Identification of a new locus for isolated familial keratoconus at 2p24

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J Med Genet 2005;42:88–94. doi: 10.1136/jmg.2004.022103

Keratoconus (KC; MIM 148300) is a non-inflammatory corneal thinning disorder. Progressive thinning and protrusion of the cornea lead to loss of visual acuity for which the only effective treatment is corneal transplantation. The estimated prevalence of KC is 1–4 per 2000 in the general population.¹ The age of onset is often at puberty, and the disorder is progressive until the third or fourth decade of life when it usually arrests. KC is a major cause of corneal transplantation in developed countries.

The cause of KC is still unknown. Histological observations have demonstrated degradation of the corneal epithelium basal membrane, diminution of the number of collagen fibrils, thinning of the corneal stroma, and keratocyte apoptosis.^{2–3} Biochemical studies describe increased activity of metalloproteinases (MMP-2 and MMP-9) and lower expression of protease inhibitors such as $\alpha 1$ -protease inhibitor.^{4–5}

Sporadic KC is the most common presentation; however, a positive familial history has been reported in at least 6–10% of patients.¹ Twin studies performed since the advent of modern computerised videokeratoscopy have reported four monozygotic pairs concordant for KC and two monozygotic pairs which were discordant,^{6–9} suggesting a genetic component for KC. Keratoconus prevalence in first degree relatives of KC patients is significantly higher than in the general population, demonstrating familial aggregation of the trait.^{10–11} Most published studies have suggested autosomal dominant inheritance of KC with incomplete penetrance or variable expression.^{1–12} Autosomal recessive inheritance as well as rare cases of X linked inheritance have also been described.^{10–13} In some rare cases, KC is associated with other genetic disorders such as trisomy 21, atopy, and Leber congenital amaurosis.^{14–15}

KC gene localisation efforts to date have been carried out in rare large families or in population isolates where genetic heterogeneity is minimised. Linkage studies include a genome scan of 20 Finnish families resulting in a multipoint LOD score of 4.1 on chromosome 16q,¹⁶ and a directed chromosome 21 scan in one large family with trisomy 21 leading to suggestive linkage.¹⁷ Association studies in eight unrelated patients in Tasmania have identified two putative loci on 18p and 20q12.^{18–19} However, these results have not yet led to the identification of a causative gene for familial KC and, although KC is present in all human populations, no studies have tested for linkage in a mixed outbred population.

In the present article, we identify a new candidate region at 2p24 mapped by linkage and haplotype analysis in a heterogeneous population.

METHODS

Families

The study population included 253 members of 28 families recruited in France, Spain, and Guadeloupe (West Indies).

Key points

- Twenty eight pedigrees with isolated familial keratoconus (MIM 148300) were investigated.
- A genome-wide search mapped a new locus for keratoconus to chromosome 2p24 with a maximal HLOD score of 5.13. Analysis of key recombination events placed the keratoconus locus in a 1.69 Mb region flanked by markers D2S305 and D2S2373. Linkage to all other known keratoconus loci on chromosomes 3, 16, 20, and 21 was excluded.
- This candidate region contains eight known genes and 10 Acembly gene predictions.
- This study reports the first genome-wide scan in an outbred population (Caucasian, Arab, and Caribbean African) and is the first step in the positional cloning of a keratoconus gene.

There were 112 affected and 104 unaffected individuals; the other 37 individuals were of undetermined phenotype. Families were of European, Arab, and Caribbean African descent. All pedigrees had a minimum of two members who met the definition of clinical KC²⁰ and no other recognised genetic disease segregated in these families. We performed ophthalmic examination of all the members of the 28 families using videokeratographic evaluation as described previously.¹¹ KC was defined according to Rabinowitz's KISA% index²⁰: patients with a KISA% over 300 have clinical KC, patients with a KISA% between 100 and 300 may have forme fruste KC and were considered as being of an undetermined phenotype, and patients with a KISA% under 100 are unaffected. Children under the age of 16 were considered of unknown phenotype unless clinical KC was observed.

This study was approved by the ethical committee "Toulouse 2" according to French law, and informed consent was obtained from each patient.

Microsatellite genotyping

Venous blood samples were collected in EDTA for DNA extraction according to standard methods.²¹ Genotyping was performed in two successive stages. An initial set of seven informative families (88 individuals of Caucasian and Arab origin) composed of both affected and unaffected individuals was genotyped with the ABI PRISM Linkage Mapping Set of microsatellite markers, version 2 (Applied Biosystems, Foster City, CA, USA). While the first analysis was underway, additional families with KC were recruited. The total sample

Abbreviations: GDF-7, growth differentiation factor 7; KC, keratoconus

Figure 1 Linkage results of the genome-wide scan for KC with 382 autosomal markers in seven informative pedigrees. Multipoint LOD scores were calculated with Merlin. The genetic locations correspond to the Marshfield genetic map²⁵ and are expressed in Kosambi cM.

size was thus increased to 28 informative pedigrees, consisting of 112 affected and 104 unaffected members available for genotyping. The second set of 21 families was only genotyped with markers for the short arm of chromosome 2.

All PCR reactions were carried out with 25 ng of genomic DNA as a template in a mixture of 1×Applied Biosystems PCR buffer II, 2.5 mM MgCl₂, 200 nM of each dNTP, 5 pmol of each primer, and 0.1 µl of AmpliTaq Gold polymerase (Applied Biosystems) in a final volume of 15 µl. The thermocycling conditions were 94°C for 10 min, followed by 33 cycles at 94°C for 30 s, 53°C for 30 s, and 72°C for 30 s, followed by a final extension step at 72°C for 5 min. PCR products were then pooled with regard to their size range and labelling and were analysed by denaturing electrophoresis on an ABI Prism 3100 DNA Sequencer (Applied Biosystems). The marker alleles were assigned with GENESCAN and GENOTYPER software (Applied Biosystems). A set of 36

additional markers obtained from online information was added for detailed analysis of the short arm of chromosome 2.

Statistical analysis

Non-parametric multipoint linkage analysis for the genome-wide screen was performed with the Merlin program.²² Non-parametric linkage analysis concentrates on allele sharing between family members with the same phenotype. Merlin outputs both NPL_{all} (Z_{lr}) scores and Kong and Cox LOD scores²³ to test for allele sharing among affected individuals, with their asymptotic p values. All Mendelian inheritance and genotype errors were checked using Merlin and resolved. Allele frequencies were calculated in unrelated founders with Merlin. Multipoint parametric linkage analysis, allowing for heterogeneity, was performed with Genehunter, version 2.1.²⁴ The frequency of the putative KC gene was set at 0.0006. No sex difference was assumed. Penetrances were 0.001, 0.5, and 0.8, respectively, for the 1/1, 1/2, and 2/2 genotypes (1, normal allele; 2, mutated allele; a penetrance of 0.001 for the 1/1 genotype corresponds to the prevalence of sporadic KC). The marker mapping information was obtained from the Marshfield map²⁵ and the NCBI database (Online Mendelian Inheritance in Man (OMIM), <http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=OMIM>). We used Genehunter for haplotype reconstruction. The program reports the most likely haplotype, marking crossovers and highlighting double crossovers. It also indicates regions of haplotype uncertainty.

RESULTS

Assignment of the KC gene to 2p24

We undertook a genome-wide search for linkage in an initial set of seven families whose results are displayed in fig 1. Non-parametric analysis by Merlin found suggestive linkage on chromosome 2p with a LOD score of 2.64 ($Z_{lr} = 4.91$, $p < 10^{-5}$) for marker D2S305. No other LOD scores > 1.5 were observed. In particular, no evidence for linkage between KC and markers on the chromosome arms 16q, 18p, or 21q was observed.

The full data set of 28 families was then typed for 36 additional markers across a 80 cM region spanning D2S319 to D2S286. Marker order is displayed in table 1. When all families were analysed we obtained suggestive evidence of linkage with a maximum LOD score at D2S305 of 3.26 ($Z_{lr} = 5.02$, $p < 10^{-5}$) (fig 2, grey line).

Figure 2 Results of multipoint linkage analysis with 48 microsatellite markers on chromosome 2p showing non-parametric LOD (grey) and HLOD (black) scores for the dominant parametric model.

Table 1 Microsatellite marker order and genetic and physical distances on chromosome 2p

Marker	cM (Marshfield map)	Mb (public location)
D2S319	7.600	5.036
D2S2211	15.610	7.493
D2S162	20.030	8.882
D2S168	27.060	11.467
D2S2200	27.600	–
D2S131	31.200	13.389
D2S2267	33.310	13.757
D2S149	34.040	14.415
D2S312	34.780	15.249
D2S2295	35.980	16.459
D2S2346	36.720	16.833
D2S272	37.380	16.851
D2S320	38.330	18.231
D2S305	38.870	19.403
D2S310	39.920	19.538
D2S2233	39.925	19.784
D2S175	39.930	19.926
D2S2342	40.200	20.314
D2S2150	40.470	20.516
D2S2373	42.650	21.097
D2S2221	44.090	23.198
D2S2168	45.300	25.063
D2S2303	45.830	–
D2S174	46.900	26.814
D2S2247	46.950	27.278
D2S165	47.430	28.578
D2S2312	47.970	28.844
D2S146	49.040	29.566
D2S352	50.650	31.476
D2S367	54.960	34.415
D2S2186	57.220	37.047
D2S177	59.360	38.001
D2S2259	64.290	42.971
D2S2294	64.840	43.850
D2S2174	67.580	45.153
D2S391	70.310	46.386
D2S2227	71.140	47.244
D2S2316	73.605	51.067
D2S123	73.600	51.263
D2S2369	73.610	53.199
D2S378	77.430	57.278
D2S2183	77.970	59.077
D2S337	80.690	61.644
D2S147	82.290	64.139
D2S2368	85.480	67.188
D2S303	88.150	70.437
D2S2110	90.820	73.047
D2S286	94.050	75.317

Markers in bold were used for the genome-wide scan.

Since segregation of KC in our families seemed in agreement with autosomal dominant inheritance, we performed multipoint parametric analysis and applied a dominant model to our data. We set gene frequency of the familial form of KC at 0.0006 and applied penetrances of 0.001, 0.5, and 0.8 corresponding to the 1/1, 1/2, and 2/2 genotypes. Allowing for genetic heterogeneity, we obtained a maximal HLOD score of 5.13 for $\alpha = 0.52$ (α = fraction of families used to obtain a maximum LOD score) at marker D2S320 (fig 2, black line). These results demonstrate significant linkage for KC at 2p24. Of note, the 1-LOD-drop support interval around the maximum HLOD score, an approximate 90% confidence interval, included a region spanning D2S2346 to D2S2342 (including D2S305 and D2S320).

Haplotype analysis

Haplotypes were established with all 48 microsatellite markers given in table 1. Since the mode of inheritance and the penetrance of KC have yet to be determined, we first examined the segregation of haplotypes at 2p24 between affected individuals. We show that marker alleles cosegregated with the KC phenotype in 17 out of the 28 families

Figure 3 Haplotypes shared by the affected members of 17 of the 28 families. Black vertical bars delimit the largest shared haplotype among all affected members of each family. Grey sections denote regions where recombination events could not be localised exactly. Haplotypes extend up to but do not include markers at the limits of the bars. Pedigree numbers are displayed above each vertical line. Two regions were shared by all 17 families: D2S305–D2S2233 and D2S2150–D2S2373.

(fig 3). Haplotypes differed between families. Haplotypes extend up to, but do not include, markers at the ends of the bars. Critical meiotic recombinants could be identified in families 3, 5, and 6. Integration of healthy individuals changed haplotypes in families 17 and 30, where a gap was found in the shared haplotype due to a double recombination event in a healthy sibling. Two intervals were therefore shared by all affected members of the 17 families (hatched area in fig 3). Detailed genotype data for families 3, 5, 6, 17, 30, and 33 are shown for 12 markers spanning the shared region in figs 4A and 4B. The first is telomeric to marker D2S2233 and centromeric to D2S305, while the second is telomeric to marker D2S2373 and centromeric to D2S2150. The overall interval covers a region of ~3.78 cM (1.69 Mb). The first interval (D2S2233–D2S305) extends over 0.4 Mb and the second interval (D2S2373–D2S2150) extends over 0.5 Mb.

Search for candidate genes or transcripts

According to data available on the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=OMIM>), UCSC Genome Browser (<http://www.genome.ucsc.edu/cgi-bin/hgGateway>), and Ensembl (<http://www.ensembl.org/>) databases, eight previously

Figure 4A Pedigrees of families 3, 5, and 33 delimiting the chromosome 2 regions and haplotypes of markers spanning this KC locus. ■ affected man; □ unaffected man; ● affected woman; ○ unaffected woman; / deceased; ? unknown phenotype; black vertical line: haplotype that segregated with disease in each family. Grey sections denote regions where recombination events could not be localised exactly. Genotypes for only 12 (out of 48) microsatellite markers are shown.

identified genes were contained between markers D2S305 and D2S2373. These genes are OSR1, KIAA1336, MATN3, LAPTM4A, SDC1, PUM1, ARHB, FLJ14249, GDF-7, and FLJ21820 (fig 5). Also, 10 Acembly (<http://www.humanangenes.org/>) spliced gene predictions have also been mapped to this region. When only the two shared intervals are considered, this list is reduced to the known genes OSR1, ARHB, FLJ14249, GDF-7, and FLJ21820, and the Acembly spliced gene predictions keekla, skylu, klodo, kerkla, klardo, plydo, and skeedo. Of this list, only ARHB (RhoB) and GDF-7 have a known cellular function (actin reorganisation and control of cell differentiation, respectively).

DISCUSSION

KC is the leading cause of corneal transplantation in developed countries. Therefore, geneticists are working to map the gene responsible for familial KC and so greatly

increase the little knowledge we have of this pathology. The results of the present genome-wide linkage and refinement studies indicate that a major gene for KC, responsible for 50–60% of familial KC cases studied, is located within a 1.69 Mb region (one of two small regions, both <0.5 Mb) at 2p24.

We performed here a genome-wide scan in seven large KC families and found a suggestive level of linkage at 2p24. The divergence of the values obtained for the LOD score and the Z_{lr} statistic at this locus (LOD = 2.64 is only evidence suggestive of linkage, whereas Z_{lr} = 4.91 is generally accepted as significant evidence) is probably due to a skewed distribution of Z_{lr} because of the small number of families included in this first step of the study (for detailed discussion see Kong and Cox²³).

Refinement of the 2p24 region using a larger set of 28 KC families resulted in a non-parametric LOD score curve with two peaks with a LOD score >3. Various hypotheses could

Figure 4B Pedigrees of families 6, 17, and 30 (see fig 4A legend).

explain the existence of these two peaks and are yet to be tested. First, one of the peaks could be artefactual, maybe due to the heterogeneity of the population and/or the phenotype. For example, linkage studies for asthma resulted in two peaks at 20p13 separated by approximately 5 cM with exactly the same LOD score values.²⁶ Refinement of the phenotype used for these linkage studies supported only the region under the second peak and led to identification of mutations in the ADAM33 gene. It is possible that there are different

phenotypes for KC that are for the moment clinically undistinguishable. Second, the two LOD peaks could be accounted for by the existence of two causal KC genes at the same locus. This has already been demonstrated in Griscelli syndrome where some patients were found to have no mutations in the MyoVa gene whilst haplotype analysis showed segregation at the same 15q21 locus.²⁷ Finally mutations were discovered in RAB27A, 1.6 cM distant from the MyoVa gene.²⁸

Figure 5 Genes localised in the KC candidate region according to UCSC Genome Browser and NCBI Mapviewer (<http://www.ncbi.nlm.nih.gov/mapview/>). Distances are shown in Mb (public location).

Since the beginning of our project, one group has published the results of their genome-wide scan suggesting the existence of a locus for familial KC on chromosome 16q22.3–q23.1 in a genetically isolated Finnish population.¹⁶ Mutations in the VSX1 gene (20p11–q11), coding for a putative homeobox domain transcription factor have also been identified in patients suffering from KC. However these mutations were only found in 4.7% of KC cases which corresponds to approximately 1% of familial KC cases.²⁹ Two additional loci (20q12 and 18p) and a founder effect have also been identified by association studies.^{18–19} Nevertheless, these results are not in conflict with our data since a high level of genetic heterogeneity is suspected for familial KC, resulting in different loci segregating with the disease in various families or populations.

Despite the use of a fairly large population including several large families, we did not obtain clear evidence (LOD = 3.26) of non-parametric linkage at commonly accepted statistical significance levels (LOD = 3.3). The segregation of KC in the majority of our families seemed largely in agreement with a dominant Mendelian mode of inheritance with incomplete penetrance. In view of this and since genetic heterogeneity is suspected for KC, we applied a dominant model to obtain parametric HLOD scores. After analysis, we obtained a significant HLOD of 5.13 with $\alpha = 0.52$.

Haplotype analysis also confirmed that a chromosomal region at 2p24 segregated with the disease phenotype in 17 out of the 28 families. This corresponds to 60% of the families and is close to the 52% used by Genehunter to optimise the HLOD score (the difference is not statistically significant). The higher percentage obtained by haplotype analysis could be due to the fact that some of the unaffected individuals used by Genehunter carry the mutated allele but display no KC phenotype because of incomplete penetrance.

In families 3 and 5, single (individual 5.II.2) and double (individual 3.III.4) recombination events allowed us to narrow down the locus to a 1.69 Mb interval. In families 17 and 30, inclusion of an unaffected sibling with a double recombination event in the haplotype analysis divided the candidate region into two smaller regions. This could lead to several different explanations. First, both apparently healthy siblings in families 17 and 30 (both are over the normal KC onset age) could be carriers of the causal mutation but display no phenotype. Second, the natural occurrence of two such double recombinations seems very unlikely and this event could perhaps be explained by spurious marker order. However, we observe a rather high recombination rate in this region in our families (95 recombination events for 97 meioses over the 80 cM region and 40% of these recombination events are restricted to two 5 cM regions surrounding markers D2S2373 and D2S367) and several small double recombinations. Microsatellite markers in the D2S272–D2S2221 region were regentyped and previously observed double recombination events were validated. This chromosomal region contains numerous repetitive sequences (Alu, LINE SINE) which could perhaps influence the stability of the 2p24 region and should be taken into account for positioning candidate regions.

We conclude that we have identified a causative locus for familial KC at 2p24 which predisposes to the disease in a significant proportion of the families. The fact that we discovered no common haplotype between the various linked families is not surprising since no relationship is known of between these families and the microsatellite markers used for haplotype analysis are probably not dense enough to prove a founder effect.

This new KC locus covers a physical distance of 1.69 Mb which was further reduced to 0.9 Mb following the analysis

of informative family 17. Eight known genes and 10 Acembly gene predictions have been mapped within its boundaries. Functional candidate KC genes have been proposed up to now on the basis of histological analysis. These include corneal collagens, proteinases, proteinase inhibitors, and interleukin-1 associated proteins. Collagen 6A1 was a major candidate after linkage; it was found on chromosome 21q in a large pedigree with Down syndrome, but this chromosome has been entirely sequenced without the discovery of any mutation.³⁰ Metalloproteinases are another candidate gene group; MMP-2 and MMP-9 have both been shown to be upregulated in patients with KC.^{3–12–31} MMP-9 is located near the 20q12 locus identified in Tasmanians and MMP-2 is near the 16q locus in a Finnish population, but both have since been excluded as candidate genes.^{16–19}

Of the five known genes (OSR1, RhoB, GDF-7, FLJ21820, and FLJ14249) in the two regions with shared haplotypes, none are known to be involved in human disease. OSR1 encodes odd-skipped related 1, a putative transcription factor which possesses a homeobox domain. RhoB is a possible candidate gene since it stimulates actin stress fibre formation and focal adhesion points. Mutations in RhoB could explain structural defects observed in KC corneas. However, this gene is expressed in many other tissues where its functions are highly important, so it seems unlikely that mutations would lead to symptoms restricted to the cornea. GDF-7 (growth differentiation factor 7) is a good candidate gene due to the existence of TGF- β domains in its sequence. TGF- β and related proteins have already been investigated for their possible implication in KC because of their importance in the corneal epithelium but to no avail. However, probable roles for GDF-7 have been identified so far in the rhesus monkey neocortex, in seminal vesicle differentiation, and in commissural interneuron growth in the mouse spinal cord where dysfunction of this protein leads to aberrant development of the mouse embryo.^{32–34} Therefore, it seems improbable that a gene essential to nervous system development could confer only a corneal phenotype when mutated in man. The functions of FLJ21820 and FLJ14249 remain unknown. The Acembly gene predictions correspond to spliced Image clones and indicate the localisation of yet unknown genes. Analysis of the expression of all these candidate genes is underway and genomic sequences are being screened for mutations.

Identification of a causative KC gene will improve our understanding of this disease and should aid discovery of other familial KC genes. Comprehension of the molecular biology and biochemistry that underlie KC will be a first step towards new therapeutic strategies to slow and prevent this blindness disorder in both the familial and sporadic forms.

ACKNOWLEDGEMENTS

We would like to thank the families for their participation in this research.

ELECTRONIC-DATABASE INFORMATION

The URLs for data presented herein are as follows:
Online Mendelian Inheritance in Man (OMIM), <http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=OMIM>; NCBI Mapviewer, <http://www.ncbi.nlm.nih.gov/mapview/>; UCSC Genome Browser, <http://www.genome.ucsc.edu/cgi-bin/hgGateway>; and Ensembl, <http://www.ensembl.org/>.

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This work was supported by grants from INSERM, l'Association Retina France, l'association Valent Haüy, and the Délégation Régionale à la Recherche Clinique des Hôpitaux de Toulouse (2003-AOL No. 0303502). HH held a Fondation de France fellowship.

Competing interests: none declared

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Received 4 May 2004

Revised version received 19 July 2004

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