A Mutational Hotspot in the 2B Domain of Human Hair Basic Keratin 6 (hHb6) in Monilethrix Patients

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Monilethrix is an inherited hair dystrophy in which affected, fragile, hairs have an unique beaded morphology. Ultrastructural studies suggest a defect in filament structure in the cortex of the hair, and the hard keratins of hair and nail are thus candidate genes. In several families with autosomal dominant monilethrix, the disorder has been linked to the type II keratin gene cluster at chromosome 12q13. Recently, causative mutations in the critical helix termination motif in the 2B domain of the human hair basic keratin 6 (hHb6) have been identified. We now report the results of sequencing this domain in 13 unrelated families or cases with monilethrix. Five of the 13 had the same mutation as previously found, a G to A transversion leading to a lysine for glutamic acid substitution (E413K) in the 2B domain (residue 117 of the 2B helix) of hHb6. The mutation was confirmed by a restriction fragment length polymorphism assay developed for this purpose, and, as this mutation is evidently a common cause of the syndrome, for use in screening other cases. In eight families or cases, however, including three in whom linkage data are consistent with a defect at the type II keratin locus, no mutation was found in this domain of hHb6. ***Key words: hair diseases/hard keratins/intermediate filaments/monilethrix. J Invest Dermatol 111:896–899, 1998***

Monilethrix ("necklace hair") is a hereditary disorder, characterized by a dystrophic alopecia. It is so named because of typical beaded hair shafts showing a regular periodicity of nodes, of normal diameter, and abnormally narrow internodal segments. Non-beaded hairs are also fragile. The effect on scalp hair is variable and ranges from normality or mild occipital hair loss to near total alopecia. In some cases alopecia persists throughout life; in others regrowth of apparently normal hair may occur around puberty or in pregnancy (Alexander and Grant, 1958). Prominent follicular keratoses are present in up to 90% of patients (Tietze, 1995) and nail defects may be manifest, for example, as a "spoon nail" (koilonychia) (Heydt, 1963; Tietze, 1995). Monilethrix-like hair dystrophies have been reported in association with mental retardation and other ectodermal dysplasias (Sfaello and Hariga, 1967; Georgmaneanu *et al*, 1976), but most families show no extracutaneous features. Most pedigrees show autosomal dominant inheritance with high, but not complete, penetrance (Deraemaeker, 1957; Birch-Machin *et al*, 1997).

Electron microscopic studies in monilethrix identify defects in the microfibrillar structure of the cortex of hair shafts, affecting both nodal and internodal regions (De Berker *et al*, 1993), and vacuolation of cortical trichocytes (Ito *et al*, 1990). Hence genes encoding structural proteins of hair are candidates for causative defects. The major structural proteins of hair are the relatively cysteine-rich "hard" keratins, also found in nail, whose genes are located in the same clusters as the "soft" keratins at 17q12-q21 (type I, acidic keratins) and 12q13 (type II, basic keratins) (Rogers *et al*, 1995). Several groups have reported that the defect in monilethrix maps to the type II keratin gene cluster at 12q13 (Healy *et al*, 1995; Stevens *et al*, 1996).[1] As yet, no linkage to the type I cluster has been shown, although in at least one family the defect does not appear to map to either major keratin gene cluster (Richard *et al*, 1996).

Knowledge of hair keratins and their patterns of expression in man is less well developed than for "soft" keratins, but the same principles of keratin biology appear to apply. Keratin intermediate filaments (IF) are composed of heterodimers containing paired acidic and basic keratins, with a highly homologous central rod domain, and functional specificity conferred by the variable N- and C-terminal domains. In man, evidence to date suggests at least seven acidic and at least four basic hair keratins (Rogers *et al*, 1997), but more may exist. Four basic keratins have been cloned (Rogers *et al*, 1995, 1997; Bowden *et al*, 1998),[1] of which human basic hair keratin 5 (hHb5) is expressed in the proliferative pool of the hair matrix, and hHb1, hHb3, and hHb6 in the cortex. A basic partner for hHa2 in the cuticle is also postulated (Rogers *et al*, 1997).

In eight families or isolated cases with monilethrix, Winter *et al* (1997a, b) have now identified mutations in hHb6, and most recently, in hHb1. The hHb6 defect in six pedigrees or cases was a G to A

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Abbreviations: hHb, human hair basic keratin; RFLP, restriction fragment length polymorphism.

[1]Korge BP, Richard G, Pünter C, *et al*: Monilethrix links to the keratin type II cluster at 12q13 and cloning of a possible candidate gene. *J Invest Dermatol* 106:843, 1996 (abstr.)

Table I. Cases studied and linkage data

Identifier	Origin	Cases	Zmax[a]	Linkage reference	Comment
S[b]	Scotland	17	9.63 (D12S96)	Healy *et al*, 1995	Family 1 in Alexander and Grant, 1958
O[b]	Ireland	9	2.65 (D12S96)	Healy *et al*, 1995	
K[b]	England	11	3.39 (D12S398)	Birch-Machin *et al*, 1997	Follicular keratoses only in some members
I	Spain	7	2.69 (D12S96)	Birch-Machin *et al*, 1997	
D	Scotland	5	1.06 (D12S339)	Birch-Machin *et al*, 1997	Linkage to type I keratin gene cluster excluded
L	Scotland	2	nd	Birch-Machin *et al*, 1997	Family 3 in Alexander and Grant, 1958
M1[b]	Germany	1	nd		Korge, unreported: neither parent affected
M2	Germany	7	2.43 (D12S96)	Footnote 1	
M3	Germany	3	nd		Tietze (1995)
M4[b]	Germany	5	nd		Tietze (1995)
M5	Germany	3	nd		Tietze (1995)
M6	Germany	4	nd		Tietze (1995)
M7	Germany	4	nd		Tietze (1995)

[a]Maximum two point lod score in linkage analysis to one or more markers at or near type II keratin gene cluster; nd, not done.
[b]Pedigrees in which E413K mutation is identified.

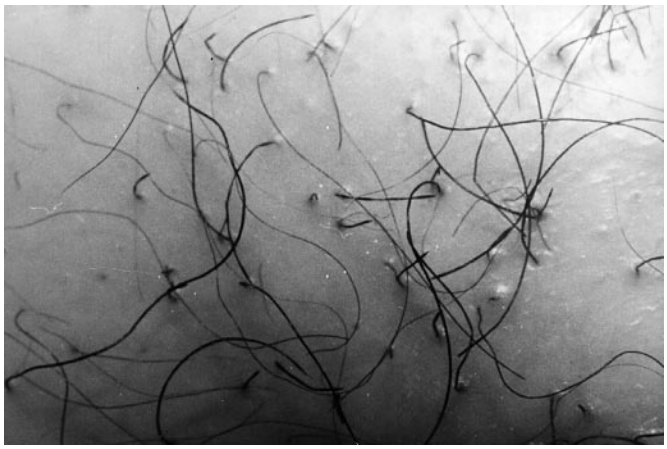

Figure 1. Severe scalp hair dystrophy in monilethrix, showing alopecia and broken scalp hairs.

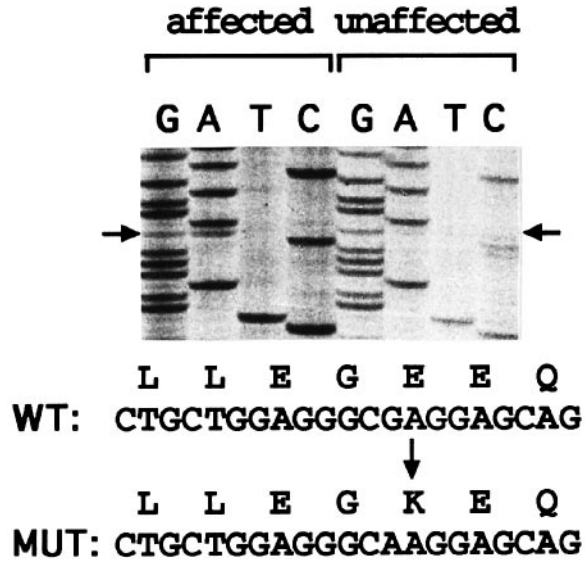

Figure 2. Part of DNA sequencing gel showing G to A transversion (*arrow*) in the 2B domain of hHb6 gene of an affected and unaffected family member of family M4. The glutamic acid to lysine substitution in the mutant allele due to this point mutation is indicate below.

transversion, encoding a lysine residue in place of a glutamic acid at position 413 (E413K) in the helix termination peptide (numbering of amino acid residues relates to the sequence published by Bowden *et al*, 1998; EMBL/Genbank AJ000263) of the keratin 2B domain (residue 117 of the 2B helix). In the seventh family, a point mutation in hHb6 at the third base of the same codon was found, causing an aspartate substitution. In this paper, we report the results of sequencing the 2B domain of hHb6 in 13 more unrelated cases or families with monilethrix. In five of these we have again identified the E413K mutation, for which we have also developed an restriction fragment length polymorphism (RFLP) assay, but in the remainder we have found no mutations in this domain.

MATERIALS AND METHODS

Subjects Most of the 13 pedigrees have previously been studied (**Table I**), and in five that have been subject to linkage analysis, this confirms or is consistent with linkage to 12q13. Affected members of all families displayed the typical phenotype of beaded and broken hairs (**Fig 1**). Linkage and DNA studies were approved by the local research ethics review committee.

Mutation detection DNA was extracted from blood or salivary samples obtained from family members, and polymerase chain reaction (PCR) amplification performed by standard methods, using the Expand Long template PCR system (Boehringer, Mannheim, Germany). The forward primer 5′-CCCT-CAGCGATGCCCGCTGCAAG-3′ was picked from the published sequence (GenBank No X99142 and EMBL/Genbank AJ000263) and the reverse from the sequence of intron 7 of hHb6: 5′-CTGGTTGCAGGGTGGGGAGGTTA-3′. PCR was performed at 94°C, then 35 cycles (94°C, 1 min; 65°C, 1 min; 68°C, 2 min) followed by a 7 min extension at 68°C. After shrimp alkaline phosphatase/exonuclease 1 treatment, PCR products were directly sequenced using the PCR Product and Sequencing Kit (USB/Amersham Life Science, Cleveland, OH).

RESULTS AND DISCUSSION

Position 413 of hHb6 is a hot spot for mutations in monilethrix In each of four families (S, O, K, and M4) and in one sporadic case with clinically normal parents (M1) we identified an identical mutation in the helix termination motif of the 2B domain of hHb6 (**Fig 2**). The G to A transversion, encoding a lysine residue in place of glutamic acid at position 413 (E413K), was the same as that identified in six families or cases by Winter *et al* (1997a, b). The presence of the E413K (numbering of amino acid residues relates to the sequence published by Bowden *et al*, 1998; EMBL/Genbank AJ000263) mutation in family K was of particular interest because in this family, follicular keratoses were a prominent or sole feature in several cases (Birch-Machin *et al*, 1997). The recurrent mutation is likely to have arisen independently, because standardization of markers for haplotyping the region of the type II keratin cluster in the British families (S, O, and K) did not suggest common ancestry (Healy *et al*, 1995; Birch-Machin *et al*, 1997), and in case M1, the mutation appears to have arisen *de novo*, being excluded in both parents. In our remaining eight pedigrees, sequencing of the whole of the 2B domain of hHb6 identified no mutations, despite the fact that in all three families in

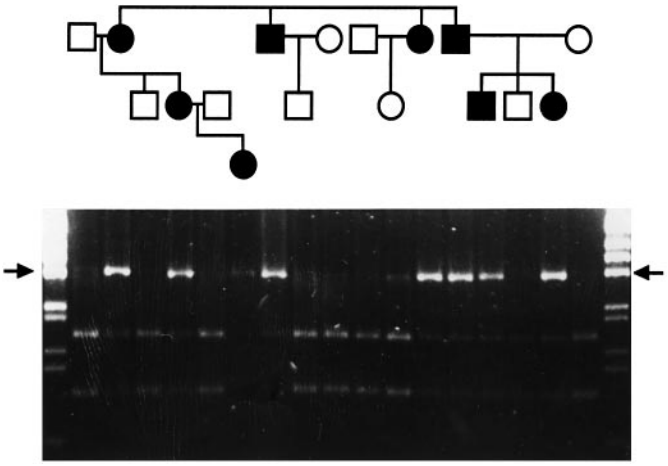

Figure 3. RFLP for the BseR1 enzyme in a 287 bp PCR fragment amplified from the 2B rod domain of hHb6 gene in part of family S, showing incomplete cleavage in affected members due to loss of the restriction site in the mutant allele. The *arrow* indicates the 298 bp fragment of the 1 kb DNA size marker (BRL/Gibco, Rockville); 2% agarose gel.

whom linkage analysis has been performed, data are suggestive of or consistent with linkage to 12q13 (**Table I**).

The domain, encoding the helix termination peptide, is thought to be critical for higher order assembly of keratin intermediate filaments (Steinert, 1995), and the vast majority of pathogenic mutations in diseases due to keratin gene defects lie in either this or the corresponding helix initiation peptide in the 1A domain (McLean and Lane, 1995; Korge and Krieg, 1996). The helix termination sequence TYR(R/K)LLEGEE is highly conserved throughout both epidermal keratins and hair keratins. The recurrent G to A transversion at position 413 in five of the 13 pedigrees of monilethrix we have studied, and six of eight other families or cases (Winter *et al*, 1997a, b), is likely to have arisen independently. Identical mutations in the corresponding nucleotide represent an even higher proportion – 10 of 14 – of reported mutations in keratin 2e in ichthyosis bullosa of Siemens (Kremer *et al*, 1994; McLean *et al*, 1994; Rothnagel *et al*, 1994; Jones *et al*, 1997), and recently, the equivalent mutation in the keratin 3 gene has been found in Meesmann's corneal dystrophy (Irvine *et al*, 1997). The common mechanism is likely to be spontaneous deamination of a methylated cytosine in a CpG dinucleotide giving rise to thymine (Holiday and Grigg, 1993). As a result of ineffective mismatch repair the opposite strand is propagated as adenine instead of guanine. In our remaining eight pedigrees, sequencing of the whole of the 2B domain of hHb6 identified no mutations, despite the fact that in all three families in whom linkage analysis has been performed, data are suggestive of or consistent with linkage to 12q13 (**Table I**).

BseRI-RFLP assay for mutation screening The 298 bp PCR fragment contains a cutting site for the restriction enzyme BseR1 that is lost following the G to A transversion. The resultant RFLP was used to confirm segregation of the mutation with the phenotype in part of one pedigree (**Fig 3**), as well as to confirm its absence in 36 normal controls (data not shown). The RFLP method we have developed will be of value in screening isolated cases or pedigrees for this common pathogenic mutation.

Protein structural implications of the E413K substitution Both the common mutant lysine residue, and the charge-conservative aspartic acid substitution found at the same codon in one other family (Winter *et al*, 1997b), replace a glutamic acid residue that occupies an internal d position in the heptad repeats of the α-helix. The d position is often occupied by glutamic acid, but relatively rarely by lysine residues, whereas the a position is often taken by lysine, but rarely by glutamic acid residues. This suggests that lysine in the d position interferes with coiled coil formation, and hence possibly with intermediate filament assembly. Whereas pathogenic mutations at the corresponding residue occur in epidermal keratin genes (see above), the precise mechanism of intermediate filament disruption may differ in hair keratins. It has been suggested (Parry, 1995, 1996) that unlike epidermal keratins, hair keratin molecules in the IF, although maintaining the major overlaps characteristic of all IF, lack the head-to-tail overlap between similarly directed molecules. The hard α-keratins are believed instead to be stabilized by numerous intermolecular disulfide bonds. Because of these differences in filament assembly, the mutant lysine residue in the d position of the type II chain may actually increase stability in monilethrix hair by interacting with an aspartate residue in the e position of the type I keratin (Parry D.A.D., Massey University, Palmerston North, New Zealand, personal communication).

Most cases of monilethrix are likely to be due to type II hair keratin defects Many keratin disorders arise from defects in either partner of a keratin pair (McLean and Lane, 1995), but no families of monilethrix have as yet been mapped to the type I keratin locus, suggesting that type II keratin defects may have a particular relevance to the disease. Certain epidermal keratins seem similarly to be uniquely associated with particular phenotypes, e.g., keratin 9 with epidermolytic palmoplantar keratoderma (Bonifas *et al*, 1994; Reis *et al*, 1994), keratin 2e with ichthyosis bullosa of Siemens (Kremer *et al*, 1994; McLean *et al*, 1994; Rothnagel *et al*, 1994; Jones *et al*, 1997), and keratin 17 with pachyonychia congenita type II (McLean *et al*, 1995; Smith *et al*, 1997). *In situ* studies indicate that hHb1 and hHb3 have similar expression patterns in the emerging cortex, but hHb6 mRNA starts and ends relatively higher in the emerging hair shaft (Rogers *et al*, 1997); however, although mutations in hHb6 appear to be commonest, they are apparently not the only cause of monilethrix. As noted above, since our work was carried out, Winter *et al* (1997a) have reported a similar mutation, E403K, in the corresponding codon of hHb1 in a family with monilethrix. It thus appears that the monilethrix phenotype is not unique to mutations in hHb6, but is a general phenomenon of defects in cortical keratins. There is a high degree of homology between hHb6 and hHb3 and particularly hHb1, even in the V domains and intron sequences (Rogers *et al*, 1997). The fact that fragility occurs, despite this apparent redundancy of expressed structural genes, suggests that these keratins have distinct roles.

Possible pathogenesis of beaded hairs The mechanism by which keratin filament defects cause the clinical phenomena of monilethrix remains obscure. The regular periodicity of beading is not diurnal and is asynchronous between follicles (Comaish, 1969; De Berker and Dawber, 1992), suggesting an intrinsic follicular clock. It has been suggested that the narrowing could be due to compression by the inner root sheath, but dystrophic change occurs below this level (Ito *et al*, 1990). It seems more likely that narrowing is intrinsic to the hair due to a periodic reduction of effective hair formation. We have previously suggested (Healy *et al*, 1995) that the periodicity results from a feedback loop in which cytolysis of dystrophic cortical keratin IF evokes a cytokine response that directly or indirectly modulates keratin gene expression, in a manner that counteracts the defect. Restoration of IF integrity would result in a reduction of cytolysis and cytokine release and hence complete the cycle.

Genotype and phenotype are poorly correlated in monilethrix Although the recurrent G to A transversion at position 413 occurs frequently in monilethrix patients, we could not correlate this with a specific clinical phenotype. The same mutation was found in affected patients within families who might have severe dystrophic alopecia, limited involvement of only follicular keratosis, or be obligate carriers with no discernible phenotype (Birch-Machin *et al*, 1997). The variable severity of monilethrix within families and with time suggests that other factors also affect gene or disease expression. Amongst these influences are likely to be sex hormones, because both puberty and pregnancy are capable of inducing changes in hair growth (Alexander and Grant, 1958). Usually patient's hair growth improves during hormonal active episodes, although a worsening has also been documented (Tietze, 1995). The prospects for improved management of monilethrix may depend on elucidation of these mechanisms, which allow normalization of hair growth despite the continued presence of the keratin gene defect.

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