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Isolation and Chromosomal Localization of a Cornea-Specific Human Keratin 12 Gene and Detection of Four Mutations in Meesmann Corneal Epithelial Dystrophy

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Summary

Keratin 12 (K12) is an intermediate-filament protein expressed specifically in corneal epithelium. Recently, we isolated K12 cDNA from a human corneal epithelial cDNA library and determined its full sequence. Herein, we present the exon-intron boundary structure and chromosomal localization of human K12. In addition, we report four K12 mutations in Meesmann corneal epithelial dystrophy (MCD), an autosomal dominant disorder characterized by intraepithelial microcysts and corneal epithelial fragility in which mutations in keratin 3 (K3) and K12 have recently been implicated. In the human K12 gene, we identified seven introns, defining eight individual exons that cover the coding sequence. Together the exons and introns span ~6 kb of genomic DNA. Using FISH, we found that the K12 gene mapped to 17q12, where a type I keratin cluster exists. In this study, four new K12 mutations (Arg135Gly, Arg135Ile, Tyr429Asp, and Leu140Arg) were identified in three unrelated MCD pedigrees and in one individual with MCD. All mutations were either in the highly conserved α-helix–initiation motif of rod domain 1A or in the α-helix–termination motif of rod domain 2B. These sites are essential for keratin filament assembly, suggesting that the mutations described above may be causative for MCD. Of particular interest, one of these mutations (Tyr429Asp), detected in both affected individuals in one of our pedigrees, is the first mutation to be identified within the α-helix–termination motif in type I keratin.

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Introduction

Keratin proteins are structurally important intermediate filaments found in all epithelial tissues. Obligate heteropolymers in which a highly conserved α-helical central rod domain is surrounded by nonhelical terminal sequences, the family of keratin proteins is further divided into type I (acidic; K9–K21) or type II (neutral or basic; K1–K8) components (Hatzfeld and Weber 1990; Lu and Lane 1990), the expression of which is both tissue specific and dependent on the stage of differentiation of the particular epithelial cell line (Moll et al. 1982).

Several inherited epidermal diseases, such as epidermolysis bullosa simplex (EBS), are caused by keratin mutations (Bonifas et al. 1991; Coulombe et al. 1991; Cheng et al. 1992; Lane et al. 1992; Chan et al. 1993; Chen et al. 1993; Hovnanian et al. 1993; Humphries et al. 1993; Rugg et al. 1993; Stephens et al. 1993; Scamsher et al. 1995), and a mutation hot spot in keratin genes is recognized to be within both the α-helix–initiation motif, at the rod domain–1A segment, and the α-helix–termination motif, at the rod domain–2B segment (Lane 1994). These segments have been shown to have functional importance (Hatzfeld and Weber 1991; Vassar et al. 1991; Geisler et al. 1992; Letai et al. 1992; Wilson et al. 1992; Steinert et al. 1993).

The corneal epithelium, a layer of five or six cells that covers the anterior corneal surface, is a hugely important barrier that protects the underlying cornea from environmental factors. We know that corneal epithelial cells specifically and predominantly express keratin 3 (K3) and keratin 12 (K12) (Moll et al. 1982; Schermer et al. 1986; Kurpakus et at. 1990; Liu et al. 1993), suggesting that these proteins form the obligate heterodimer of corneal epithelial keratin intermediate filaments.

The human K3 gene has been cloned and characterized (Klinge et al. 1987 [GenBank accession numbers X05418, X05419, X05420, and X05421]), and in recent years the rabbit K3 gene (Wu et al. 1994 [GenBank accession number X74371]) and the murine K12 gene (Liu et al. 1994 [GenBank accession number U08095])

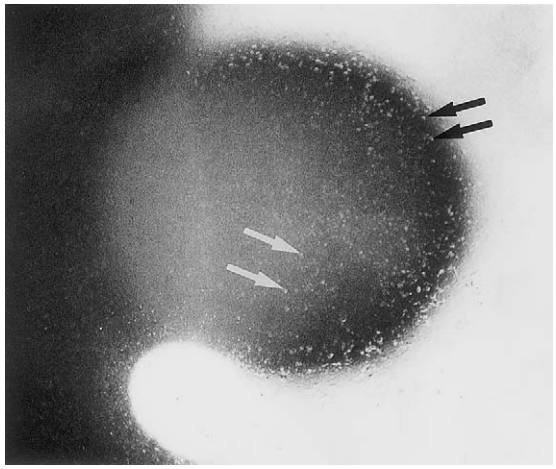

Figure 1 Slit-lamp photograph of patient FM (see fig. 3) with MCD, showing numerous intraepithelial microcysts (*arrows*) in the central cornea.

have also been isolated and characterized. In addition, our group has isolated human K12 cDNA from a human corneal epithelial cDNA library and has determined its complete sequence (Nishida et al. 1996 [GenBank accession number D78367]). Recently, the importance of K12 in the corneal epithelium has been highlighted by the finding that K12 knockout mice possess fragile corneal epithelia (Kao et al. 1996).

Meesmann corneal epithelial dystrophy (MCD; MIM 122100), also known as "juvenile hereditary epithelial dystrophy," is a rare, bilateral, and symmetric dystrophy that is inherited as an autosomal dominant trait (Meesmann and Wilke 1939). The clinical appearance of MCD is characterized by intraepithelial microcysts (fig. 1), which are visible on slit-lamp examination within the 1st year of life. These increase in number with age, although the patients tend to remain asymptomatic until late adolescence when the cysts can break through the epithelial surface, causing lacrimation, photophobia, and pain. Histopathologically, Periodic acid Schiff-positive materials are found intracytoplasmically in the abnormal epithelial regions, via impression cytology. Also, a "peculiar substance," thought to be the result of cytoplasmic filament degeneration (Kuwabara and Ciccarelli 1964), is occasionally found in the epithelial cells, and myeloid structures (documented in one of our patients; fig. 2) have been found intracytoplasmically (Nakanishi and Brown 1975).

Since, clinically, MCD has a number of features in common with various epidermal keratin diseases that have been linked to mutations in keratin genes, we sought to determine the human K12 exon-intron boundary structure, document its chromosomal localization, and search for K12 mutations in three unrelated Japanese families and one individual with MCD.

Material and Methods

Genomic Cloning , Exon/Intron Structure, and Chromosomal Localization of the Human K12 Gene

cDNA clones that we previously had isolated (Nishida et al. 1996) were used as probes to screen a human genomic library in λ-FIX II (Stratagene). Primers for the K12 cDNA sequences were designed for reference to the murine K12 gene structure as reported elsewhere (Liu et al. 1994). Both K12 cDNA and a genomic clone F327 were used as templates for PCR. Positions of introns were identified by comparing the products from both cDNA and genomic DNA. Fragments that were larger after amplification from genomic DNA were sequenced directly, by use of PCR primers. Sequencing was performed with the *Taq* dideoxy terminator cycle-sequencing kit (Applied Biosystems), according to the manufacturer's protocol. Sequence reactions were resolved on an ABI PRISM 377 automated sequencer (Applied Biosystems).

FISH was used to identify the chromosomal localization of the human K12 gene. DNA from clone F327 was labeled with digoxigenin dUTP (Boehringer Mannheim) by nick translation. Labeled probe was combined with sheared human DNA and hybridized to normal metaphase chromosomes derived from phytohemagglutinin-stimulated peripheral blood lymphocytes from a male donor, in a solution containing 50% formamide, 10% dextran sulfate, and 2 × SSC. Specific hybridization signals were detected by incubating the hybridized

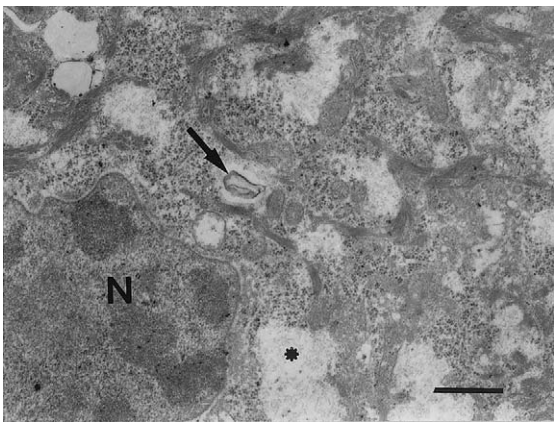

Figure 2 Micrograph of a portion of a basal epithelial cell from MCD patient TM (see fig. 3). Numerous electron-lucent intracytoplasmic spaces (*) are evident, and an electron-dense "myeloid structure," as reported by Nakanishi and Brown (1975), is also documented (*arrow*). N = nucleus. Scale bar = 720 nm.

Table 1

Primers for PCR Direct Sequencing

Presumptive Exon	5′ Pimer	3′ Primer
1	GCATGTCTGCTTCCAGTGTT	TAAAGTGGAGTGAAACAACC
2	TAGTATTTTAGGGCTTCAATC	AGGCAGGACAGTAGGACAGA
3	GCAAGAAATAGCCCTGAAGA	ATTTTTGGGTTTGGGAGGAA
4	GGCCCAAGAGGACAAAAGTA	GCAGGCCTTTCTGTGAATGT
5	AGAAAGGCCTGCGAAACGAG	AAAGAGGAGGGTAGCCAACG
6	GTCCCCTCCATCGTTATTTC	CTATTTCTGCTGCCCACTCT
7	TCCATTAAAAGCCAGGTTGT	GGTCTTACAGGTTTTGCATT
8	GCCTACATTAAACAACCAGT	TGACCAAAATGACTTGTGACTG

slides in fluoresceinated antidigoxigenin antibodies, followed by counterstaining with DAPI.

Reverese Transcriptase–PCR (RT-PCR) and K12/K3 cDNA Sequence

This study was conducted in accordance with the principles of the Declaration of Helsinki regarding research involving human subjects and was approved by the Institutional Review Board on Human Experiments, Kyoto Prefectural University of Medicine. Informed consent was obtained from all our human subjects. With a Quick-Prep mRNA Purification kit (Pharmacia-LKB), mRNA was purified from the corneal epithelium of an MCD patient (TM, an affected individual in our MCD-T family) who had received a corneal transplant because of a secondary corneal opacity unrelated to dystrophic abnormality. cDNA was generated as described elsewhere (Nishida et al. 1995). RT-PCR was performed by use of primer pairs covering an open reading frame of K3 and K12. PCR products were directly sequenced by use of several sequencing primers designed for determining the full sequence of K3 and K12 cDNA. With proper informed consent, cDNA from normal human corneal epithelia obtained at autopsy was analyzed as a positive control.

Mutation Analysis

We studied K12 as a candidate gene for MCD in four families—denoted as "N," "K," "T," and "F"—in whom a clinical diagnosis of MCD was confirmed histopathologically via impression cytology. We searched for a mutation in the α-helix rod domain by SSCP analysis and sequencing of amplified genomic DNA. For SSCP, PCR was performed by use of the following oligonucleotide primer pairs: For helix initiation, we used K12/347F (GCTCTCTAGGTATTCTCTCG) and K12/556R (CCTGAGTCTTCAATCAGTGG), and, for helix termination, we used the exon 6 5′ and exon 6 3′ primers shown in table 1. These regions are thought to possess functional importance (Hatzfeld and Weber 1991; Vassar et al. 1991; Geisler et al. 1992; Letai et al. 1992; Wilson et al. 1992; Steinert et al. 1993), and mutations there are known to cause keratin diseases other than MCD (Lane 1994). PCR for the helix-termination motif was followed by restriction-enzyme *Eco*47I (Toyobo) digestion. To look at other regions, we used primers for exon-specific amplification, shown in table 1. Denatured samples were electrophoresed for 12–20 h at 4 W in a cooled room. Migration was performed on a 7% polyacrylamide gel containing 10% glycerol. After migration, the gel was stained with 1 × SYBR green nucleic-acid gel-stain solution (Molecular Probes) and was analyzed by use of a laser-scanning image analyzer (FluorImager 595; Molecular Dynamics).

The sequencing was performed by use of an automatic fluorescent DNA sequencer (ABI PRISM 377; Applied Biosystems). Primers for amplification of individual exons, except for the 5′ primer for presumptive exon 1 and the 3′ primer for presumptive exon 8, were designed from adjacent intron sequences within 100 bp of the splice site. The 3′ primer for exon 8 was designed in the 3′ UTR region. Exon 1 spans the head domains (E1, V1, and H1) and the 1A segment of the α-helix domain. H1 and 1A are mutation hot spots, whereas E1 and V1 are polymorphic; therefore, the 5′ primer for exon 1 was designed in the E1/V1 region. Genomic DNA (50 ng) was amplified with Ampli*Taq* gold polymerase (Perkin Elmer) in 1 × PCR buffer supplied by the manufacturer. The primers (5′→3′) are shown in table 1.

Restriction-enzyme digestion (i.e., RFLP) analysis was performed to confirm the mutations, to test for cosegregation, and to show that the mutations do not occur in 100 normal unrelated chromosomes. Since, among the four mutations that we report, three do not alter any known restriction site, we synthesized primers that contained mismatches; these are given here with the mismatch bases underlined. For the mutation Arg135Gly in our MCD-N family, we used the primers K12/NF (GGAGGTGGCTCTCTAGGTATT) and K12/NR (CTTATCCAGGTAGGAAGCTGGT), which generate an *Eco*47I (Toyobo) site in the mutated allele but not in the normal allele; for the mutation Arg135Ile in our

Table 2

Exon/Intron Boundary Sequences of K12

Presumptive Exon	intron/EXON . . . EXON/intron	K12 cDNA (Nucleotide Positions[a])	Exon Length (bp)
1	. . . CAGGAATAAGgtaagatctc	<1–567	>567
2	cttttgccagATCATTTCAG . . . TCAGGATGAAgtgagtcgaa	568–650	83
3	tggttttcagGTATGAGAAT . . . CCACGAGGATgtgagtcaga	651–807	257
4	ctgtccccagGAGCTCCAAA . . . CATTGAAAAGgtaacacaaa	808–969	162
5	gctgttgcagAGCGGGGAGC . . . GCTCGCCATGgtaggctcgt	970–1095	126
6	ccacgtgcagAAGAAATCCC . . . AGGCCCAAGGgtgagcagac	1096–1316	221
7	tttcttacagTGATGGTTTG . . . TCCTCTAAAGgtatgcagaa	1317–1387	71
8	ttatctttagACCCAACCAA . . .	1388–1844<	>457

[a] Numbers refer to positions of the cDNA, with the first nucleotide of the open reading frame being +1.

MCD-K family, we used the primers K12/KF (GAAAAAGAAACTATGCAAAATCTTAATGAAA) and K12/KR (CATTCTCGAATTTTATTTTCTAGCTCAGTA), which generate a *Ssp*I (Toyobo) site in the mutated allele but not in the normal allele; for the mutation Tyr429Asp in our MCD-T family, we used the primers K12/TF (TGGAGCTGGAGATTGAGGTC) and K12/TR (ACTCTTGGTCAGCCCCTGAA), which generate a *Sal*I (Takara) site in the mutated allele but not in the normal allele; and, for the mutation Leu140Arg in our MCD-F family, we used the primers K12/FF (GCAATGATGGAGGCCTTCTT) and K12/FR (AGCTCAGTATTAGCCTCTTCT), which generate a *Hap*II (Takara) site in the mutated allele but not in the normal allele. Migration was performed on a 4% NuSieve agarose gel.

Results

Genomic clones of human K12 were isolated from a human genomic library. One of the clones, F327, was analyzed by PCR amplification and subsequent direct sequencing. Seven introns were identified, thereby defining eight individual exons that cover the coding sequence. Together the exons and introns span ~6 kb of genomic DNA. The exon-intron boundary sequences will appear in the DDBJ, EMBL, and GenBank nucleotide-sequence databases, with the following accession numbers: AB007115, AB007116, AB007117, AB007118, and AB007119. These all conform to the canonical splice-donor and -acceptor sequences. Partial nucleotide sequences are given in table 2.

Using FISH, we mapped the human K12 gene to chromosome 17q12 (data not shown), where a type I keratin cluster exists. This supports the recent finding by Irvine et al. (1997), who assigned the human K12 gene to chromosome 17q (in the interval between D17S800 and D17S930), using the Genebridge 4 radiation-hybrid panel.

We were able to sequence K12 and K3 cDNAs obtained from the corneal epithelium of one MCD patient (an affected individual denoted as "TM" in our MCD-T family), who had a corneal transplant because of a secondary corneal opacity unrelated to the dystrophic abnormality. This analysis showed a T→G transversion in codon 429 in K12, whereas no mutation was detected in K3 cDNA (data not shown).

Using a combination of SSCP (data not shown) and sequencing of amplified genomic DNA (fig. 3), we found K12 mutations in all our MCD families. In family MCD-N, all affected individuals heterozygously had an A→G transition at the first nucleotide position in codon 135. This transition predicts the substitution of an Arg (AGA) by a Gly (GGA) at codon 135 (a substitution designated as "Arg135Gly") within the highly conserved helix-initiation motif of rod domain 1A (fig. 3*a*). Similarly, in family MCD-K, all affected individuals heterozygously had a G→T transversion at the second nucleotide position in codon 135, resulting in an Arg135Ile substitution (fig. 3*c*). In family MCD-T, both affected individuals had a T→G transversion at the first nucleotide position in codon 429. This transversion is within the highly conserved helix-termination motif of rod domain 2B and results in a Tyr429Asp substitution (fig. 3*e*). SSCP, direct sequencing of genomic DNA, and RFLP analyses showed that the mutations in all three families completely cosegregated with the disease phenotype. Finally, the MCD-F individual had a T→G transversion at the second nucleotide position in codon 140 within the conserved domain 1A, resulting in a Leu140Arg substitution (fig. 3*g*). A cosegregation study in this family could not be performed, because of the patient's refusal to give consent. By repeating the amplification procedures and sequence analyses, we verified that the documented transition or transversions were not due to polymerase errors during PCR. All mutations were detected when we sequenced in both the forward and the reverse directions, and, when the PCR product was subcloned, both mutant and normal alleles were detected. Also, we sequenced all

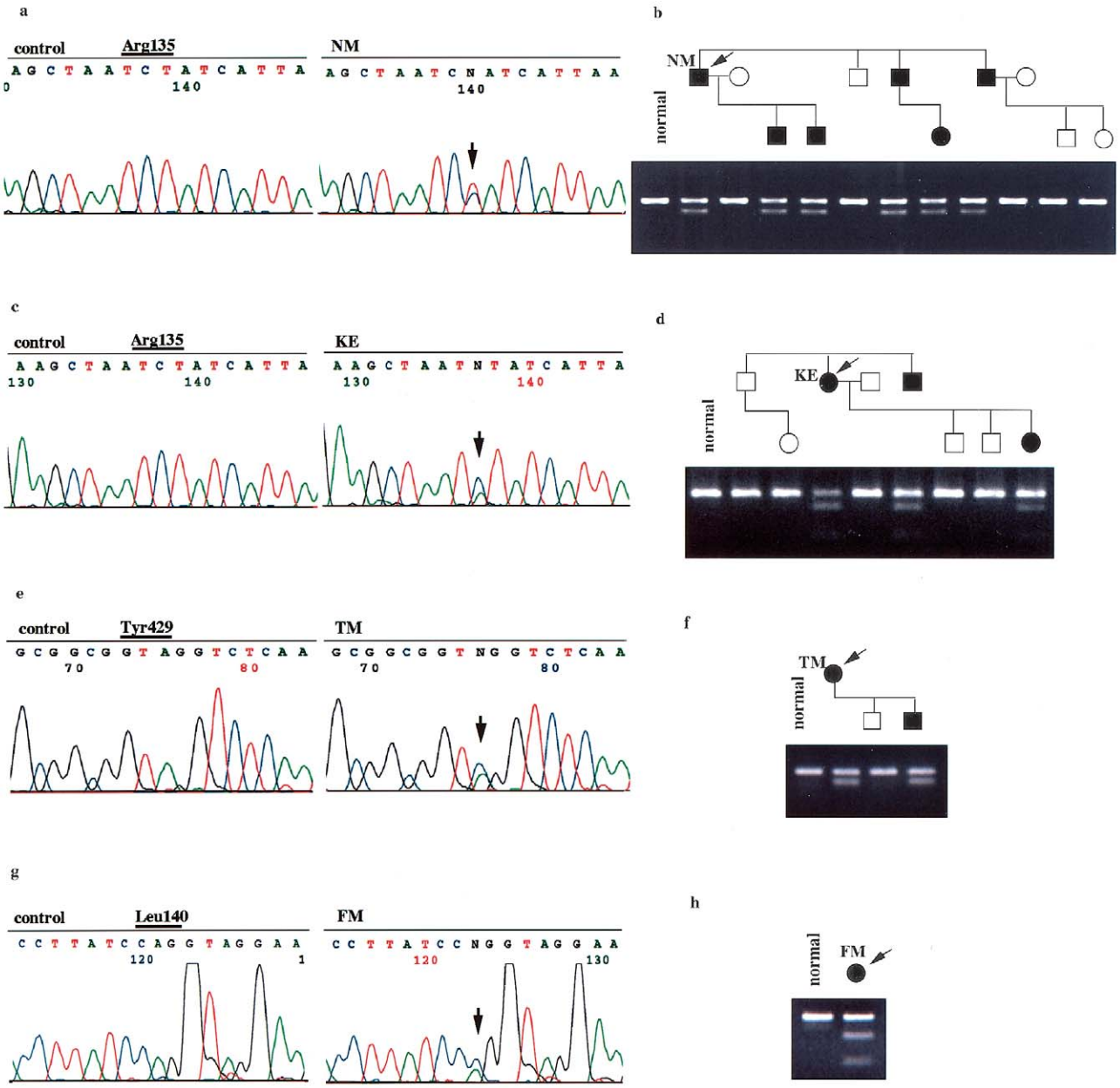

Figure 3 Direct sequencing and restriction-enzyme–digestion analysis of the K12 gene in MCD. In the pedigrees males are represented by squares, females by circles. Affected individuals are represented by blackened symbols, and the proband in each family is indicated by an arrow. Sequencing was performed in both directions; the results shown (*a*, *c*, *e*, and *g*) are in the antisense direction. The left-hand portion of each sequencing panel shows results for an unaffected control, whereas the right-hand portion shows results for a family member affected with MCD. *a*, K12 sequence within the helix-initiation motif of rod domain 1A in family MCD-N (results for patient NM are shown), showing an A→G transition at the first nucleotide position in codon 135 that results in an Arg135Gly substitution. *b*, RFLP analysis, using a mismatch primer, in family MCD-N. The K12 mutation creates a new *Eco*47I site. This mutation-specific *Eco*47I digestion pattern cosegregated with the disease phenotype. *c*, K12 sequence within the helix-initiation motif of rod domain 1A in family MCD-K (results for patient KE are shown), showing a G→T transversion at the second nucleotide position in codon 135 that results in an Arg135Ile substitution. *d*, RFLP analysis, using a mismatch primer, in family MCD-F. The K12 mutation creates a new *Ssp*I site. This mutation-specific *Ssp*I digestion pattern cosegregated. *e*, K12 sequence within the helix-termination motif of rod domain 2B in family MCD-T (results for patient TM are shown), showing a T→G transversion at the first nucleotide position in codon 429 that results in a Tyr429Asp substitution. *f*, RFLP analysis, using a mismatch primer, in family MCD-T. The K12 mutation creates a new *Sal*I site. This mutation-specific *Sal*I digestion pattern cosegregated. *g*, K12 sequence within the helix-initiation motif of rod domain 1A in family MCD-F (results for patient FM are shown), showing a T→G transversion at the second nucleotide position in codon 140 that results in a Leu140Arg substitution. *h*, RFLP analysis in family MCD-F. The K12 mutation creates a new *Hap*II site. This mutation-specific *Hap*II digestion pattern was detected in affected patient FM but not in normal individuals.

coding regions of the K12 gene in affected individuals from each family and checked individual exons covering the regions other than the α-helix–initiation or –termination motif. Apart from the mutations described, no other substitutions or polymorphisms were found in the rod domain of K12.

RFLP analyses showed that the mutations that we report completely cosegregated with the disease phenotype within each of the three families (MCD-N, MCD-K, and MCD-T) that participated in our cosegregation study (fig. 3*b, d,* and *f*). Although we were not permitted to perform a cosegragation study of family MCD-F, the mutation detected in the affected individual was confirmed by RFLP (fig. 3*h*). All four mutations described were excluded from 100 normal unrelated chromosomes.

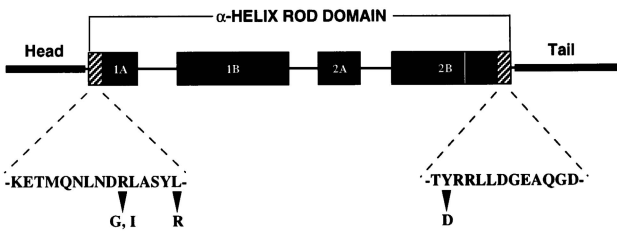

Figure 4 Depiction of the domain structure of K12 and the mutations found in MCD. The rod domain comprises α-helical segments 1A, 1B, 2A, and 2B, flanked by the nonhelical head and tail domains. The vertical line in the middle of segment 2B represents the "stutter" that is an interruption in the phasing of the heptad repeat. The helix-initiation and -termination motifs are denoted by hatched boxes at the beginning and end of the rod domain, respectively. The amino acid sequences for the helix-initiation and -termination motifs are shown.

Discussion

MCD corneas possess a fragile epithelium, and the clinical presentation of the cornea closely resembles several blistering epidermal diseases caused by mutations in epidermal keratins. This clinical picture, along with several pathological features shared by MCD and some epidermal diseases, led us to postulate that MCD might be caused by a mutation in K12 or K3, keratins that are expressed specifically in the corneal epithelium. Recently, our suspicions were proved true by a study that documented K3 and K12 mutations in MCD (Irvine et al. 1997). This showed that K12 mutations exist at positions 135 (Arg135Thr) and 143 (Val143Leu) in MCD patients from an Irish pedigree and from Meesmann's original German kindred. We have also found mutations at position 135 (Arg135Gly and Arg135Ile) in two of our four Japanese pedigrees, although no mutation at position 143 was detected in any of our MCD patients. In addition, the present study identifies two extra mutations (Tyr429Asp and Leu140Arg) not present in the Irish or German MCD families that recently have been reported (Irvine et al. 1997).

The mutations that we detected are located in the helix-initiation motif of the 1A segment (mutations Arg135Gly, Arg135Ile, and Leu140Arg) in two Japanese families and one individual with MCD and in the helix-termination motif at the end of the 2B segment (mutation Tyr429Asp) in another family (fig. 4). When family members could be tested, these mutations completely cosegregated and were absent from normal individuals. The regions in which the MCD mutations occur are highly conserved among intermediate-filament chains and are thought to play important roles in filament assembly and stability. Several lines of evidence show that the regions in which our mutations occur represent a mutation hot spot in the keratin gene, in several inherited epidermal diseases. Substitutions of the Arg residue at position 135 in K12 in families MCD-N and MCD-K are in some ways analogous to (*a*) the Arg-residue substitution in codon 125 in K14 in EBS–Dowling-Meara (EBS-DM) (Coulombe et al. 1991; Stephens et al. 1993), (*b*) the Arg-residue substitution in codon 156 in K10 in bullous ichthyosiform erythema (BCIE) (McLean et al. 1994) and in epidermolytic hyperkeratosis (EHK) (Rothnagel et al. 1992), (*c*) the Arg residue substitution in codon 162 in K9 in patients with epidermolytic palmoplatar keratoderma (EPPK) (Reis et al. 1994), and (*d*) the Arg residue substitution in codon 127 in K16 in nonepidermolytic palmoplanter keratoderma patients (Scamsher et al. 1995). It is curious that, in families MCD-N and MCD-K, the Arg substitution at codon 10 in the helix-initiation motif (position 135 in the case of K12) does not occur in a CpG dinucleotide, whereas, in the aforementioned diseases, Arg substitutions in this position in K9, K10, K14, and K16 all occur in CpG dinucleotides; the significance of this remains unkown.

We know that the more severe phenotypes of EBS-DM are caused by mutations in the helix-initiation or helix-termination motif (Coulombe et al. 1991; Stephens et al. 1993), whereas the milder forms (EBS–Weber-Cockayne and EBS-Köbner) are associated with mutations at other sites (Rugg et al. 1993). Drawing analogies with MCD, we can infer that all affected individuals in the four MCD families that we studied should represent the severe phenotype. Indeed, this would seem to be the case, because, although vision is not diminished enough to require a corneal graft (it rarely is with MCD), intraepithelial microcysts in all individuals are extensive, and subjective findings such as photophobia, lacrimation, and itching are strong. As with EBS, we cannot rule out the possibility that a mild form of MCD may exist, caused by mutations at sites other than the helix-initiation or -termination motif as documented here.

In assessing the importance of the K12 mutations in the MCD families that we studied, it is useful to consider some ultrastructural aspects of keratins more closely. These proteins, of which types 3 and 12 are found in cornea, are intermediate filaments, whose structure depends on the positions of polar and nonpolar residues in their α-helical segments. Basically, keratins are composed of two parallel α-helical segments (one type I and one type II) that lie in register. The amino acid sequence in the helical rod domains comprises repeating amino acid sequences possessing seven residues, $(a–g)_n$, where a and d are usually nonpolar residues and the others are usually polar or charged residues (Parry et al. 1977; Coulombe and Fuchs 1990; Hovnanian et al. 1993). This heptad model of keratin ultrastructure dictates that the polar residues a and d form a hydrophobic core at the center of the heterodimer. In addition, the polar residues e and g, located adjacent to the hydrophobic core, stabilize the dimer via ionic interactions. Since all the mutations found in our study change a polar residue into a nonpolar residue, or vice versa, they should result in the loss of either an ionic bond or a hydrophobic interaction, thereby destabilizing the keratin filament and possibly giving rise to MCD.

Of particular interest here is the discovery of a substitution, in the helix-termination motif in type I keratin, that was found in both of the affected individuals in family MCD-T. This region is the most highly conserved motif in intermediate-filament sequences. In family MCD-T, the affected individual (TM) whose cornea was grafted showed no other mutations in the α-helix domain in either K12 or K3, by RT-PCR and subsequent direct sequence analysis. These K12 data were confirmed at the genomic level. Taking these results together with those showing that this new mutation (Tyr429Asp) was present in TM's affected sibling but not in either his unaffected sibling or 100 normal individuals, we propose that it may be causative for MCD. To the best of our knowledge, no investigators have documented substitutions in the helix-termination motif in a type I keratin that are causative for epidermal keratin diseases. Nevertheless, the possibility that a mutation in the helix-termination motif in type I keratin may cause MCD is not totally unexpected, since mutations in this motif are known, for example, in type II keratins in patients with EBS (Lane et al. 1992; Rothnagel et al. 1992). Also, it is worth noting that previous in vitro experiments showing that the helix-termination motif in type I keratin is important for keratin filament assembly/function (Hatzfeld and Weber 1991; Letai et al. 1992; Wilson et al. 1992) are indirectly supported by our finding that in family MCD-T a Tyr residue in codon 429 in the helix-termination domain in K12 may well be important for the interaction of K3 and K12.

Acknowledgments

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References

Bonifas JM, Rothman AL, Epstein EH (1991) Epidermolysis bullosa simplex: evidence in two families for keratin gene abnormalities. Science 254:1202–1205

Chan Y-M, Yu Q-C, Fine J-D, Fuchs E (1993) The genetic basis of Weber-Cockayne epidermolysis bullosa simplex. Proc Natl Acad Sci USA 90:7414–7418

Chen MA, Bonifas JM, Matsumura K, Blumenfeld A, Epstein EH (1993) A novel three nucleotide deletion in the helix 28 region of keratin 14 in epidermolysis bullosa simplex. Hum Mol Genet 2:1971–1972

Cheng J, Syder AJ, Yu Q-C, Letai A, Patter A, Fuchs E (1992) The genetic basis of epidermolytic hyperkeratosis: a disorder of differentiation-specific epidermal keratin genes. Cell 70: 811–819

Coulombe PA, Fuchs E (1990) Elucidating the early stages of keratin filament assembly. J Cell Biol 111:153–169

Coulombe PA, Hutton ME, Letai A, Hebert A, Paller AS, Fuchs E (1991) Point mutations in human keratin 14 genes of epidermolysis bullosa simplex patients: genetic and functional analysis. Cell 66:1301–1311

Geisler N, Schunemann J, Weber K (1992) Chemical crosslinking indicates a staggered and antiparallel protofilament of desmin and characterizes one higher-level complex between protofilaments. Eur J Biochem 206:841–852

Hatzfeld M, Weber K (1990) The coiled coil of in vitro assembled keratin filaments is a heterodimer of type I and II keratins: use of site-specific mutagenesis and recombinant protein expression. J Cell Biol 110:1199–1210

——— (1991) Modulation of keratin intermediate filament assembly by single amino acid exchanges in the consensus sequence at the C-terminal end of the rod domain. J Cell Sci 99:351–362

Hovnanian A, Pollack E, Hilal L, Rochat A, Prost C, Barandon Y, Goossens M (1993) A missense mutation in the rod domain of keratin 14 associated with recessive epidermolysis bullosa simplex. Nat Genet 3:327–332

Humphries MM, Sheils DM, Farrar GJ, Kumar-Singh R, Kenna PF, Mansergh FC, Jordan SA, et al (1993) A mutaion (Met-to-Arg) in the type 1 keratin (K-14) gene responsible for autosomal dominant epidermolysis bullosa simplex. Hum Mutat 2:37–42

Irvine AD, Corden LD, Swensson O, Swensson B, Moore JE, Frazer DG, Smith FJD, et al (1997) Mutations in keratin K3 or K12 genes cause Meesmann's corneal dystrophy. Nat Genet 16:184–187

Kao WWY, Liu CY, Converse RL, Shiraishi A, Kao CWC, Ishizaki M, Doetschman T, et al (1996) Keratin 12–deficient mice have fragile corneal epithelia. Invest Ophthalmol Vis Sci 37:2572–2584

Klinge EM, Sylvestre YR, Freedberg IM, Blumenberg M (1987) Evolution of keratin genes: different protein domains evolve by different pathways. J Mol Evol 24:319–329

Kurpakus MA, Stock EL, Jones JC (1990) Expression of the 55-kD/64-kD corneal keratins in ocular surface epithelium. Invest Ophthalmol Vis Sci 31:448–456

Kuwabara Y, Ciccarelli EC (1964) Meesmann's corneal dystrophy. Arch Ophthalmol 71:676–682

Lane EB (1994) Keratin disases. Curr Opin Genet Dev 4: 412–418

Lane EB, Rugg EL, Navsaria H, Leigh IM, Heagerty AHM, Ishida-Yamamoto A, Eady RAJ (1992) A mutation in the conserved helix termination peptide of keratin 5 in hereditary skin blistering. Nature 356:244–246

Letai A, Coulombe PA, Fuchs E (1992) Do the ends justify the mean? proline mutations at the ends of the keratin coiled-coil rod segment are more disruptive than internal mutations. J Cell Biol 116:1181–1195

Liu CY, Zhu G, Converse R, Kao CWC, Nakamura H, Tseng SCG, Mui MM, et al (1994) Characterization and chromosomal localization of the cornea-specific murine keratin gene Krt1.12. J Biol Chem 269:24627–24636

Liu CY, Zhu G, Westerhausen-Larson A, Converse R, Kao CWC, Sun TT, Kao WWY (1993) Cornea-specific expression of K12 keratin during mouse development. Curr Eye Res12:963–974

Lu X, Lane EB (1990) Retrovirus-mediated transgenic keratin expression in cultured fibroblasts: specific domain functions in keratin stabilization and filament formation. Cell 62: 681–696

McLean WHI, Morley SM, Eady RAJ, Dopping-Heppenstal PJC, McMillan JR, Leigh IM, Navsaria HA, et al (1994) Mutations in the rod 1A domain of keratins 1 and 10 in bullous congenital ichthyosiform erythroderma (BCIE). J Invest Dermatol 102:24–30

Meesmann A, Wilke F (1939) Klinische und anatomische Untersuchungen uber eine bisher Unbekannte, dominant verebte Epitheldystrophie der Hornhaut. Klin Mbl Augenheilkd 103:361–391

Moll R, Franke WW, Schiller DL, Geiger B, Krepher R (1982) The catalog of human cytokeratins: patterns of expression in normal epithelia, tumor and cultured cells. Cell 31:11–24

Nakanishi I , Brown SI (1975) Ultrastructure of the epithelial dystrophy of Meesmann. Arch Ophthalmol 93:259–263

Nishida K, Adachi W, Shimizu-Matsumoto A, Kinoshita S, Mizuno K, Matsubara K, Okubo K (1996) A gene expression profile of human corneal epithelium and the isolation of human keratin 12 cDNA. Invest Ophthalmol Vis Sci 37: 1800–1809

Nishida K, Sotozono C, Adachi W, Yokoi N, Kinoshita S (1995) Transforming growth factor-β1, -β2, -β3 mRNA expression in human cornea. Curr Eye Res 14:235–241

Parry DAD, Crewther WG, Fraser RDB, MacRae TP (1977) Structure of α-keratin: structural implications of the amino acid sequences of the type I and type II chain segments. J Mol Biol 113:449–454

Reis A, Hennies HC, Langbein L, Digweed M, Mischke D, Drechsler M, Schröck E, et al (1994) Keratin 9 gene mutations in epidermolytic palmoplantar keratoderma (EPPK). Nat Genet 6:174–179

Rothnagel JA, Dominey AM, Dempsey LD, Longley MA, Greenhalgh DA, Gagne TA, Huber M, et al (1992) Mutations in the rod domains of keratins 1 and 10 in epidermolytic hyperkeratosis. Science 257:1128–1130

Rugg EL, Morley SM, Smith FJD, Boxer M, Tidman MJ, Navsaria H, Leigh IM, et al (1993) Missing links: keratin mutation in Weber-Cockayne EBS families implicate the central L12 linker domain in effective cytoskeleton function. Nat Genet 5:294–300

Scamsher MK, Navsaria HA, Stevens HP, Ratnavel RC, Purkis PE, Kelsell DP, McLean WHI, et al (1995) Novel mutations in keratin 16 gene underly focal non-epidermolytic palmoplantar keratoderma (NEPPK) in two families. Hum Mol Genet 4:1875–1881

Schermer A, Ganvin S, Sun TT (1986) Differentiation-related expression of a major 64K corneal keratin in vivo and in culture suggests limbal location of corneal epithelial stem cells. J Cell Biol 103:49–62

Steinert PM, Marekov LN, Fraser RDB, Parry DAD (1993) Keratin intermediate filament structure: crosslinking studies yield quantitative information on molecular dimensions and mechanism of assembly. J Mol Biol 230:436–452

Stephens K, Sybert VP, Wijsman EM, Erlich P, Spencer A (1993) A keratin 14 mutational hot spot for epidermolysis bullosa simplex, Dowling-Meara: implication for diagnosis. J Invest Dermatol 101:240–243

Vassar R, Coulombe PA, Degenstein L, Albers K, Fuchs E (1991) Mutant keratin expression in transgenic mice causes marked abnormalities resembling a human genetic skin disease. Cell 64:365–380

Wilson AK, Coulombe PA, Fuchs E (1992) The roles of K5 and K14 head, tail, and R/KLLEGE domains in keratin filament assembly in vitro. J Cell Biol 119:401–414

Wu RL, Chen TT, Sun TT (1994) Functional importance of an Sp1- and NFkB-related nuclear protein in an keratinocyte-specific promotor of rabbit K3 gene. J Biol Chem 269: 28450–28459