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We hope that making available the relevant information on Pachyonychia Congenita will be a means of furthering research to find effective therapies and a cure for PC.
Mutations in cornea-specific keratin K3 or K12 genes cause Meesmann's corneal dystrophy

Alan D. Irvine1, Laura D. Corden2,3, Ole Swensson4, Beate Swensson5, Jonathan E. Moore6, David G. Frazer6, Frances J.D. Smith2, Robert G. Knowlton7, Enno Christophers4, Rainer Rochels5, Jouini Uitto2,8 & W.H. Irwin McLean2

The intermediate filament cytoskeleton of corneal epithelial cells is composed of cornea-specific keratins K3 and K12 (refs 1,2). Meesmann's corneal dystrophy (MCD) is an autosomal dominant disorder causing fragility of the anterior corneal epithelium2,4, where K3 and K12 are specifically expressed6. We postulated that dominant-negative mutations in these keratins might be the cause of MCD. K3 was mapped to the type-II keratin gene cluster on 12q, and K12 to the type-I keratin cluster on 17q using radiation hybrids. We obtained linkage to the K12 locus in Meesmann's original German kindred (Zmax = 7.53; θ = 0) and we also showed that the phenotype segregated with either the K12 or the K3 locus in two Northern Irish pedigrees. Heterozygous missense mutations in K3 (E599K) and in K12 (V143L; R135T) completely co-segregated with MCD in the families and were not found in 100 normal unrelated chromosomes. All mutations occur in the highly conserved keratin helix boundary motifs, where dominant mutations in other keratins have been found to severely compromise cytoskeletal function, leading to keratocyte fragility phenotypes. Our results demonstrate for the first time the molecular basis of Meesmann's corneal dystrophy.

Epithelia function primarily as specialized barrier tissues and to ensure expression of keratins in a tissue-specific manner, particularly cytokeratins, the intermediate filament proteins of epithelial cells6,7. Mutations have been reported in eleven human keratin genes, each causing a disease characterized by fragility of specific epithelial tissues8,9. The most anterior portion of the cornea consists of a stratified non-cornified epithelium where suprabasal corneal keratinocytes specifically express the keratin pair K3 and K12, which are thought to be specialized to cater for the particular trauma encountered by the cornea10-12.

Meesmann's corneal dystrophy (MCD; MIM #122100) is a bilaterally symmetrical, autosomal dominant disorder of the corneal epithelium3,4. MCD has a characteristic slit-lamp appearance of a myriad of fine round epithelial cysts which become visible by 12 months of age and increase in number throughout life. Patients are usually asymptomatic until adulthood when rupture of the corneal microcysts may cause erosions, producing clinical symptoms such as photophobia, contact lens intolerance and intermittent diminution of visual acuity. Rarely, subepithelial scarring causes irregular corneal astigmatism and permanent visual impairment. Histological examination shows a disorganized and thickened epithelium with widespread cytoplasmic vacuolization and numerous small, round, debris-laden intraepithelial cysts4.

McKusick recognizes several disorders where corneal dystrophy forms all or part of the phenotype12. Various loci have been identified on chromosomes 1p34, 5q13.13, 16q17, and 20p15.19. Furthermore, pathogenic mutations have been identified in the LCAT gene at 16q22.1 causing FISH-eye disease10 and in the gelsolin gene at 9q34 causing lattice type-II corneal dystrophy, which is part of the amyloidosis phenotype21. Recently, mutations in the kerato-epithelin gene have been identified in the four corneal dystrophies which map to 5q (ref. 22). Of the uncharacterized diseases, we considered MCD a good candidate for a corneal keratin disease: i) MCD is autosomal dominant and as keratins form polymeric structures, dominant mutations are most common in these genes2 and ii)

Family M

Family K

Family D

Fig. 1 Pedigrees of MCD families under study, showing typical autosomal dominant inheritance. All individuals who participated in the study (*) were examined by slit lamp to ensure correct diagnosis (see Fig. 2). Family M represents the descendents of the original German kindred studied by Meesmann in the 1930s; families D and K are unrelated Northern Irish kindreds.

1 Department of Dermatology, Belfast City Hospital, Lisburn Road, Belfast BT9 7AB, UK. 2 Epithelial Genetics Group, Department of Dermatology and Cutaneous Biology, Jefferson Medical College, 233 South 10th Street, Philadelphia, Pennsylvania 19107, USA. 3 CRC Cell Structure Research Group, Cancer Research Campaign Laboratories, Department of Anatomy and Physiology, Medical Sciences Institute, University of Dundee DD1 4HN, UK. 4 Department of Dermatology, and 5 Department of Ophthalmology, Christian-Albrechts-Universität, Kiel 24105, Germany. 6 Department of Ophthalmology, Royal Group of Hospitals, Grosvenor Road, Belfast BT12 6BA, UK. 7 Departments of Dermatology and Cutaneous Biology, and 8 Biochemistry and Molecular Pharmacology, Jefferson Institute of Molecular Medicine, 233 South 10th Street, Philadelphia, Pennsylvania 19107, USA. A.D.L. & L.D.C. contributed equally to this work. Correspondence should be addressed to W.H.I.M.
MCD affects only the anterior epithelium where K3 and K12 are specifically expressed. Furthermore, the reported cytoplasmic densities in MCD are reminiscent of the tonofilament clumping seen in other dominant keratin disorders and complete loss of the K3/K12 cytoskeleton results in extreme fragility of corneal keratinocytes in K12 knockout mice.

We studied the descendants of Meesmann’s original German family (M) and two Northern Irish MCD kindreds (D and K), all demonstrating typical autosomal dominant inheritance (Fig. 1). We examined all members of the families by slit lamp for correct clinical ascertainment. The slit-lamp appearance of the cornea of an affected individual (family D proband) is shown in Fig. 2, with the numerous epithelial microcysts and gray lines characteristic of MCD clearly visible. The location of the genes encoding K3 (ref. 26) and the recently cloned K12 mRNA were not known with certainty. Thus, we developed intronic STSs and mapped these using the Genebridge 4 radiation-hybrid panel. We mapped the K3 gene (KRT3) to the type II keratin gene cluster on 12q between markers CHLC.GATA111B02 and D12S325. Similarly, we mapped the K12 gene (KRT12) within the type I keratin cluster on 17q in the interval between D17S800 and D17S930. Linkage analysis using markers close to both genes revealed highly significant linkage to the K12 locus in family M (Zmax = 7.53; θ = 0; Table 1). Although families D and K were too small to achieve significant lod scores, the disease was observed to cosegregate with K12 in family D and with K3 in family K, the reciprocal keratin locus being excluded in each case (Table 1).

In families K and D, the regions of the K3 or K12 genes encoding the highly conserved helix boundary peptides were amplified and directly sequenced as these are structurally important regions of the protein where mutations have been shown to cause keratin filament aggregation and severe phenotypes in other keratin diseases. In affected members of family K, we found a heterozygous missense mutation, G1525A (base numbers from the presump-

tive K3 mRNA sequence, ATG = 1, Fig. 3a, b). This purine transition is a potential CpG deamination mutation and leads to the amino acid substitution E309K, located in the highly conserved helix termination motif of the keratin K3 polypeptide. This mutation abolishes a BseGI restriction site which we used to confirm that the mutation completely cosegregates with the disease in this family (Fig. 3c). Interestingly, an identical CpG mutation in the analogous residue of epidermal K2e is the most commonly reported mutation in ichthyosis bullosa of Siemens.

We identified a heterozygous missense mutation, G451C (base numbers from the K12 mRNA sequence) in affected members of family D (Fig. 3d, e). This causes amino acid substitution V143L in the highly conserved helix initiation motif of K12, the region where the majority of severe dominant

Table 1 Two-point lod scores for keratin markers

<table>
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<tr>
<th>Family</th>
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<th>Keratin cluster</th>
<th>Zmax values</th>
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<td>———</td>
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<td>KRT9 Type I</td>
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<tr>
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<td>D12S36 Type II</td>
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Fig. 3 K3 and K12 mutation detection by direct sequencing and confirmation by restriction enzyme digestion. a, Normal K3 sequence within the helix termination motif (presumptive mRNA base numbers 1516-1530; strand shown), derived from family K, individual III.3. b, The same region of K3 as in (a) derived from family K, MCD affected individual III.1, showing heterozygous missense mutation G1525A, predicting the amino-acid change E509K. c, K3 mutation E509K disrupts a BseRI site. Loss of this site is seen to co-segregate with MCD in members of family K. d, Normal K12 sequence within the helix-initiation motif (mRNA base numbers 442-458; strand shown), derived from family D, individual III.4. e, The same region of K12 as shown in (d), derived from family D, individual III.3 (proband), showing heterozygous missense mutation G451C, predicting amino-acid transition V143L. f, K12 mutation V143L, destroys BsiII site. Loss of this restriction site is seen to co-segregate with MCD in family D. g, Normal K12 sequence within the helix-initiation motif (mRNA base numbers 420-436; strand shown), derived from family M, individual IV.17. h, The same region of K12 as shown in (g), derived from family M, individual III.7 (proband), showing heterozygous missense mutation G428K, predicting amino-acid transition R135T. i, Mutation R135T does not alter any known restriction site and so a mismatch primer was used for PCR which, in combination with the mutation, creates a BsrGI site. This RFLP was used to show that the mutation completely co-segregates with the MCD phenotype in the family. All mutations were excluded from 100 normal unrelated individuals by the appropriate digests.

hereditary epidermal blistering disease due to mutations in basal keratins K5 and K14, in that the lesions are in both cases due to keratinocyte fragility and largely heal without scarring, resulting in a comparatively mild phenotype. Mutations in other cutaneous basement membrane zone molecules lead to more severe junctional and dystrophic forms of epidermolysis bullosa. By analogy, it is possible that mutations in cornea-specific basement membrane molecules might be responsible for more serious corneal dystrophies.

Of general interest, MCD patients are intolerant of contact lenses, as these devices directly traumatize the corneal epithelium. The families studied here carry mutations in the highly conserved helix boundary peptides of K3 and K12, analogous to those mutations seen in K5 and K14 in the severe Dowling-Meara variant of EBS and in many other keratin diseases. However, mutations in other domains of K5 and K14 cause milder EBS phenotypes and so one would predict milder phenotypes for similar mutations in corneal keratins. Contact lens intolerance with epithelial microcyst formation is not uncommon in the 'normal' population (D.G.F., unpublished observations) and we speculate that one possible cause could be K3 or K12 mutations causing a milder phenotype than MCD. We are currently determining the full genomic sequence and intron-exon organization of the K3 and K12 genes to allow development of a comprehensive mutation detection strategy to help address this issue as well as the study of additional MCD families.

Methods

Radiation hybrid mapping. A 390-bp human-specific fragment of the K3 gene (GenBank X54201) was amplified using primers K3e7L (5'-TCC TAC CTC CCT TGC CCC AGA ATG-3') and K3e7R (5'-TGG GAG GGA GTG GCC TGG TAG AGG-3'). A 'touchdown' program was used (94°C 5 min x1); (94°C 30 s, N°C 45 s, 72°C 45 s, x2; where N = 65, 63, 61; (94°C 30 s, 57°C 45 s, 72°C 45 s x30); (72°C 5 min x1). The position of intron 1 of K12 was determined by alignment of the human mRNA sequence (GenBank D78367) to the murine genomic sequence (GenBank U80895). This 382-bp intron was amplified and fully sequenced using primers K12e7L (5'-ATG GAT GTC TCG AAC ACC ATG-3') and K12e7R (5'-GGA GTG AGT TTC GCA GGA ATG-3') and intron 1 primer K12sp1 (5'-CTC TAG GTA TTC TCT CCG GGA ATG-3') and intron 1 primer K12sp2 (5'-TGC TGC AAG TAC GTC TAA ATT GGA-3'), using the following PCR program: (94°C 5 min x1); (94°C 30 s, 55°C 45 s, 72°C 45 s x35); (72°C 5 min x1). Both K3 and K12 STs were used to screen the Genebridge 4 radiation hybrid panel (Research Genetics). Mapping was computed to a lod score of >15 using the RH Mapper program.
Genotyping and linkage analysis. Microsatellite markers were PCR amplified using [32P]-labelled primers, analysed on standard 6% sequencing gels and visualized by autoradiography. Two-point lod scores were computed using the MLINK algorithm of LINKAGE version 3.1 assuming a maternal allele frequency of 0.001 and 100% penetrance. Marker allele frequencies were assumed to be equal in the population and in the case of family M, recalculation using a population frequency of 50% for the linked allele yielded the same lod score of $Z_{max} = 7.53; \theta = 0$. Similarly, recalculation at 90% penetrance still gave a significant lod score of $Z_{max} = 7.23; \theta = 0$.

Mutation analysis. Most of the presumptive K12 exon 1 was amplified from affected and unaffected members of families D and M using primers K12e1.L (above) and K12e1.R (5'-GTA AGC GTG TGA AGC ATC TGC-3'), derived from the mRNA sequence. PCR products were purified using QIAquick columns (Qiagen) and sequenced with both primers using the ABI PRISM Ready Reaction system (Perkin-Elmer). Sequencing ladders were analysed on an ABI 377 automated sequencer. Mutation K12 V143L destroys a Bsl restriction site. To confirm the mutation within family M and D and exclude it from 100 normal unrelated chromosomes, PCR products generated with primers K12sp1 and K12sp2 (above) were digested overnight with 15 U BslI and analysed on 3% NuSieve/TBE minigels. Loss of the single BslI site in this fragment was only observed in affected members of family D. Mutation K12 R135T does not alter any known restriction site and so a mismatch primer was designed, K12M (5'--AAG AAA CTA TGC AAA ATC TTA ATT GTA-3', mismatch bases underlined), which creates a BsrGI site only in conjunction with the mutation. For PCR, this primer was used in combination with K12sp2 as above, except an annealing temperature of 50 °C was used. PCR products were digested overnight with 10 U of BsrGI and analysed on 3% NuSieve/TBE gels. This RFLP was used to show that the mutation completely co-segregated with the phenotype in family K and to exclude the mutation from 100 normal unrelated individuals. Exon 7 of the K3 gene, encoding the telomere termination motif, was amplified with intronic primers K3e7.L (5'-TCC TAG CTC TTC TGG CCG AGA ATG-3') and K3e7.R (5'-TGG GGA GTG GGC TGG TAG AGG-3') derived from the published partial genomic sequence. Sequencing was performed with both primers as above. Mutation K3 E509K disrupts a single BseK1 site in this PCR fragment, which was used to confirm the mutation in all affected members of family K and to exclude it from normal unrelated chromosomes.


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