

A Novel Keratocan Mutation Causing Autosomal Recessive Cornea Plana

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PURPOSE. Mutations in keratocan (*KERA*), a small leucine-rich proteoglycan, have recently been shown to be responsible for cases of autosomal recessive cornea plana (*CNA2*). A consanguineous pedigree in which cornea plana cosegregated with microphthalmia was investigated by linkage analysis and direct sequencing.

METHODS. Linkage was sought to polymorphic microsatellite markers distributed around the *CNA2* and microphthalmia loci (*arCMIC*, *adCMIC*, *NNO1*, and *CHX10*) using PCR and non-denaturing polyacrylamide gel electrophoresis before *KERA* was directly sequenced for mutations.

RESULTS. Positive lod scores were obtained with markers encompassing the *CNA2* locus, the maximum two-point lod scores of 2.18 at recombination fraction $\theta = 0$ was obtained with markers D12S95 and D12S327. Mutation screening of *KERA* revealed a novel single-nucleotide substitution at codon 215, which results in the substitution of lysine for threonine at the start of a highly conserved leucine-rich repeat motif. Structural modeling predicts that the motifs are stacked into an arched β -sheet array and that the effect of the mutation is to alter the length and position of one of these motifs.

CONCLUSIONS. This report describes a novel mutation in *KERA* that alters a highly conserved motif and is predicted to affect the tertiary structure of the molecule. Normal corneal function is dependent on the regular spacing of collagen fibrils, and the predicted alteration of the tertiary structure of *KERA* is the probable mechanism of the cornea plana phenotype. (*Invest Ophthalmol Vis Sci.* 2001;42:3118-3122)

Cornea plana is a rare condition in which there is a degree of flattening of the normally convex corneal surface.¹ It is inherited as either an autosomal dominant (*CNA1*; [OMIM] 121400) or autosomal recessive trait (*CNA2*; OMIM 217300) both of which map to 12q21.^{2,3} (Online Mendelian Inheritance in Man [OMIM]; provided in the public domain by the National Center for Biotechnology Information, <http://www.ncbi.nlm.nih.gov/omim>) The autosomal recessive form of the condition

exhibits the more severe phenotype and includes the presence of a degree of corneal opacity and sclerocornea (OMIM 181700). Two mutations in keratocan (*KERA*; OMIM 603288), a small leucine-rich proteoglycan (SLRP), have recently been shown to be responsible for autosomal recessive cornea plana in a series of patients of Finnish and American extraction. The cause of the autosomal dominant form of the disease remains to be determined.

The combined phenotype of cornea plana and microphthalmia has not been previously described. Microphthalmia is a heterogeneous group of developmental anomalies in which the eye fails to attain its normal dimensions with the axial diameter reduced to less than the age-adjusted 5th centile.⁴ The condition occurs either in a nonsyndromic ocular form or in conjunction with a diverse range of systemic malformations, including mental retardation (OMIM 251500), esophageal atresia (OMIM 600992), and dwarfism (OMIM 309700). Autosomal dominant, autosomal recessive, and X-linked inheritance patterns of microphthalmia have been reported and one developmental gene, *CHX10*, (OMIM 142993) has so far been shown to cause the phenotype in humans.⁵ Three loci have also been identified on 11p,⁶ 14q32,⁷ and 15q12-q15,⁸ and syndromic forms of microphthalmia and anophthalmia⁹⁻¹² that do not map to these loci suggest the existence of further genetic heterogeneity.

We report the mapping of a family with cornea plana to the *CNA2* locus on 12q21 in which the phenotype is caused by a novel mutation in *KERA*, leading to an amino acid substitution in a highly conserved portion of the protein. Protein modeling suggests that this mutation alters the tertiary structure of the protein resulting in the disease phenotype. An interesting feature of this family is that reduced axial length cosegregates with the cornea plana phenotype. The implications of this observation are discussed.

MATERIALS AND METHODS

Family and Clinical Data

The family studied was a consanguineous pedigree (Fig. 1) originating from Bangladesh which displays an autosomal recessive pattern of inheritance. The parents are first cousins and neither they nor their relatives (apart from four of their offspring) are affected. Family members were examined in the United Kingdom and ophthalmic examination included slit lamp biomicroscopy, gonioscopy, measurement of intraocular pressure, optic disc assessment, and B-scan ultrasonography (in affected individuals) to determine axial length. The presence of cornea plana and sclerocornea were the criteria used to determine that an individual was affected, and axial length measurements were used to diagnose microphthalmia. None of the systemic features associated with microphthalmia, such as mental retardation, were present in this pedigree. The study had the approval of the Moorfields Eye Hospital ethics committee and conformed to the tenets of the Declaration of Helsinki. Informed consent was obtained from all participants.

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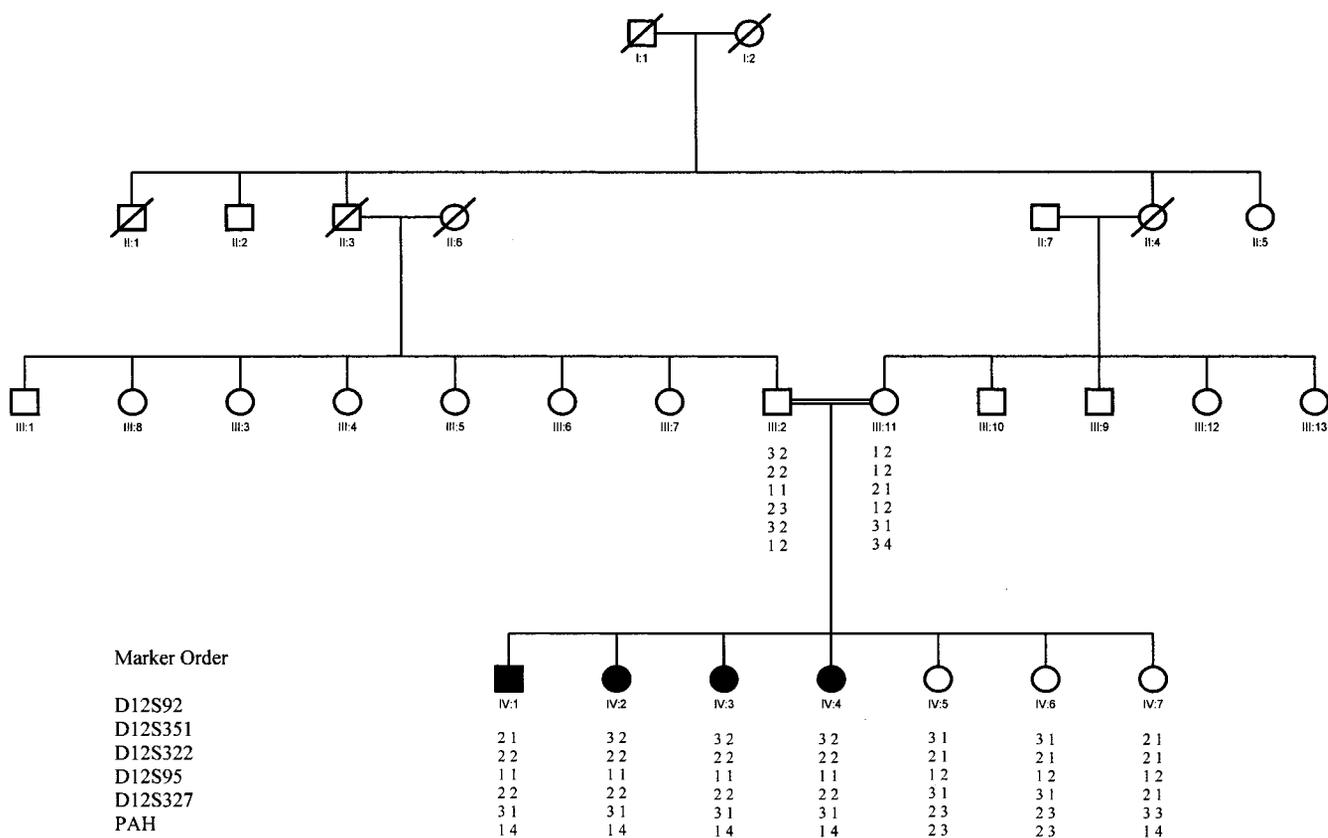


FIGURE 1. Pedigree of consanguineous family, showing haplotypes for the polymorphic markers on chromosome 12q21. Affected individuals are denoted by *solid symbols*.

Genotyping

Genomic DNA extracted from venous blood or buccal mucosa samples using standard techniques (Nucleon Biosciences, Glasgow, UK; Qiagen DNA minikit; Qiagen, Hilden, Germany) was amplified using the following tri- and tetranucleotide repeat markers: D12S92, D12S351, D12S322, D12S95, D12S327, PAH (*CNA2*); D14S62, D14S987, D14S65, D14S267, D14S78 (*arCMIC*); D11S1313, D11S903, D11S4191, D11S987 (*NNO1*); D14S77, D14S1025 (*CHX10*); AFMb293yg1 and AFM312zd9 (*adCMIC*), all from Research Genetics, Huntsville, AL (available at <http://www.resgen.com>). PCR reactions were performed in 20- μ l volumes with 50 ng genomic DNA, 10 pmol of each primer, 200 mM dNTPs, 1.5 mM MgCl and 1 U *Taq* DNA polymerase (Promega, Madison, WI). The PCR conditions consisted of 35 cycles of amplification (94°C, 1 minute; 50°C to 60°C, 1 minute; and 72°C, 1 minute). The amplified PCR products were resolved by 6% nondenaturing polyacrylamide gel electrophoresis (Protogel; National Diagnostics, Atlanta, GA) and were visualized by staining with ethidium bromide. Subsequent genotyping was performed manually.

Linkage Analysis and Mutation Detection

Pedigree data were collated with pedigree mapping software (Cyrillic, Setauket, NY) and two-point linkage analysis performed using the MLINK component of the LINKAGE program, version 5.1¹³ using an autosomal recessive model, equal allele frequencies, a gene frequency of 10⁻⁴, and a mutation rate of 10⁻⁶ (Table 1).

PCR amplification of *KERA* gene sequences was performed in 20- μ l reactions using published primers.¹⁴ After purification with columns (QIAquick; Qiagen, Crawley, UK) samples were sequenced bidirectionally with fluorescent dideoxynucleotides (PE Biosystems, Foster City, CA) on an automated sequencer using standard conditions (model 373; PE Applied Biosystems, Foster City, CA).

Protein Modeling

A three-dimensional structure of keratocan was modeled based on a theoretical structure of the acid-labile subunit (ALS) of a serum insulin-like growth factor¹⁵ using the Swiss Model server program^{16,17} and the viewing programs (Swiss Pdb Viewer ver. 3.7b2¹⁸ and Rasmol ver. 2.6 (available at: <http://ca.expasy.org/spdbv/mainpage.htm> and <http://www.bio.cam.ac.uk/doc/rasmol.html> respectively).

RESULTS

The clinical characteristics of the four affected individuals were as follows: Their ages ranged from 2 to 12 years (mean, 5 years) and all exhibited bilateral cornea plana with a varying degree of corneal opacity, sclerocornea, and microphthalmia (mean axial length 19.3 mm; range 18.2-20.3 mm; normal axial length 23.0 mm, range 22-24 mm¹⁹). All four also had reduced visual acuity, ranging from Snellen 6/24 to 6/60 in the better eye, and none had undergone ocular surgery.

Positive lod scores were obtained with markers in a region on chromosome 12q21 spanning approximately 3 centimorgans (cM), whereas the *arCMIC*, *adCMIC*, *CHX10*, and *NNO1* loci were excluded from linkage. The maximum two-point lod scores were obtained with markers D12S95 and D12S327 (2.18 at $\theta = 0$); the markers and the lod scores are summarized in Table 1. This interval includes a region of homozygosity of 1 cM between markers D12S351 and D12S95 that encompasses the *CNA2* locus.

Sequencing of *KERA* revealed a single-nucleotide substitution in exon 2 that segregated with the disease phenotype. The sequence change (ACA→AAA at codon 215) results in an amino acid substitution from threonine (T) to lysine (K). All

TABLE 1. Two-Point Lod Scores between the *CNA2* Locus and Six Markers on 12q21 Ordered from the Telomere

	Locus					
	0.00	0.05	0.10	0.2	0.3	0.4
D12S92	−∞	0.15	0.31	0.32	0.21	0.07
D12S351	1.28	1.16	1.03	0.75	0.45	0.15
D12S322	1.28	1.16	1.03	0.75	0.45	0.15
D12S95	2.18	1.98	1.77	1.3	0.78	0.24
D12S327	2.18	1.98	1.77	1.3	0.78	0.24
PAH	−∞	0.99	1.09	0.93	0.6	0.21

Data are lod scores at θ .

four affected siblings were homozygous for the mutated allele, whereas both parents and one of the unaffected siblings were heterozygous. The other two siblings were homozygous for the normal ACA allele. The mutated sequence does not generate or alter a restriction enzyme site. Primary sequence analysis indicates that the T215K substitution occurs at the start of a leucine-rich repeat (LRR) motif, the seventh of 10 such motifs present in the sequence.²⁰

A relatively high degree of sequence identity (30.5%) was found with the protein ALS, which also contains a series of LRR motifs. The availability of a theoretical structural model for ALS facilitated the generation of a partial three-dimensional model for keratocan (Fig. 2a). Although the model is incomplete at the N- and C-termini (the homology was limited to the region of the protein comprising LRRs 1–9), nonetheless it was possible to examine the structural implication of the mutation at site 215, which is located at the start of LRR7. The model indicates that in the protein the LRR motifs form a series of parallel β -strands, which stack into an arched β -sheet array. The mutation reduces the effective length of LRR7 and causes the loop connecting LRR6 to LRR7 to be laterally displaced (Fig. 2b).

DISCUSSION

Mutations in keratocan have recently been shown to cause autosomal recessive cornea plana in both a large cohort of Finnish patients and an American patient.¹⁴ The changes identified to date (AAC→AGC transition at codon 247 and a homozygous CAG→TAG transversion causing a stop mutation) both occurred in a conserved domain and resulted in the typical cornea plana phenotype. Our data demonstrate that a novel ACA→AAA mutation at codon 215 from a family of different ethnicity also causes cornea plana. This mutation substitutes the polar residue threonine for the positively charged amino acid lysine at the start of the seventh LRR motif. Threonine is conserved at this position in all described KERA proteins (human, bovine, murine, and chicken) and the substitution of this highly conserved amino acid may explain the different phenotype observed in this family.

In common with other members of the keratan sulfate proteoglycan subgroup of the SLRP family, KERA includes a series of LRR motifs with a consensus sequence of LXXLXLLXX-NXL.²⁰ Similar LRR motifs are found in the structure of porcine RNase inhibitor whose crystalloid structure has been deter-

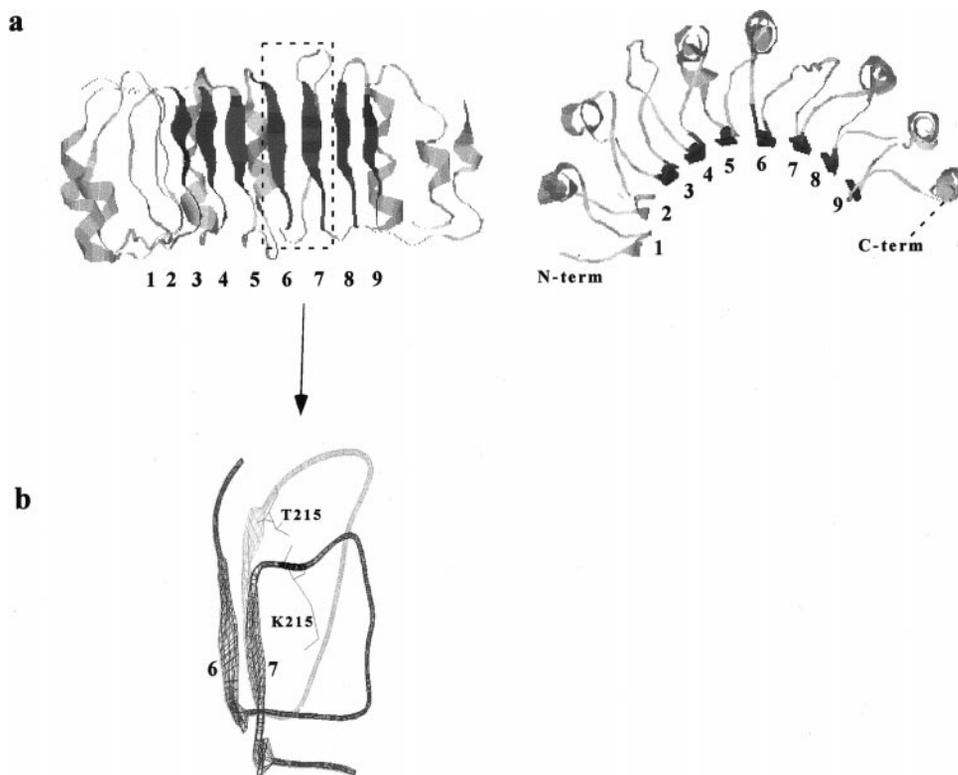


FIGURE 2. Three-dimensional modeling of wild-type and T215K mutant keratocan. (a) Partial structure of the wild type represented in ribbon form with LRR motifs 1 to 9 numbered and highlighted in dark gray. The model is presented from two perpendicular viewpoints. (b) An enlargement of the boxed region in (a) showing the superimposition of the wild type (light gray) and T215K mutant (dark gray). The side chains of residues 215 are shown projecting from the protein backbone and the lateral displacement of the loop connecting LRRs 6 and 7 is clearly visible.

mined.²¹ This defined protein structure has been used to model the structures of other proteins containing analogous LRR motifs, such as the protein ALS.¹⁵ It has previously been suggested that the structure of keratocan includes a similar β -sheet array²² and the model presented here supports this. The modeling predicts that the structural consequence of the mutation is to cause substantial displacement of one of the loops holding together the β -sheet structure. However, it should be noted that this conclusion is based on theoretical considerations only. The regular LRR motif arrangement has been proposed to be important in the spacing of collagen fibrils on which corneal transparency depends, and this is compatible with the observation that mutations in *KERA* result in corneal opacity.

A particularly interesting feature of this consanguineous pedigree is the cosegregation of cornea plana with reduced axial length. This may indicate that a single *KERA* mutation causes both phenotypes although the alternative hypothesis—that this represents the chance cosegregation of the two phenotypes due to separate mutations, cannot be excluded, even though no linkage was found to the known microphthalmia loci. The hypothesis that a single mutation in *KERA* causes the combined cornea plana and microphthalmia is supported, but not proven, by the novel nature of the mutation observed in a conserved portion of *KERA*; the low probability that the two phenotypes cosegregate by chance in four out of seven offspring: 1 in 607 or 0.16% [$(1/4)^4 \times (3/4)^3$]; and the observation of *KERA* expression in bovine^{23,24} and murine sclera.²⁰

SLRPs are important mediators of normal connective tissue assembly that influence, through collagen binding, the rate of assembly and diameter of collagen fibrils.²⁵ In decorin, one of several corneal-expressed SLRPs, this collagen-regulating activity is believed to be mediated by the central LRR region²⁶⁻²⁸ and it is likely that the evolutionarily conserved structure of SLRPs reflects a common mechanism of collagen interaction.²⁵ The T215K substitution in keratocan is predicted to induce a conformational change in the LRR domain and may also affect collagen binding. This would provide a potential mechanism by which the observed corneal and scleral phenotypes could be caused by alterations in the diameter or in the spacing of collagen fibrils.

Microphthalmia has a wide spectrum of disease severity with minor degrees of congenital microphthalmia to anophthalmia coexisting in the same pedigree.⁴ Because *KERA* is only (transiently) expressed in early embryonic murine sclera,²⁰ it may not be possible to verify whether the same applies to human embryonic tissue. The generation of a murine model with the T215K substitution may represent an alternative means of testing the hypothesis. The phenotype reported is compatible with reports of altered scleral thickness and abnormal collagen bundles in histologic specimens from microphthalmia cases²⁹ and although they lie at the milder end of the disease spectrum, the axial lengths overlap those of the *arCMIC*³⁰ and *NNO1*⁶ pedigrees. The milder degree of microphthalmia observed may reflect *KERA*'s presumed structural role, and contrasts with the more severe phenotype observed with mutations in the developmental gene *CHX10*.⁵

In summary, we have identified a novel mutation in *KERA* in a large Bangladeshi family with cornea plana. The mutation alters a highly conserved motif and is predicted to affect the tertiary structure of the molecule. This change is responsible for the corneal phenotype observed. The cosegregation of cornea plana with reduced axial length raises the intriguing possibility that mutations in keratocan may have a wider phenotype than one solely involving the cornea.

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