Two-Stage Genome-Wide Linkage Scan in Keratoconus Sib Pair Families

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Purpose. To identify susceptibility gene loci for keratoconus.

Methods. A genome-wide linkage analysis was performed with data from 67 keratoconus sib pair families with 110 affected sib pairs of white or Hispanic origin. A total of 351 subjects were genotyped for 380 microsatellite markers along the genome at ~10-cM density. An additional 58 microsatellite markers at ~2-cM density in the identified linkage regions on chromosomes 4, 5, 9, 12, and 14 were also genotyped. Multipoint linkage analysis was performed in all pedigrees by nonparametric methods and maximum likelihood estimates of identity by descent sharing as implemented in GeneHunter (http://linkage.rockefeller.edu/soft/gh/ provided in the public domain by Rockefeller University, New York, NY).

Results. The strongest evidence of linkage was observed at the telomere (159 cM) of chromosome 9 (lod = 4.5) in all pedigrees. Other regions suggestive of linkage were identified at 176 cM of chromosome 4 (lod = 2.7), 143 cM of chromosome 5 (lod = 2.0), 7 cM of chromosome 9 (lod = 2.8), 12 cM of chromosome 11 (lod = 2.3), 27 cM of chromosome 12 (lod = 2.3), and 14 cM of chromosome 14 (lod = 2.9). Two significant linkage regions were also observed on chromosomes 17 at 86 cM (lod = 3.9) and 9 at 34 cM (lod = 3.8) in the Hispanic subjects only. After fine mapping these regions (with the exception of chromosomes 11 and 17), most linkage peaks remained similar (lod = 2.2 at 176 cM on chromosome 4; lod = 1.7 at 146 cM on chromosome 5; lod = 3.5 at 160 cM on chromosome 9; lod = 2.5 at 7 cM on chromosome 12; and lod = 2.6 at 19 cM on chromosome 14).

Conclusions. These results indicate that one or more loci may contribute to keratoconus susceptibility. (*Invest Ophthalmol Vis Sci.* 2006;47:3791–3795) DOI:10.1167/iovs.06-0214

Keratoconus is a clinical term used to describe a condition in which the cornea assumes a conical shape as a result of noninflammatory thinning and protrusion. Keratoconus is detected clinically by slit lamp evaluation, which demonstrates stromal corneal thinning. Other clinical signs may include any one or a combination of the following: Vogt's striae, an iron ring, scarring, and retroillumination signs such as the "Charleaux oil droplet reflex" and scissoring on retinoscopy. Videokeratography is exquisitely accurate for confirming the diagnosis, particularly in subtle cases in which slit lamp findings or clinical signs may not be obvious.

The estimated prevalence of keratoconus ranges from 50 to 230/100,000 in the general population.[1] The age of onset is at puberty and the disorder is progressive until the third to fourth decades of life, when it usually arrests. It is a major cause of cornea transplantation in developed countries. The underlying biochemical processes and its cause remain poorly understood.[1]

Although the etiology of keratoconus is still unknown, genetic factors may play an important role, as indicated by the association of keratoconus with genetic syndromes, segregation analyses, genetic epidemiologic data, and gene mapping studies.[2–6] Approximately 6% to 23.5% of cases reported in the literature demonstrate clinically recognized familial transmission.[1,2,4] The estimated prevalence of keratoconus in first-degree relatives is 3.34%, 15 to 67 times higher than in the general population.[4] Both dominant and recessive models have been observed in keratoconus pedigrees.[4–6] Although most other families do not fit any typical mode of inheritance, subjects with keratoconus exhibit bilateral disorder almost universally (in excess of 90%). In many cases, the disorder may initially be evident unilaterally, but over time the other eye becomes involved.[7] This bilaterality also lends support to a genetic basis for this disease. Twin studies are another line of evidence of genetic contribution in keratoconus.[2,3,8] At least 18 sets of monozygotic twins showing 54% concordance of keratoconus in both twins have been reported in the literature, whereas there are insufficient data on dizygotic twins to analyze concordance.

To date, gene loci for keratoconus have been mapped by linkage analysis to the after chromosomal regions: 2p24, 3p, 5q, 16q22, 20q12[9–13]; and a mutation in the visual system homeobox gene 1 (*VSX1*; mapped to 20p11-q11) has been reported in 4% to 9% of patients with keratoconus in two studies.[14,15] Most linkage studies on keratoconus have been performed in white families under the dominant model.

These results from gene mapping efforts imply the importance of genetic determinants, but the lack of consistent chromosomal loci among different studies indicate genetic heterogeneity and illustrates the complex nature of the genetic contribution to keratoconus. Genes identified from the Mendelian form of the disease have implications for disease pathogenesis, but the role of rare mutations may be limited, as noted by the mutations reported in the *VSX1* gene in a small number of patients with keratoconus. Therefore, identification of susceptibility genes that contribute to non-Mendelian forms of keratoconus and other ethnic groups is still necessary in understanding its pathogenesis.

The goal of this study was to perform a two-stage genome scan in different ethnic groups and to identify susceptibility genes for keratoconus by using the nonparametric method.

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TABLE 1. Samples in Keratoconus Sib Pair Families

Affected Sibship Size	Families (*n*)	Parents (*n*)	Affected Sibs (*n*)	Unaffected Sibs (*n*)	Total Subjects (*n*)	Total Affected Sib Pairs (*n*)
2	57	69	122	92	283	59
3	7	10	12	14	36	21
4	1	3	4	3	10	8
5	1	1	6	7	14	10
6	1	2	6	0	8	12
Subtotal	67	85	150	116	351	110

METHODS

Subjects

We enrolled families ascertained through a clinically affected keratoconus proband as a part of the longitudinal videokeratography and genetic study at the Cedars-Sinai Medical Center (Los Angeles, CA).[4] Study subjects included 351 individuals from 67 sib pair families with 110 affected sib pairs. Among these pedigrees, 40 families with 217 individuals were white, and 17 families with 93 individuals were Hispanic. Seven African-American families, 2 Asian families, and 1 mixed-origin family were also studied.

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 examine whether there was stromal corneal thinning, Vogt's 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 retroillumination signs of keratoconus, such as the oil droplet sign and scissoring of the red reflex. Videokeratography evaluation was also performed on each eye (Topographic Modeling System; Computed Anatomy, New York, NY). Subjects were deemed to have 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.[1] The (AB/SRAX) pattern is the most common pattern on videokeratography in patients with keratoconus and has been demonstrated the most useful pattern for differentiating subtle cases of keratoconus from the normal population.[1,4] Any affected individual or family members were excluded if they had any other genetic disease or disease known to be associated with keratoconus.[1]

Institutional Review Board approval was obtained at Cedars-Sinai Medical Center. Written informed consent was obtained from all subjects. Venous blood was drawn from all study subjects for molecular genetic analysis. All study procedures were within the tenets of the Declaration of Helsinki.

Genome Scan

Subjects were genotyped for 380 microsatellite markers along the genome at ~10-cM density, using the Marshfield screen set 13 (http://www.marshfieldclinic.org/genetics). The genotyping was performed through the Mammalian Genotyping Services (MGS) at the Marshfield Medical Research Foundation (Marshfield, WI). Mean marker density was 9.2 cM, and mean heterozygosity was 0.74 (range, 0.31–0.90). Relcheck and Pedcheck were performed to identify errors in the familial relationships and Mendelian inconsistencies.[16,17] Any problematic individuals, families, and markers were corrected for further analysis.

To delineate further any suggestive linkage near chromosomes 4, 5, 9, 12, and 14, we genotyped an additional 58 microsatellite markers at ~2 cM density in the identified linkage regions. The information content ranged from 0.64 to 0.87 with an average of 0.77.

Statistical Methods

Multipoint linkage analysis for each pedigree was performed by both the nonparametric method and maximum-likelihood estimates of identity by descent sharing, as implemented in the GeneHunter program (http://linkage.rockefeller.edu/soft/gh/ provided in the public domain by Rockefeller University, New York, NY).[18] Multipoint estimates of identity-by-descent (ibd) were obtained as weighted average of ibd for individual markers.

We further estimated empiric probabilities for linkage analysis by performing a simulation study using the SIMULATE program under the null hypothesis that there was no linkage between a trait and a marker.[19]

RESULTS

We genotyped and analyzed 351 individuals in 67 sib pair families. Among these pedigrees, 40 families with 217 individuals were white and 17 families with 93 individuals were Hispanic. Table 1 summarizes the families studied. Studied subjects included 85 parents and 150 affected and 116 unaffected sibs, which resulted in 110 affected sib pairs. Using the family structure from our study, we simulated 1000 replicates on the selected chromosomes with identified linkage peaks with frequencies and maps identical with the real data. The empiric probabilities were computed by dividing the number of replicates that exceeded the particular lod score threshold by the total number of the replicates (1000).

Genome-Wide Multipoint Linkage Analyses

Figure 1 shows the linkage results for the white pedigrees, the Hispanic pedigrees, and all 67 pedigrees along the whole genome, except for chromosome X. The nonparametric method found several regions suggestive of linkage in the white, Hispanic, and all pedigrees. Table 2 shows the selected chromosomes from the genome scan linkage analysis which have a maximum lod score >2.0 observed in the white, Hispanic, and/or all pedigrees. In general, the peaks identified in the white or all pedigrees were similar, although the magnitude of the lod score varied. The strongest evidence of linkage was observed at the telomere (159 cM) of chromosome 9 (lod = 4.5) in all pedigrees. Other suggestive linkages were identified at 176 cM of chromosome 4 (lod = 2.7), 143 cM of chromosome 5 (lod = 2.0), 7 cM of chromosome 9 (lod = 2.8), 12 cM of chromosome 11 (lod = 2.3), 27 cM of chromosome 12 (lod = 2.3), and 14 cM of chromosome 14 (lod = 2.9) (Table 2). Three (chromosomes 4, 9, and 14) of 22 autosomal chromosomes showed the maximum lod ≥2.5, either in all or in white families, whereas two significant linkage regions were identified on chromosomes 17 at 86 cM (lod = 3.9) and 9 at 34 cM (lod = 3.8) for Hispanics only. Of note, we had identified evidence of linkage at 103 cM on chromosome 5 in a four-generation pedigree and now obtained the maximum lod with 2.0 at 143 cM in sib pair families.[12] Although these two peaks

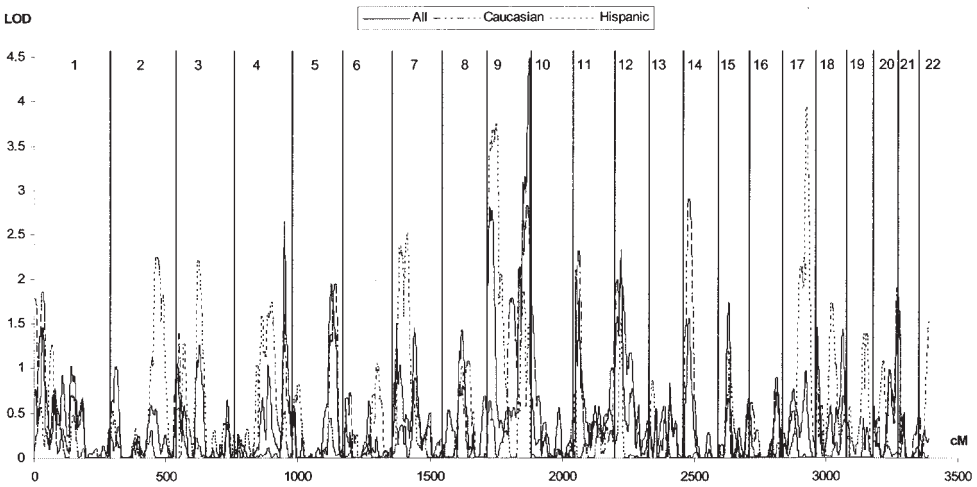

FIGURE 1. The linkage results in keratoconus families in pedigrees, Hispanic, and all pedigrees throughout the genome, except for chromosome X.

are not in perfect overlap, their close proximity indicates that these may represent linkage to the same locus.

Fine Mapping on Chromosomes 4, 5, 9, 12, and 14

Based on the linkage regions identified, we selected the regions with either lod > 2.5 or NPL > 2.5 for whites and/or all pedigrees to do the fine mapping. Thus, an additional 58 markers on chromosomes 4, 5, 9, 12, and 14 along the previous identified linkage regions were genotyped, and multipoint linkage analysis was then performed using all and the white and Hispanic pedigrees, by both the nonparametric method and maximum likelihood estimate of ibd. Most linkage peaks were similar after fine mapping. Figure 2 shows the lod scores before and after fine mapping for these chromosomes. In general, the linkage peaks remained similar, but most lod scores in these regions dropped slightly. Significant linkage was still detected at 160 cM on chromosome 9 with lod = 3.5. Other suggestive linkages were on chromosomes 4 (lod = 2.2 at 176 cM), 12 (lod = 2.5 at 7 cM), and 14 (lod = 2.6 at 19 cM).

DISCUSSION

We have conducted a two-stage genome-wide study to identify the susceptibility genes for keratoconus using the nonparametric method. We observed evidence of linkage for keratoconus on chromosomes 4, 5, 9, 12, and 14 for white and/or all pedigrees. With the use of Hispanic pedigrees only, an additional significant linkage region on chromosome 17 was identified. The results of the fine mapping in regions with suggestive linkage were consistent with the linkage peaks we had previously identified. These results indicate that several loci may contribute to keratoconus susceptibility.

Only several genome scan studies of keratoconus have been conducted in recent years and mostly in whites.[12–15] Evidence of linkage was not consistent among these studies. Because many factors can affect the ability to detect trait loci (e.g., study population and genetic models), a disease locus affecting a complex trait may not be detected in all studies. Our study used a sample of mixed populations with most of the whites ascertained from probands with clinical keratoconus, and provided evidence of linkage on several chromosomes. In addition, we identified some consistent linkage regions in both whites and Hispanics; however, some regions were identified in whites or Hispanics only (Table 2).

Although there are hundreds of known or predicted genes in the linkage regions we identified, there are only a few keratoconus candidate genes in these regions. The most promising one is lysyl oxidase, a gene responsible for collagen cross linking, which is thought to be defective in patients with

TABLE 2. Suggestive Linkage Peaks for Keratoconus

	White Pedigrees				Hispanic Pedigrees				All Pedigrees			
Chr	Position (cM)	Maximum Lod	NPL	*P*	Position (cM)	Maximum Lod	NPL	*P*	Position (cM)	Maximum Lod	NPL	*P*
2	—*	—*	—*	—*	184	2.3	0.96	0.008	—	—	—	—
3	—	—	—	—	74	2.2	1.69	0.006	—	—	—	—
4	176	1.6	1.82	0.023	127	1.7	1.08	0.025	176	2.7	2.68	0.001
5	157	2.0	2.03	0.013	—	—	—	—	143	2.0	2.90	0.013
7	—	—	—	—	42	2.5	2.64	0.002	69	1.5	1.86	0.022
9†	—	—	—	—	34	3.8	3.55	<0.001	7	2.9	2.63	0.004
9†	152	2.8	2.80	0.001	—	—	—	—	159	4.5	2.83	<0.001
11	12	2.3	1.98	0.003	—	—	—	—	1	1.9	2.16	0.018
12	27	2.0	2.47	0.009	27	1.6	2.25	0.054	27	2.3	2.64	0.010
14	14	2.9	2.91	0.003	—	—	—	—	15	1.6	2.23	0.032
17	—	—	—	—	86	3.9	3.32	<0.001	—	—	—	—

Chr, chromosome.

* Maximum lod < 1.3; all others, maximum lod ≥ 2.0.

† Chromosome 9 is listed twice. The first row of data illustrates results of analysis for Hispanics only. The second row illustrates results for all families. Both have a positive lod score.

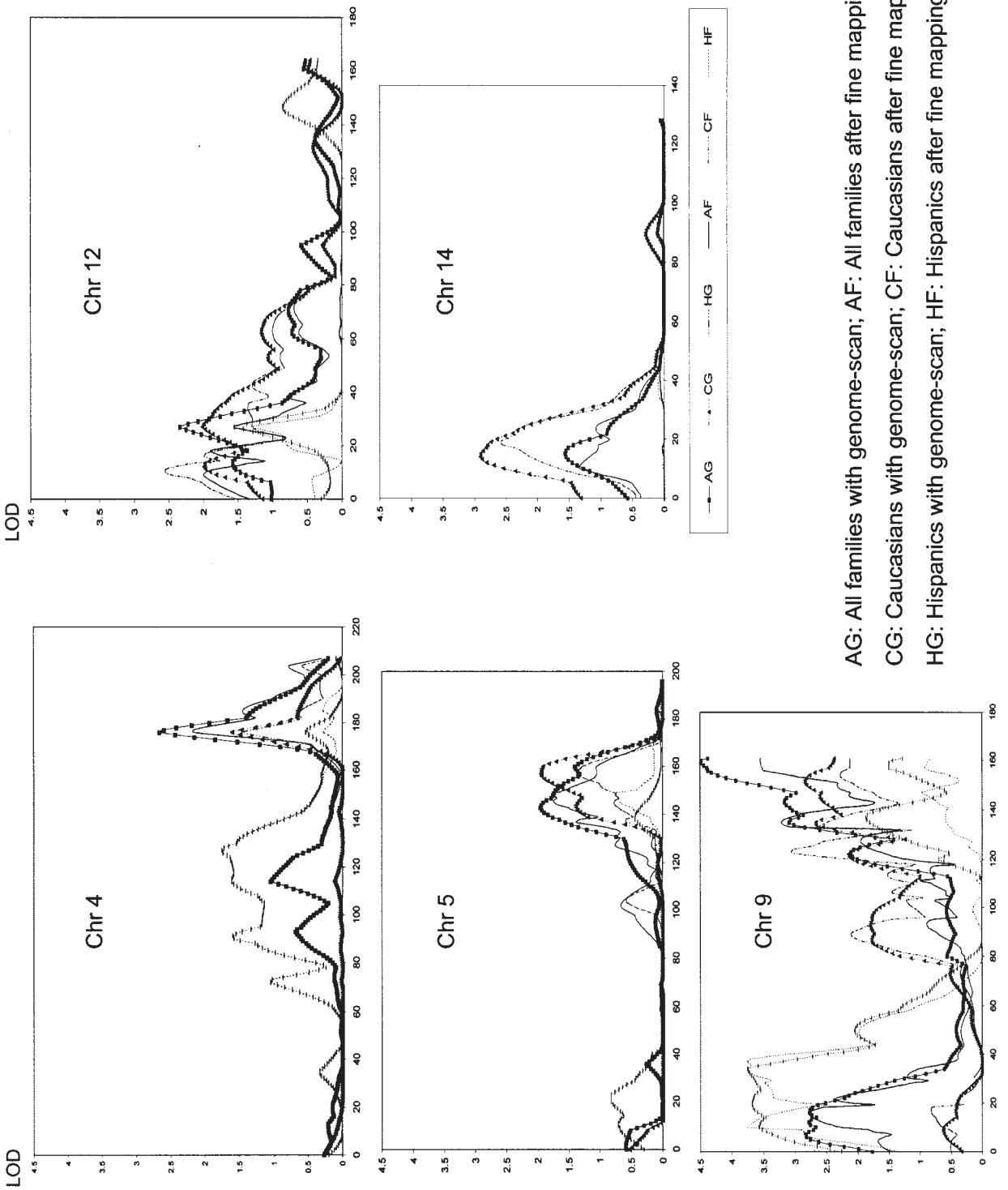

FIGURE 2. The linkage results on chromosomes selected for fine mapping.

keratoconus.[20] This gene is located at 5q23.2 and is located beneath the linkage peak we identified in this study. It is intriguing that the 5q region observed in our study falls in a region within 30 cM reported by our previous study which identified the linkage of the 5q14-21 region to keratoconus with lod scores of 3.3 in a four-generation white pedigrees under a dominant model.[12] Although these two peaks are not in perfect overlap, their close proximity indicates that these may be the same locus.

Another promising candidate gene for studying keratoconus is an apoptosis gene, cell death-inducing DEFA-like effector b (*CIDEB*), located at 14q11.2 under our linkage peak on chromosome 14 in this genome-wide scan of keratoconus families. Several previously reported histochemical studies suggest that apoptosis may play an important role in the pathogenesis of keratoconus.[21,22] This was subsequently confirmed by a gene expression study that demonstrated the presence of 67 apoptosis genes in keratoconus corneal buttons, some of which are expressed at much higher levels than those in the normal corneal epithelium.[23]

The only significant corneal gene in the region of most significant linkage in this study (9q34) is gelsolin (*GSN*) which has been reported to be causative of another corneal dystrophy, familial amyloidosis.[24,25]

This study is the first to attempt to identify susceptibility genes for keratoconus in a Hispanic population. We identified two significant linkage regions on chromosomes 7 and 17 in this ethnic group; however, the modest sample size studies (approximately 90 individuals) may limit the interpretability of our results.

In all genetic studies of keratoconus, there are limitations that should be taken into account when interpreting linkage data. Subclinical forms of keratoconus may exist that may not be detectable using our and keratoconus may occur or progress at any age. Future linkage studies, with quantitative videokeratography indices, may identify a quantitative trait locus for assigning early disease providing more statistical power and may be helpful in confirming our results.

Keratoconus is a complex disease, and the regulation and mechanisms of gene effects are poorly understood. Our study is the first to identify linkage using subpopulations in a relatively large study sample.

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