Epidermolytic Hyperkeratosis and Epidermolysis Bullosa Simplex Caused by Frameshift Mutations Altering the V2 Tail Domains of Keratin 1 and Keratin 5

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The cytoskeleton of epithelial cells is formed by hetero-polymeric keratin proteins characterized by a central α-helical rod flanked by nonhelical head and tail domains of variable sequence. Most mutations described in 18 distinct keratins disrupt highly conserved regions at the boundaries of the rod, which have been recognized as zones of overlap during keratin alignment and assembly into intermediate filaments. We recently reported the first mutation located in a keratin tail domain (V2) in ichthyosis hystrix Curth–Macklin. In this study, we report two novel frameshift mutations that are predicted to alter the tail of keratin 1 or keratin 5, leading to an atypical form of epidermolytic hyperkeratosis and a mild form of epidermolysis bullosa simplex, respectively. Mutation analysis of the patient with epidermolytic hyperkeratosis revealed a de novo hetero-zygous nucleotide insertion (1752insG) in exon 9 of KRT1, predicted to result in an aberrant 69 residue keratin 1 tail. In the patient with mild epidermolysis bullosa simplex, we identified a single nucleotide deletion (1635delG) in exon 9 of KRT5 leading to frameshift and translation of an abnormal V2 domain, 35 amino acids longer than the native keratin 5 tail. Our results, together with previous observations, establish the existence of a subgroup of keratin disorders due to frameshift mutations altering the keratin tail domains that are characterized by phenotypic heterogeneity. *Key words: epidermolysis bullosa simplex/ epidermolytic hyperkeratosis/keratin intermediate filament/ palmoplantar keratoderma. J Invest Dermatol 120:623–626, 2003*

E pidermolysis bullosa simplex (EBS; OMIM131900) and epidermolytic hyperkeratosis (EHK; OMIM113800) are inherited disorders, each characterized by a variable degree of cell fragility and disturbed cornification due to a weakened keratin intermediate filament (KIF) cytoskeleton. These genodermatoses are caused by mutations in genes encoding epidermal keratins, whose differentiation-specific expression profile determines the site of disease pathology. For example, K5/K14 are abundant in basal keratinocytes, and pathogenic mutations in either gene result in cell lysis and intraepidermal blistering within the basal layer characteristic for EBS (Irvine and McLean, 1999). Similarly, in EHK, underlying mutations have been identified in the genes for the suprabasal keratins K1/K10, leading to structural abnormalities and altered differentiation in the upper epidermis (Chipev *et al*, 1992; Rothnagel *et al*, 1992).

Keratin polypeptides typically consist of a central α-helical rod that is flanked by nonhelical head (V1) and tail (V2) domains (Fuchs and Cleveland, 1998). Investigations of naturally occurring keratin mutations and their functional consequences have significantly enhanced our understanding of the pivotal function of the rod domain for alignment, assembly, and stabilization of KIF (Fuchs and Cleveland, 1998). The structure and intrinsic functions of the nonhelical end domains, however, have just begun to emerge, in part, through the identification of a small but growing number of pathogenic mutations altering these regions. In this study, we describe two novel keratin mutations affecting the V2 domains of K1 and K5 and resulting in an atypical form of EHK and in a Weber–Cockayne type of EBS, respectively.

MATERIALS AND METHODS

Patients and biologic material All participants gave their written informed consent to take part in this study. Peripheral blood samples or buccal swabs were collected and prepared for DNA analysis from each family member.

Mutation analysis The coding sequences and flanking intronic boundaries of the K1 and K5 genes were polymerase chain reaction (PCR) amplified from genomic DNA with Taq polymerase and 10% Q-solution (Qiagen, Valencia, CA) using previously published primer pairs (Chipev *et al*, 1992; Whittock *et al*, 2000) and standard PCR conditions at an annealing temperature of 60°C. Gel-purified PCR

fragments were subjected to bidirectional direct sequencing using the BigDye terminator system on an ABI Prism 310 sequencer (Applied Biosystems, Foster City, CA).

Paternity testing To confirm paternity, we obtained the genotypes for each member of the families for eight polymorphic microsatellite markers on different chromosomes. The markers were PCR amplified from genomic DNA using fluorescently labeled and tailed primers and standard conditions for AmpliTaq Gold (Applied Biosystems). The PCR products were separated by polyacrylamide gel electrophoresis on an ABI Prism 377 sequencer, and genotypes were established using the GeneScan 3.1 and GenoTyper 2.0 software (Applied Biosystems). Assuming a prior probability of 50%, we calculated the probability of paternity (Bayesian calculation).

Electronic databases Online Mendelian Inheritance in Man: http://www3.ncbi.nlm.nih.gov/Omim/
 Human Intermediate Filament Mutation Database: http://www.interfil.org/

RESULTS

A *de novo* insertion mutation (1752insG) altering the V2 domain of K1 causes an atypical form of EHK The first proband was a 17-y-old male of Chinese ancestry born to nonconsanguineous parents (**Fig 1***a*). His younger sibling, both parents, and other relatives had no evidence of any skin disorder. His skin appeared normal at birth and during infancy. At 2 y of age, the skin of palms and soles became thickened (**Fig 1***b*, *d*). Concurrently, he developed well-demarcated, yellowish hyperkeratotic plaques over the ankles, elbows, and knees (**Fig 1***c*, *e*). Some islands of superficial peeling reminiscent of the "mauserung" phenomenon in ichthyosis bullosa of Siemens (IBSOMIM146800) (Traupe *et al*, 1986) were observed on the skin of the trunk and the extensor surface of the legs (**Fig 1***c*). The disease progressively worsened during childhood. Histologic examination of a skin biopsy revealed marked orthokeratotic hyperkeratosis, papillomatosis, and acanthosis. Occasional foci of vacuolated cells and binucleated cells were observed in the upper spinous and granular layers (**Fig 1***f*, insert). Electron microscopy demonstrated fractured and shortened KIF that remained connected to the desmosomes, occasional KIF clumping, abnormalities of the extracellular lamellar bilayers, but no shell formation (Schmuth *et al*, 2001).

Scrutiny of the entire coding sequence and flanking intronic boundaries of the KRT1 gene disclosed in the affected individual two sequence aberrations: a frequent, heterozygous 21 bp deletion polymorphism that eliminates a complete glycine loop (Korge *et al*, 1992), and a heterozygous insertion of a guanine base at position 1752 downstream of the ATG start codon (**Fig 1***g*). This insertion mutation is predicted to result in a frameshift and to introduce a delayed termination codon (**Fig 1***h*). It was not observed in DNA sequences of the proband's parents or unaffected sister or in 200 control alleles. Parentage was confirmed (probability of paternity p = 0.99985 assuming a prior probability of 0.5). Collectively, these data demonstrate that the insertion mutation 1752insG has most probably arisen *de novo* in the proband, although parental germline mosaicism cannot be excluded. No mutations were identified in K2e and the mutational hot spot regions of K10 (not shown).

As a result of the 1752insG mutation, the variable K1 end domain is predicted to be replaced by an aberrant sequence of 69 amino acids very rich in arginine and tryptophan residues that is 8 amino acids longer than the wild-type protein (**Fig 1***h*). The mutation eliminates two of the 10 glycine loops that are thought to be crucial for interactions of the K1 tail with proteins of the cornified cell envelope (Sprecher *et al*, 2001). These changes are likely to alter significantly the structural and chemical characteristics of the K1 tail.

A spontaneous deletion mutation (1635delG) in the V2 domain of K5 causes EBS Weber−Cockayne The second patient was a 25-y-old male of Ashkenazi−Jewish origin, who

has had a history of blister formation predominantly on palms, soles, and knees during infancy and childhood. No mucosal or nail involvement, no seasonal variation, and no palmoplantar keratoderma (PPK) were noticed. His skin fragility markedly improved with age, and at the time of the last skin examination at 25 y of age no skin lesions were observed. Histologic evaluation of lesional skin at the age of 7 y showed marked cytolysis of the basal cell layer, and electron microscopy confirmed intraepidermal separation above the basement membrane zone without evidence for keratin clumping. The initial clinical presentation, structural abnormalities of lesional skin, and course of disease were consistent with a very mild form of EBS Weber−Cockayne (Irvine and McLean, 1999). The patient's nonconsanguineous parents and his sister were unaffected (**Fig 2***a*).

DNA sequence analysis of the complete coding sequence of K5 disclosed in exon 9 a heterozygous guanine deletion at position 1635 downstream of the ATG start codon of K5 (1635delG) (**Fig 2***b*). This mutation introduces a frameshift and delayed stop codon 80 amino acids downstream of the mutation site (**Fig 2***c*). Restriction fragment analysis confirmed the mutation, which was not present in the patient's parents, his sister (**Fig 2***a*), and 50 unrelated, unaffected control individuals. Together with results confirming paternity (probability of paternity p = 0.99816 assuming a prior probability of 0.5), these observations demonstrate that 1635delG represents a new sporadic mutation. The deletion is predicted to lead to the translation of an aberrant K5 protein carrying an elongated tail domain (**Fig 2***c*).

DISCUSSION

A growing number of inherited disorders of the skin, nails, hair, mucous membranes, and other epithelial tissues have been recognized as resulting from germline or somatic mutations in 18 different keratin genes (Irvine and McLean, 1999). The majority of mutations result in replacement of conserved amino acid residues, disrupting the highly conserved boundaries of the central α-helical rod domain, the so-called helix initiation and termination motifs. These changes in turn perturb KIF alignment and assembly as evident by perinuclear KIF clumping. The mutant proteins destabilize the cellular KIF network, compromise cell integrity and mechanical strength, and eventually result in blistering and/or perturbed epithelial differentiation (Irvine and McLean, 1999). In general, these "hot spot" mutations are associated with typical and relatively severe clinical phenotypes. Recently, keratin gene mutations located outside the helix boundaries have drawn attention for their unusual clinical presentations, revealing unforeseen genotype−phenotype correlations. A recurrent mutation (P25L) in KRT5 affecting the variable head domain (V1) of K5 has been associated with EBS and mottled pigmentation of the skin (OMIM131960) (Uttam *et al*, 1996). Another mutation affecting the V1 domain of K16 was found in a mosaic form of epidermolytic PPK (Terrinoni *et al*, 2000). The phenotypic spectrum of dominant K1 mutations is even more puzzling. Whereas those affecting the K1 rod domain cause EHK, a missense mutation disrupting a conserved amino-terminal sequence motif (the so-called ISIS box), which has been implicated in K1-cornified envelope interactions (Steinert and Marekov, 1995), was found to segregate with diffuse, nonepidermolytic PPK (Kimonis *et al*, 1994). In contrast, a frameshift mutation (GG1609 → A) disturbing most of the K1 tail domain (Sprecher *et al*, 2001) was identified in a family with ichthyosis hystrix Curth−Macklin (MIM146590), a rare disorder characterized by nonepidermolytic hyperkeratosis and mutilating PPK (Curth and Macklin, 1954). The mutation was shown to impair KIF bundling and KIF-loricrin interactions (Sprecher *et al*, 2001). Another V2 frameshift mutation in a similar location (1628delG) was recently described in a family with striate PPK (Whittock *et al*, 2002). Although the molecular consequences of 1628delG differed little from those seen for GG1609 → A, the clinical phenotype was much less severe and
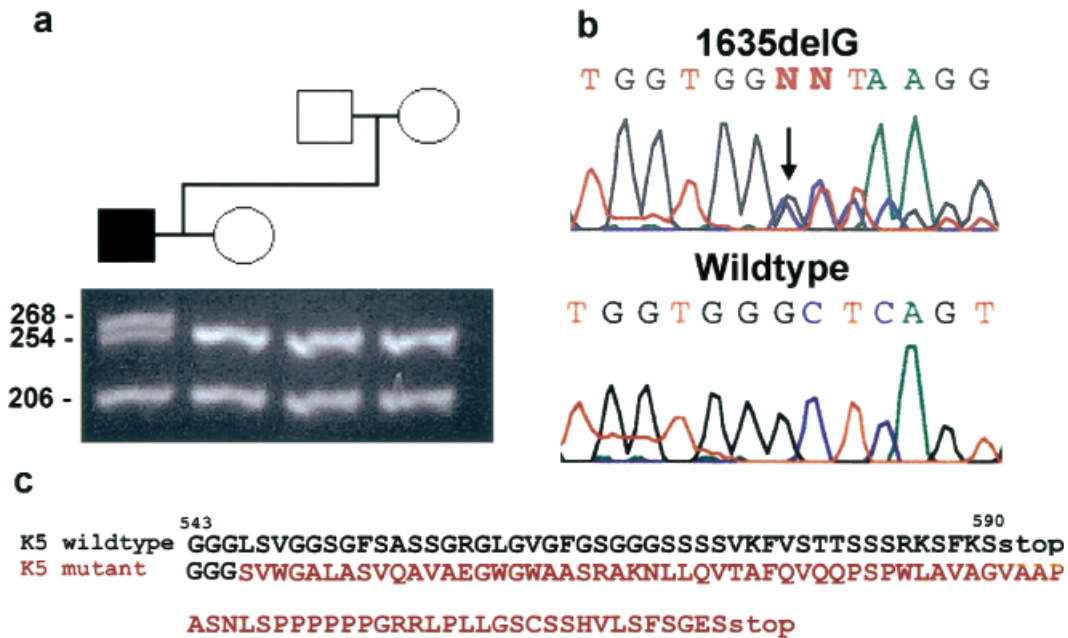
Figure 1. A mutation affecting the K1 tail causes atypical EHK. (*a*) Pedigree of the affected family. The filled symbol indicates the affected individual. (*b*) Thickened hyperkeratotic plaques with underlying erythema and fissuring on the palms of both hands. (*c*) Well-demarcated hyperkeratotic plaques with a cobblestone surface pattern over the right knee. Note several islands of superficial peeling (*arrows*). (*d*) Diffuse plantar keratoderma with prominent fissuring. (*e*) Thick, well-demarcated hyperkeratotic plaques with fine scales over the elbows. (*f*) Histopathologic examination reveals orthokeratotic hyperkeratosis, hypergranulosis, church-spire-like papillomatosis, and areas suggestive of vacuolar degeneration (*arrows*) (hematoxylin−eosin, 400 ×). A few binucleated cells are present in the granular layer (*insert*; 630 ×). (*g*) Mutation analysis of the proband reveals a G insertion at nucleotide position 1752 (*arrow*) in exon 9 of K1 (*central panel*) compared to the wild-type sequence in the mother (*right panel*) and father (*left panel*) of the proband. Sequence chromatograms depict the antisense strand because of the presence of a heterozygous 21 bp deletion polymorphism (Korge *et al*, 1992) upstream of the mutation. (*h*) Comparison of the amino acid sequence of wild-type (*black*) and mutant (*red*) K1 spanning the V2 domain. Numbers denote the amino acid positions. The recurrent glycine loop motifs (Korge *et al*, 1992) are shown in boxes.

ultrastructural studies revealed only a diminished density of KIF in the suprabasal epidermis. Whittock *et al* (2002) observed in both cases aberrant nuclear localization of KIF due to the loss of the wild-type V2 domain.

In this study, we report two new frameshift mutations perturbing the carboxy-terminal end of K1 or K5. The phenotype associated with 1752insG (K1) was distinct from the previously reported V2 mutations and instead broadly overlapped with mild EHK and IBS. Unusual features included the late age of onset and complete lack of skin blistering, whereas virtually all EHK patients present disease symptoms at or soon after birth (DiGiovanna and Bale, 1994). In contrast to mutations GG1609→A in ichthyosis hystrix Curth−Macklin and 1628delG in striate PPK that eliminate most of the K1 glycine loop motifs in V2, mutation 1752insG is located closer to the carboxy-terminus and leaves most of these motifs intact.

The 1635delG mutation reported here represents the first pathogenic alteration of the variable tail domain of K5. The clinical features were consistent with EBS Weber−Cockayne, although unusually mild and associated with complete regression during adulthood. These findings contrast with the severe phenotype observed in two unrelated EBS patients, who were found to carry nonsense mutations truncating K5 within the conserved L L E G E motif at the end of the rod domain (Muller *et al*, 1999; Livingston *et al*, 2001). In our case, the mutation did not involve rod sequences, which might explain the much milder phenotype. Nevertheless, the mutation is predicted to modify the tail domain of K5 into an aberrant and elongated peptide, which might interfere in a dominant negative manner with the protein function, as suggested by *in vitro* assembly experiments (Wilson *et al*, 1992).

Summarizing the four mutations affecting V2 tail domains of keratins observed to date, a recognizable pattern emerges. Disease-causing mutations of V2 are usually small insertions/deletions leading to frameshift and translation of an aberrant polypeptide, thus partially eliminating the normal tail. They are associated with a broad spectrum of differing, sometimes unusual,

Figure 2. A mutation affecting the K5 tail causes EBS. (*a*) Pedigree of the affected family. The *filled symbol* indicates the affected individual. Mutation 1635delG abolishes a recognition site for the endonuclease *Ban*II. PCR fragments amplified from exon 9 were digested with *Ban*II. A novel 268 bp fragment is observed in the affected patient only. (*b*) Mutation analysis. The proband was found to carry a heterozygous G deletion at position 1635 of KRT5 (*upper panel*). The wild-type sequence is shown for comparison (*lower panel*). (*c*) Comparison of the amino acid sequence of wild-type (*black*) and mutant (*red*) K5 spanning part of the V2 domain. Numbers denote amino acid positions. The 1635delG mutation leads to the generation of an aberrant and elongated K5 tail domain.

phenotypes, which, at least for now, escape prediction. Their specific location, the extent of the remaining wild-type sequence, and characteristics of the mutant peptide, as well as the specific functions of these tail regions that are compromised by mutations, are all likely to contribute to the phenotypic heterogeneity of keratin tail disorders. Pathogenic keratin gene mutations affecting keratin head and tail domains might be more prevalent than realized so far because of the common practice to screen solely for mutations in mutational hot spot regions. Thus, systematic screening of the entire coding sequences of candidate keratin genes seems warranted in patients presenting with a disorder of cornification and atypical clinical features.

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