due to different genotyping strategies in the two cohorts.

Table 4. Mean Serum HGF Concentration by Genotype of rs3735520 and Results of Nonparametric Tests under Genotypic, Dominant (CC vs. TC + TT), and Recessive (CC + TC vs. TT) Models

Genotype	*n*	Mean Serum HGF ± SE	Mean Rank by Genotype*	Mean Rank Dominant†	Mean Rank Recessive‡
CC	31	486.8 ± 39.0	33.35	33.35	37.66
TC	35	527.5 ± 28.7	41.36		
TT	12	632.0 ± 68.4	53.08	44.29	53.08

The mean rank of the serum HGF measurements is given by genotype group, as input for the nonparametric statistical tests.

* Kruskal-Wallis test $P = 0.036$.

† Mann-Whitney U test $P = 0.039$.

‡ Mann-Whitney U test $P = 0.032$.

inflated in this study because of the "winner's curse."[49] Importantly, all odds ratios (including those from US2 that did not reach significance) indicate an association in the same direction, providing further confidence in the validity of the association.

The *HGF* gene has been reported to be associated with refractive error in multiple populations including myopia in Han Chinese[50] and both myopia and hypermetropia in Caucasians.[36,51] The refractive power of the eye is determined at least in part by the shape of the cornea, which is severely altered in keratoconus, thus suggesting overlap between the genetic determinants of these complex ophthalmic conditions.

HGF (OMIM 142409; Online Mendelian Inheritance in Man; http://www.ncbi.nlm.nih.gov/Omim/, National Center for Biotechnology Information, Bethesda, MD), also known as scatter factor, is an inducible cytokine involved in a variety of cellular processes.[52] In general, HGF is produced and excreted by mesenchymal cells, such as fibroblasts. It remains bound to the extracellular matrix until it becomes activated by cleavage into its α and β chains by plasminogen activators.[52,53] It is then active on cells expressing the c-MET receptor, which are typically epithelial cells that do not express *HGF*.[53] The signals transduced via c-MET are varied and can include proliferation, cell scattering or changes in morphology, dependent on the context. The intracellular signals appear to be mediated through autophosphorylation of the c-MET intracellular docking region followed by docking of phosphatidylinositol-3-kinase (PI3K) or growth factor receptor–bound protein (Grb2).[52,53]

The *HGF* gene and its receptor c-MET are both known to be expressed in the cornea by all three cellular layers, although at lower levels in the epithelium than in the stroma and endothelium.[54] The protein is also produced by the lacrimal glands and is found in tears.[55] *HGF* expression in corneal keratinocytes is upregulated in response to corneal injury, although levels of the receptor are unchanged.[55] Primary cultures of epithelial and endothelial cells are both responsive to the proliferative signals of HGF, whereas stromal fibroblasts are not, despite expressing the receptor.[54] Thus, the interplay of HGF production and responsiveness in the cornea is complex. The observed genetic association and evidence that the same genetic variants are associated with elevated serum levels of the protein suggests HGF may be directly involved in the development of keratoconus.

The inflammatory cytokines IL-6 and TNFα have been detected at elevated levels in tears of patients with severe keratoconus[56] and also in eyes with subclinical keratoconus.[57] The human and mouse *HGF* gene promoters both contain binding sites for the proinflammatory cytokine IL-6 that have been shown to be active in murine cell culture models.[58] Thus, the mechanism of HGF association with keratoconus may be through inflammatory pathways.

The associated SNP rs3735520 is located 1.5 kb upstream of the transcription start site of the *HGF* gene. It is not known at this time whether this particular SNP is directly involved in the transcription of *HGF* or tags other variants that may affect transcription factor binding sites and alter the efficiency or timing of transcription of this gene. In this study, we did detect a significant association of this SNP with serum HGF levels in control participants. Carriers of the minor T allele (which is the at-risk allele for keratoconus) have, on average, a higher concentration of serum HGF than the CC homozygotes. Additional studies in a larger cohort of controls and, more important, in patients with keratoconus, are needed to further our understanding of the relationship between rs3735520 genotypes and serum HGF levels. In addition, HGF levels may be more relevant in tears or the corneas of patients than circulating serum levels. However, these data indicate a relationship between the SNPs associated with keratoconus and expression of the *HGF* gene.

In summary, association of common variations in *HGF* with keratoconus brings new insight in its pathogenesis by identifying a signaling pathway not previously directly implicated in this disease.

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References

1. Rabinowitz YS. Keratoconus. *Surv Ophthalmol.* 1998;42(4):297–319.
2. Rabinowitz YS. The genetics of keratoconus. *Ophthalmol Clin North Am.* 2003;16(4):607–620, vii.
3. Brancati F, Valente EM, Sarkozy A, et al. A locus for autosomal dominant keratoconus maps to human chromosome 3p14–q13. *J Med Genet.* 2004;41(3):188–192.
4. Tang YG, Rabinowitz YS, Taylor KD, et al. Genomewide linkage scan in a multigeneration Caucasian pedigree identifies a novel locus for keratoconus on chromosome 5q14.3-q21.1. *Genet Med.* 2005;7(6):397–405.
5. Hughes AE, Dash DP, Jackson AJ, Frazer DG, Silvestri G. Familial keratoconus with cataract: linkage to the long arm of chromosome 15 and exclusion of candidate genes. *Invest Ophthalmol Vis Sci.* 2003;44(12):5063–5066.
6. Burdon KP, Coster DJ, Charlesworth JC, et al. Apparent autosomal dominant keratoconus in a large Australian pedigree accounted for by digenic inheritance of two novel loci. *Hum Genet.* 2008;124(4):379–386.
7. Gajecka M, Radhakrishna U, Winters D, et al. Localization of a gene for keratoconus to a 5.6-Mb interval on 13q32. *Invest Ophthalmol Vis Sci.* 2009;50(4):1531–1539.
8. Hutchings H, Ginisty H, Le Gallo M, et al. Identification of a new locus for isolated familial keratoconus at 2p24. *J Med Genet.* 2005;42(1):88–94.
9. Tyynismaa H, Sistonen P, Tuupanen S, et al. A locus for autosomal dominant keratoconus: linkage to 16q22.3-q23.1 in Finnish families. *Invest Ophthalmol Vis Sci.* 2002;43(10):3160–3164.

10. Li X, Rabinowitz YS, Tang YG, et al. Two-stage genome-wide linkage scan in keratoconus sib pair families. *Invest Ophthalmol Vis Sci.* 2006;47(9):3791–3795.
11. Bisceglia L, De Bonis P, Pizzicoli C, et al. Linkage analysis in keratoconus: replication of locus 5q21.2 and identification of other suggestive loci. *Invest Ophthalmol Vis Sci.* 2009;50(3): 1081–1086.
12. Fullerton J, Paprocki P, Foote S, et al. Identity-by-descent approach to gene localisation in eight individuals affected by keratoconus from north-west Tasmania, *Australia Hum Genet.* 2002;110(5): 462–470.
13. Dash DP, George S, O'Prey D, et al. Mutational screening of VSX1 in keratoconus patients from the European population. *Eye* (*Lond*). 2010;24(6):1085–1092.
14. Eran P, Almogit A, David Z, et al. The D144E substitution in the VSX1 gene: a non-pathogenic variant or a disease causing mutation? *Ophthalmic Genet.* 2008;29(2):53–59.
15. Heon E, Greenberg A, Kopp KK, et al. VSX1: a gene for posterior polymorphous dystrophy and keratoconus. *Hum Mol Genet.* 2002; 11(9):1029–1036.
16. Mok JW, Baek SJ, Joo CK. VSX1 gene variants are associated with keratoconus in unrelated Korean patients. *J Hum Genet.* 2008; 53(9):842–849.
17. Paliwal P, Singh A, Tandon R, Titiyal JS, Sharma A. A novel VSX1 mutation identified in an individual with keratoconus in India. *Mol Vis.* 2009;15:2475–2479.
18. Aldave AJ, Yellore VS, Salem AK, et al. No VSX1 gene mutations associated with keratoconus. *Invest Ophthalmol Vis Sci.* 2006; 47(7):2820–2822.
19. Bisceglia L, Ciaschetti M, De Bonis P, et al. VSX1 mutational analysis in a series of Italian patients affected by keratoconus: detection of a novel mutation. *Invest Ophthalmol Vis Sci.* 2005; 46(1):39–45.
20. Tang YG, Picornell Y, Su X, et al. Three VSX1 gene mutations, L159M, R166W, and H244R, are not associated with keratoconus. *Cornea.* 2008;27(2):189–192.
21. Liskova P, Ebenezer ND, Hysi PG, et al. Molecular analysis of the VSX1 gene in familial keratoconus. *Mol Vis.* 2007;13:1887–1891.
22. Udar N, Atilano SR, Brown DJ, et al. SOD1: a candidate gene for keratoconus. *Invest Ophthalmol Vis Sci.* 2006;47(8):3345–3351.
23. Stabuc-Silih M, Strazisar M, Hawlina M., Glavac D. Absence of pathogenic mutations in VSX1 and SOD1 genes in patients with keratoconus. *Cornea*. 29(2):172–176.
24. Kim SH, Mok JW, Kim HS, Joo CK. Association of −31T>C and −511 C>T polymorphisms in the interleukin 1 beta (IL1B) promoter in Korean keratoconus patients. *Mol Vis.* 2008;14:2109–2116.
25. Stabuc-Silih M, Ravnik-Glavac M, Glavac D, Hawlina M, Strazisar M. Polymorphisms in COL4A3 and COL4A4 genes associated with keratoconus. *Mol Vis.* 2009;15:2848–2860.
26. Burdon KP, Macgregor S, Hewitt AW, et al. Genome-wide association study identifies susceptibility loci for open angle glaucoma at TMCO1 and CDKN2B-AS1. *Nat Genet.* 2011;43(6):574–578.
27. Kokotas H, Grigoriadou M, Petersen MB. Review: age-related macular degeneration: genetic and clinical findings. *Clin Chem Lab Med.* 2011;49(4):601–611.
28. Young TL, Metlapally R, Shay AE. Complex trait genetics of refractive error. *Arch Ophthalmol.* 2007;125(1):38–48.
29. 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(5), e1000947.
30. 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(21):4304–4311.
31. Vithana EN, Aung T, Khor CC, et al. Collagen-related genes influence the glaucoma risk factor, central corneal thickness. *Hum Mol Genet.* 2011;20(4):649–658.
32. Rabinowitz,YS. Videokeratographic indices to aid in screening for keratoconus. *J Refract Surg.* 1995;11(5):371–379.
33. Brown KM, Macgregor S, Montgomery GW, et al. Common sequence variants on 20q11.22 confer melanoma susceptibility. *Nat Genet.* 2008;40(7):838–840.
34. Macgregor S, Zhao ZZ, Henders A, et al. Highly cost-efficient genome-wide association studies using DNA pools and dense SNP arrays. *Nucleic Acids Res.* 2008;36(6):e35.
35. Macgregor S, Visscher PM, Montgomery G. Analysis of pooled DNA samples on high density arrays without prior knowledge of differential hybridization rates. *Nucleic Acids Res*. 2006;34(7):e55.
36. Yanovitch T, Li YJ, Metlapally R, et al. Hepatocyte growth factor and myopia: genetic association analyses in a Caucasian population. *Mol Vis.* 2009;15:1028–1035.
37. Purcell S, Neale B, Todd-Brown K, et al. PLINK: a tool set for whole-genome association and population-based linkage analyses. *Am J Hum Genet.* 2007;81(3):559–575.
38. Wang Y, Rabinowitz YS, Rotter JI, Yang H. Genetic epidemiological study of keratoconus: evidence for major gene determination. *Am J Med Genet.* 2000;93(5):403–409.
39. Fried LP, Borhani NO, Enright P, et al. The Cardiovascular Health Study: design and rationale. *Ann Epidemiol.* 1991;1(3):263–276.
40. Psaty BM, O'Donnell CJ, Gudnason V, et al. Cohorts for Heart and Aging Research in Genomic Epidemiology (CHARGE) Consortium: design of prospective meta-analyses of genome-wide association studies from five cohorts. *Circ Cardiovasc Genet.* 2009;2(1):73–80.
41. Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res.* 1988;16(3):1215.
42. Gunderson KL, Steemers FJ, Lee G, Mendoza LG, Chee MS. A genome-wide scalable SNP genotyping assay using microarray technology. *Nat Genet.* 2005;37(5):549–554.
43. Gunderson KL, Steemers FJ, Ren H, et al. Whole-genome genotyping. *Methods Enzymol.* 2006;410:359–376.
44. Gunderson KL, Kuhn KM, Steemers FJ, et al. Whole-genome genotyping of haplotype tag single nucleotide polymorphisms. *Pharmacogenomics.* 2006;7(4):641–648.
45. DeBakker, P. Tagger: selection and evaluation of tag SNPs. Available at http://www.broad.mit.edu/mpg/tagger/. Accessed July 2010.
46. Barrett JC, Fry B, Maller J, Daly MJ. Haploview: analysis and visualization of LD and haplotype maps. *Bioinformatics.* 2005;21(2): 263–265.
47. The International HapMap Project. *Nature*. 2003;426(6968):789–796.
48. Frazer KA, Ballinger DG, Cox DR, et al. A second generation human haplotype map of over 3.1 million SNPs. *Nature.* 2007; 449(7164):851–861.
49. Kraft P. Curses—winner's and otherwise—in genetic epidemiology. *Epidemiology.* 2008;19(5):649–651, discussion 657–658.
50. Han W, Yap MK, Wang J, Yip SP. Family-based association analysis of hepatocyte growth factor (HGF) gene polymorphisms in high myopia. *Invest Ophthalmol Vis Sci.* 2006;47(6):2291–2299.
51. Veerappan S, Pertile KK, Islam AF, et al. Role of the hepatocyte growth factor gene in refractive error. *Ophthalmology.* 2010; 117(2):239–45, e1–e2.
52. Stuart KA, Riordan SM, Lidder S, et al. Hepatocyte growth factor/scatter factor-induced intracellular signalling. *Int J Exp Pathol.* 2000;81(1):17–30.
53. Grierson I, Heathcote L, Hiscott P, et al. Hepatocyte growth factor/scatter factor in the eye. *Prog Retin Eye Res.* 2000;19(6):779–802.
54. Wilson SE, Walker JW, Chwang EL, He YG. Hepatocyte growth factor, keratinocyte growth factor, their receptors, fibroblast growth factor receptor-2, and the cells of the cornea. *Invest Ophthalmol Vis Sci.* 1993;34(8):2544–2561.
55. Li Q, Weng J, Mohan RR, et al. Hepatocyte growth factor and hepatocyte growth factor receptor in the lacrimal gland, tears, and cornea. *Invest Ophthalmol Vis Sci.* 1996;37(5):727–739.
56. Lema I, Duran JA. Inflammatory molecules in the tears of patients with keratoconus. *Ophthalmology.* 2005;112(4):654–659.
57. Lema I, Sobrino T, Duran JA, Brea D, Diez-Feijoo E. Subclinical keratoconus and inflammatory molecules from tears. *Br J Ophthalmol.* 2009;93(6):820–824.
58. Liu Y, Michalopoulos GK., Zarnegar R. Structural and functional characterization of the mouse hepatocyte growth factor gene promoter. *J Biol Chem.* 1994;269(6):4152–4160.