Novel Splice Site Mutation in Keratin 1 Underlies Mild Epidermolytic Palmoplantar Keratoderma in Three Kindreds

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We report a novel mutation in the exon 6 splice donor site of keratin 1 (G4134A) that segregates with a palmoplantar keratoderma in three kindreds. The nucleotide substitution leads to the utilization of a novel in-frame splice site 54 bases downstream of the mutation with the subsequent insertion of 18 amino acids into the 2B rod domain. This mutation appears to have a milder effect than previously described mutations in the helix initiation and termination sequence on the function of the rod domain, with regard to filament assembly and stability. Affected individuals displayed only mild focal epidermolysis in the spinous layer of palmoplantar epidermis, in comparison with cases of bullous congenital ichthyosiform erythroderma also due to keratin 1 mutations, which show widespread and severe epidermolysis. This study describes a novel mutation in KRT1 that results in a phenotype distinct from classical bullous congenital ichthyosiform erythroderma. ***Key words: intermediate filaments/keratin genes/rod domain. J Invest Dermatol 116:606–609, 2001***

The palmoplantar keratodermas (PPKs) are a highly heterogeneous group of diseases that present as thickening of the palmoplantar epidermis. These disorders have previously been classified according to their phenotypic characteristics. The diffuse PPKs show uniform thickening of the skin on the palms and soles, compared to the different patterns of keratoderma seen in focal and punctate PPK. The two simple forms of diffuse PPK are epidermolytic PPK (EPPK: OMIM 144200) and nonepidermolytic PPK (NEPPK: OMIM 148400) (Hatsell and Kelsell, 2000). These two disorders are particularly difficult to separate when using only clinical findings. Histologically they differ, however, with EPPK showing epidermolysis of keratinocytes in the suprabasal layers, a characteristic not observed in NEPPK. In mild cases of EPPK, however, diagnosis can still be problematic as detection of epidermolysis can be difficult.

The genetic background of EPPK has been well studied since the first identified mutation in the palmoplantar specific keratin 9 (Reis *et al*, 1994). EPPK has so far been shown to be a homogeneous disease, with KRT9 mutations identified in the majority of patients investigated. The genetic basis of diffuse NEPPK is less well described, with only one mutation being identified in KRT1 as yet (Kimonis *et al*, 1994). Other families with diffuse NEPPK have been mapped outside the keratin cluster on 12q13, implying a non keratin background to the disease in several families from Sweden and the U.K. (Kelsell *et al*, 1999).

Keratins constitute the intermediate filament proteins of keratinocytes. Type I and type II keratins form specific heterodimers that are expressed in a differentiation and tissue specific manner (Moll *et al*, 1982). Many epidermal diseases have been associated with keratin mutations, which are proposed to disrupt the cytoskeleton of the cell leading to collapse of the cell and loss of adhesion (Corden and McLean, 1996).

The majority of KRT1 mutations have been identified in bullous congenital ichthyosiform erythroderma (BCIE). Specific mutations in keratin 1, however, have been implicated in disorders with different phenotypes. Here we describe three Scottish families in which a mild epidermolytic diffuse PPK segregates with microsatellite markers mapping to the type II keratin cluster on 12q13. Following the elucidation of the correct genomic structure of KRT1, a novel mutation in the 2B rod domain causing the insertion of 18 amino acids into the protein was detected in these families. This adds to the evidence that KRT1 mutations can result in a heterogeneous phenotype.

MATERIALS AND METHODS

Haplotype analysis using microsatellite markers Microsatellite analysis was performed by polymerase chain reaction (PCR), using one ^{32}P end-labeled and one unlabeled primer. The primer was labeled with 0.25 mCi (g-^{32}P)-dATP per pmol primer and T4 polynucleotide kinase. Genomic DNA (15 ng) was added to each PCR reaction, together with 1–5 pmol of each primer, 100 mM dNTP, Taq DNA polymerase buffer, and 0.15 units Taq DNA polymerase (Life Technologies, Gibco, Paisley,

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Abbreviations: BCIE, bullous congenital ichthyosiform erythroderma; EPPK, epidermolytic palmoplantar keratoderma; NEPPK, nonepidermolytic palmoplantar keratoderma; PPK, palmoplantar keratoderma.

Table I. Primer sequences for amplification of all exons of keratin 1

Exons amplified	Primer sequence 5′–3′
Exon 1	1F- ctgactctagaagaccaagc
	1R- actgcaccgatcccgagtgg
Exon 2	2F- ctccatggctatgagactcc
	2R- catgctgcttcatgatcttagc
Exons 3–4	3F- ttagaatgctggacttcagg
	4R- tctagatcgttgtgggttcc
Exons 5–6	5F- ggactgcttctctgtctaag
	6R- gaaagcactcgccctttacc
Exons 7–9	7F- tattggcctcactggagtgg
	9R- atccaggttagacagagccg
Exon 8 sequencing primer	8F- ggttaagtgcatggggaggc

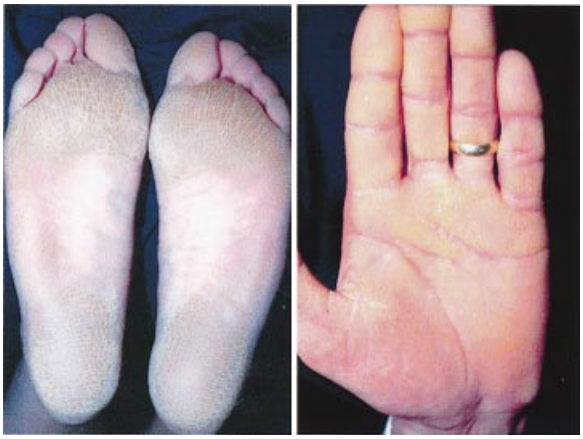

Figure 1. Clinical presentation of the disease, showing a diffuse keratoderma restricted to the palms and soles.

U.K.). The PCR program was one cycle of 95°C for 4 min, 55°C for 30 s, 72°C for 30 s, and then 95°C for 30 s, 55°C for 30 s, 72°C for 30 s during 26 cycles, followed by 95°C for 30 s, 55°C for 30 s, 72°C for 4 min. The PCR products were separated on 6% denaturing polyacrylamide gels and detected by autoradiography.

For further haplotype analysis between families, microsatellite markers were amplified by PCR with M13 tagged forward primers and a third M13 fluorescently labeled primer using standard cycling conditions. Products were visualized and analyzed using an LI-COR 4200 GeneReadIR automated sequencer (MWG, Milton Keynes, U.K.).

Electron microscopy A biopsy sample of clinically affected skin was processed for electron microscopy as described previously (Ishida-Yamamoto *et al*, 1992) with few modifications. Briefly, the samples were fixed first in phosphate-buffered gluteraldehyde/formaldehyde fixative, then in aqueous osmium tetroxide, dehydrated in ethanol, and embedded in epoxy resin (Taab Laboratories, Aldermaston, Berkshire, U.K.). Semithin sections were cut and stained with methylene blue and azure II for light microscopy and orientation purposes. Ultrathin sections were stained with lead citrate and uranyl acetate and viewed in a JEOL 100 CX transmission electron microscope, operating at 80 kV. Sections of normal epidermis were examined for comparative purposes.

Mutation detection The keratin 1 gene was amplified from genomic DNA using intronic primers (**Table I**), with conditions of 35 cycles of 95°C for 30 s, 60°C for 30 s, 72°C for 1 min, to obtain the full exonic sequence. PCR fragments were directly sequenced using the same primers.

Reverse transcriptase PCR (RT-PCR) RT-PCR was performed on total RNA extracted from a snap-frozen skin biopsy obtained from an affected individual using RNAzol, following the manufacturer's protocol (Biogenesis, Poole, U.K.). PCR products, using primers spanning exons 2–7, were amplified using GeneAmp RNA PCR kit (Perkin Elmer, Warrington, U.K.). These were subcloned using a Topo TA cloning kit (Invitrogen, Groningen, The Netherlands) and positive clones were sequenced.

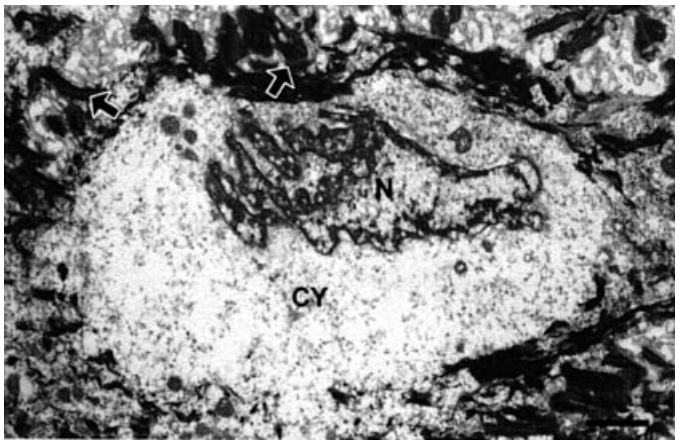

Figure 2. Electron microscopy shows early cytolysis in an epidermal keratinocyte. The nucleus (N) of a cell in the spinous layer of a patient with a KRT1 mutation has an irregular, notched surface and the perinuclear cytoplasm (CY) lacks the normal complement of tonofilaments. Tonofilament aggregates are present at the cell periphery, where they appear to associate with desmosomes (*arrows*). *Scale bar*: 2 μm.

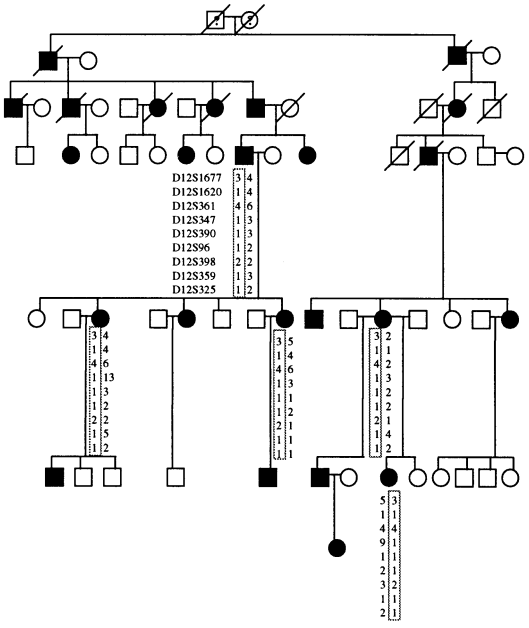

Figure 3. Affected individuals in pedigree 1 show a common haplotype for microsatellite markers spanning the type II keratin cluster on 12q13.

RESULTS

Clinical description of families Three Scottish pedigrees were studied, in which autosomal dominant inherited PPK was segregating. Two families were from Glasgow, and although no relationship was identified after 1830, their antecedents at this period shared an unusual surname, suggesting an earlier common ancestry. The third pedigree, from Ayrshire, was not known to be related. Affected family members displayed hyperkeratosis restricted to the palms and soles, but varying in severity, which had been present since childhood (**Fig 1**). In the most severely affected cases, there was confluent plantar keratoderma on weight-bearing surfaces, but sparing the instep. Also in severe cases, the edge of the normal skin bordering the keratoderma displayed a violaceous erythema. In milder cases there was patchy plantar keratoderma in

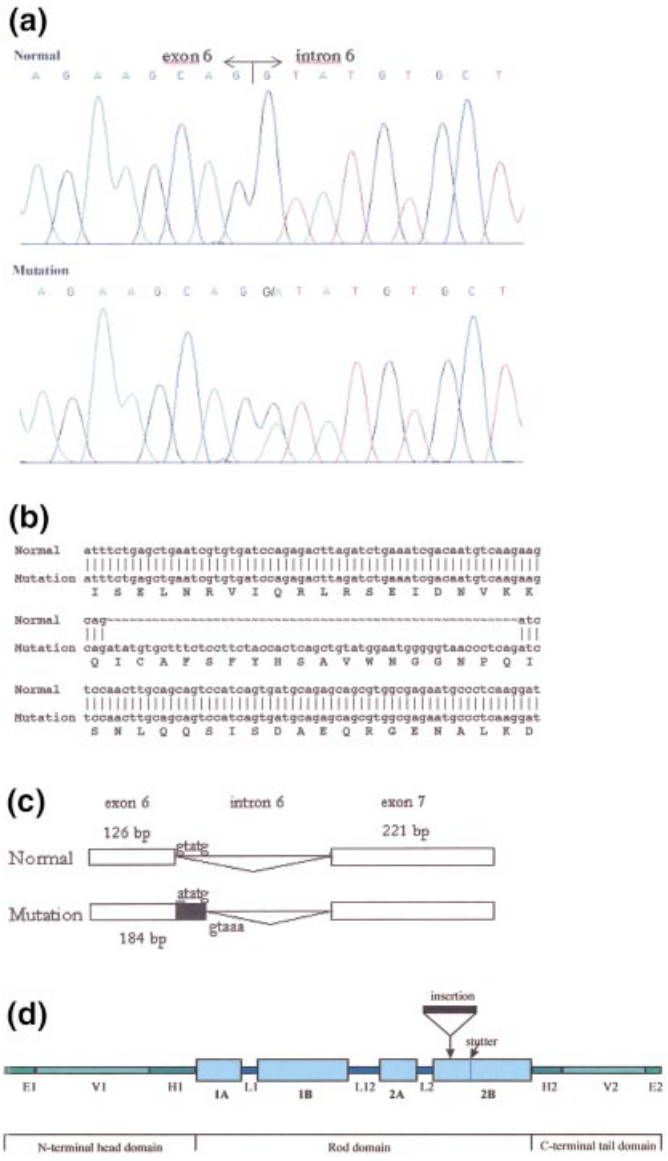

Figure 4. Identification of the G4134A mutation in keratin 1 and its effect on KRT1 mRNA. (*a*) Sequencing of keratin 1 revealed a heterozygous G–A substitution in the exon 6 splice donor site. (*b*) Alignment of cDNA sequence from an affected individual compared to normal; the mutation leads to the insertion of 54 nucleotides. (*c*) Schematic representation of abnormal splicing of exon 6 of KRT1, showing the mutated splice site and new utilized splice site. (*d*) Eighteen amino acids are inserted into the 2B rod domain of the keratin 1 molecule.

weight-bearing areas, resembling focal keratoderma. Confluent palmar keratoderma was seen only in manual workers, and even then was the exception; most cases had patchy keratoderma of the palmar midlines of the fingers and of pressure areas of the palm. There was no generalized transgradient keratoderma, but several individuals had developed marked local callosities on dorsal toes or fingers at sites of rubbing. The remaining skin looked entirely normal except for mild hyperkeratosis of the knees in one man. Nails, hair, and teeth were also normal, but another man complained of rapidly growing cuticles of the nailfolds.

Electron microscopy Although epidermolysis had not been identified by light microscopy in biopsies from three individuals, electron microscopy show that a minority of keratinocytes in the spinous and granular layers appeared to be undergoing cytolysis. The nuclei of affected cells had infolded nuclear membranes and the perinuclear cytoplasm was largely devoid of tonofilaments. Instead, the tonofilaments formed tight clumps or aggregates at the cell surface, often adjacent to desmosomes. These changes did not appear to affect clusters of adjacent cells, so the changes were less disruptive than those typically seen in bullous congenital ichthyosiform erythroderma (epidermolytic hyperkeratosis) where blistering is associated with extensive cell lysis (**Fig 2**).

Haplotype analysis The chromosomal regions harbouring the type I keratins on 17p21–q12 and type II keratins on 12q13 were investigated in pedigree 1, using microsatellite markers mapping to these regions. Six affected individuals from three generations of the family were screened. A common haplotype was seen in all six individuals for markers D12S1620, D12S361, D12S347, D12S270, D12S390, D12S96, D12S398, D12S359, and D12S325 spanning the type II keratin cluster (**Fig 3**). No linkage was seen for microsatellite markers mapping to 17q12–q21.

Identification of a novel splice site mutation in the 2B rod domain Having shown a common haplotype across the type II keratin cluster on 12q13 in all affected individuals investigated from pedigree 1, we carried out mutation analysis of the type II keratin, KRT1. Mutations in KRT1 have previously been associated with NEPPK and BCIE. KRT1 is expressed in the suprabasal layers of both palmoplantar and interfollicular epidermis. As discrepancies had previously been detected in the KRT1 genomic sequence (GenBank accession no. M77890), the gene was resequenced (GenBank accession no. AF304164) and primers were designed to amplify all exons (**Table I**).

Using PCR, each exon of KRT1 was amplified from genomic DNA of affected family members from pedigree 1. These PCR products were then directly sequenced. Affected individuals showed a single base substitution G–A in the exon 6 splice donor site, G4134A (with reference to GenBank accession no. AF304164) (**Fig 4*a***).

Two other families with diffuse PPK, from a similar region of Scotland, were screened and shown to harbour the same mutation. Genotyping of an affected individual in pedigree 2 showed the same genotype as in pedigree 1. This was not seen in an unaffected individual from the same pedigree. In pedigree 3, this common genotype only extended to D12S96, KRT1 maps between D12S96 and D12S390 (**Table II**). These data suggest that all three families may indeed have the same ancestral mutation. We were unable to obtain enough samples for haplotype analysis in these families, however. This variant was not found in 50 unrelated unaffected individuals. Also no mutation was found in KRT1 in two other families with diffuse PPK. The absence of a KRT9 mutation in the helix initiation and termination regions in these families further suggests heterogeneity in the diffuse PPKs; however, more investigations need to be carried out.

Prediction of the effect of the abolition of this splice site was made using a splice predictor program (http://genomic.sanger.ac.uk/gf/gf.html). This gave a potential splice site 54 bases downstream of the mutation with a probability of 0.81, compared with a probability of 0.90 for the wild type splice site. To confirm the effect of this splice site mutation on the KRT1 mRNA, RT-PCR was performed on total RNA extracted from a skin biopsy taken from an affected individual. Using primers spanning exons 2–7, two different sized alleles were amplified in the affected individual, which were subsequently subcloned and sequenced. This revealed the utilization of the predicted in-frame splice site (**Fig 4*b***, ***c***). Sequencing of further clones did not show the use of any other splice sites. This mutation results in the insertion of 18 residues into the 2B rod domain (**Fig 4*d***).

DISCUSSION

The novel mutation described results in the insertion of 18 amino acids into the rod 2B domain of the protein, which could disrupt the heptad repeat structure of the rod. This may have a detrimental

Table II. Genotype of individuals in a further two families at 12q13

Microsatellite marker	Pedigree 1[a]	Pedigree 2[b]		Pedigree 3[b]
		Affected	Unaffected	Affected
D12S347	**3**	**3** 3	2 2	1 2
D12S270	**2**	**2** 3	3 3	3 2
D12S390	**3**	**3** 2	2 2	1 2
D12S96	**2**	**2** 1	1 2	**2** 1
D12S398	**2**	**2** 2	2 2	**2** 1
D12S359	**5**	**5** 4	1 3	**5** 3
D12S325	**4**	**4** 1	1 3	**4** 1

[a]Common haplotype seen in **Fig 1.**
[b]Alleles shared with pedigree 1 shown in bold.

effect on the function of the rod domain, which is predicted to be important in intermediate filament assembly and stability.

Most previously described dominant KRT1 mutations result in BCIE, also known as epidermolytic hyperkeratosis (Cheng *et al*, 1992; Chipev *et al*, 1992; Rothnagel *et al*, 1992). In BCIE lesions usually appear within the first year of life as blistering, erythroderma that improves with age, and hyperkeratosis that generally persists and can deteriorate. Histologically, epidermolysis is seen in suprabasal cells with keratin clumping. This disorder can also be due to mutations in KRT10, but palmoplantar involvement is reported to occur more often in the KRT1 form (DiGiovanna and Bale, 1994).

The phenotype in these families is distinct from BCIE as they lack the generalized hyperkeratosis distributed over the entire body. Hyperkeratosis is restricted to the palmoplantar surfaces in all but one of the patients examined. This milder phenotype is reflected in the histologic findings, with only infrequent focal epidermolysis in the upper spinous layer, instead of the widespread epidermolysis of suprabasal cells common to BCIE.

The majority of mutations in KRT1 have been found in either the helix initiation or termination sequence of the rod domain. These regions are highly conserved among all keratins and other intermediate filament proteins. These sites are preferentially mutated in other keratin disorders, possibly occurring by CpG methylation and deamination, such as keratin 5 and 14 in Dowling–Meara epidermolysis bullosa simplex. Mutations in these regions tend to result in a more severe disease phenotype (Letai *et al*, 1992). Specific mutations in the helix termination sequence of keratin 1, however, are capable of causing distinct disorders, such as polycyclic erythematous (Michael *et al*, 1999) and psoriasiform lesions (Sybert *et al*, 1999) in conjunction with epidermolytic palmoplantar keratoderma.

A mutation in the V1 subdomain of KRT1, however, results in a distinct phenotype, being associated with NEPPK and limited hyperkeratosis of the areola, umbilicus, and knuckle pads of the dorsal aspects of the finger joints. This mutation changes residue lysine-72 to isoleucine, which is found in a highly conserved 22 residue sequence present in all type II keratins except KRT7 and KRT8 (Kimonis *et al*, 1994). This region has been shown to be essential for keratin filament attachment to the cornified cell envelope (Candi *et al*, 1998). A mutation in the L12 linker domain of the rod results in an atypically mild form of BCIE (Kremer *et al*, 1998).

This study supports the findings that mutations more central in the rod domain of keratin 1 do not disrupt the function of filament assembly and stability as much as those in the helix initiation and termination sequence. It also confirms that mutations in keratin 1 can cause a wide range of phenotypes other than classical BCIE, including PPK without obvious epidermolysis.

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