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Two Cases of Primarily Palmoplantar Keratoderma Associated with Novel Mutations in Keratin 1

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Mutations in keratin 1 were initially described in the classical form of bullous congenital ichthyosiform erythroderma (also known as epidermolytic hyperkeratosis). More recently the range of phenotypes associated with mutations in this gene has been extended to include annular ichthyosiform erythroderma and mild epidermolytic palmoplantar keratoderma. Here we present two novel mutations in the keratin 1 gene (*KRT1*): a 5′ donor splice site mutation in exon 1 (591 + 2T > A) that predicts a 22 amino acid in-frame deletion in the keratin 1 1A domain; and an in-frame deletion in exon 7 (1376del24) that predicts a foreshortened 2B coiled-coil domain of keratin 1. In each case these mutations are associated with palmoplantar keratoderma and mild ichthyosis, largely limited to the flexural areas. These mutations appear to have a less damaging effect than previously reported mis-sense mutations sited in the helix boundary motifs. This report extends the range of phenotypes associated with mutations in *KRT1*. ***Key words: disorder of keratinization/genetics/genodermatosis/intermediate filaments/mutation. J Invest Dermatol 119:966–971, 2002***

The traditional classification of palmoplantar keratodermas (PPK) has been by morphologic appearance, i.e., diffuse, focal, or punctate, and the presence or absence of extracutaneous features (Stevens *et al*, 1996). Recent advances in understanding the molecular basis of these disorders have facilitated a molecular-based classification (Irvine and Paller, 2002). Keratins are structural proteins found in all epithelial cells (Lane, 1995). They are divided into type I (K9–20) and type II (K1–8) keratins, and are normally expressed in pairs consisting of one of each type in a tissue- and differentiation-specific manner. Specific pairs of type I and type II keratins form heteropolymeric 10 nm intermediate filaments, which serve to protect epithelial cells from physical damage (Irvine and McLean, 1999). In the highly specialized palmoplantar epidermis the keratin expression pattern is tightly regulated with specific expression of keratins K9, K16, and K17 (Swensson *et al*, 1998). K1 is the putative expression partner of both K9 and K10 in the palmoplantar epidermis and the partner of K10 in interfollicular epidermis in nonglabrous skin. Pathogenic mutations in keratin genes are now known to underlie a range of human epithelial fragility disorders with the majority of mutations occurring in the helix boundary motifs, highly conserved regions at either end of the rod domain (Irvine and McLean, 1999). Following the first reports of mutations in the suprabasal cytokeratin K1 and its expression partner K10 in patients with classical bullous congenital ichthyosiform erythroderma (BCIE; also known as epidermolytic hyperkeratosis, EHK) (Cheng *et al*, 1992; Chipev *et al*, 1992; Rothnagel *et al*, 1992), a diverse range of subtly different phenotypes has been ascribed to mutations in K1. PPK is a more prominent feature of the BCIE patients with mutations in K1 than those with K10 mutations (DiGiovania and Bale, 1994), possibly because K1 is the major expression partner of K9 in palmoplantar epidermis. K1 mutations have also been shown to underlie the annular ichthyosis variant of BCIE (Michael *et al*, 1999; Sybert *et al*, 1999), and a mutation in K1 has been associated with nonepidermolytic PPK in a single family (Kimonis *et al*, 1994). More recently a novel splice site mutation leading to the insertion of 54 nucleotides into the K1 2B domain was shown to cause a mild epidermolytic PPK (Hatsell *et al*, 2001). At the time of writing a total of 23 different mutations have been reported in K1 (Cassidy *et al*, 2002). Here we report two novel mutations in K1, each associated with a well-demarcated diffuse PPK and very limited involvement elsewhere. This report extends the knowledge of genotype–phenotype correlation in K1 disorders.

MATERIALS AND METHODS

K1exon 7 mutation detection and confirmation Buccal cell DNA was used to amplify a 390 bp fragment of the *KRT1* gene encompassing all of exon 7 (collected and prepared using the GeneDx Buccal Cell collection kit as previously described; Richards *et al*, 1993). Amplification consisted of one cycle at 95°C, 15 min, 37 cycles of 94°C, 30 s, 62°C, 60 s, 72°C, 90 s, and a final cycle at 72°C, 5 min. Reactions contained 1 µl buccal DNA, 200 nM each primers K1ex7F and K1ex7R (**Table I**), 0.2 nM deoxyribonucleoside triphosphate, 0.15 U HotStart Taq polymerase and 1 × polymerase chain reaction (PCR) buffer (Qiagen, Valencia, CA). The amplified fragments were gel-purified through 2% agarose (Seakem LE, BioWhittaker, North Brunswick, NJ) and isolated using EZNA DNA column purification (Omega-Bio-Tek,

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Abbreviations: dHPLC, denaturing high-pressure liquid chromatography; PPK, palmoplantar keratoderma; EHK, epidermolytic hyperkeratosis

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Table I.

Primer	Sequence: 5′–3′
K1ex7F	CTCACTGGAGTGGGGAGACC
K1ex7R	AAGGAAGGACCAGGGGAAGAG
K1e1.F	GAGGAGTGTTTAGCTCCTTCCCTT
K1e1.R	CTACTGTGTTTTGACTGCACCAATC
K1-H1	ATGGTCCTGTCTGCCCTCCT
K1sp1	GCACCATGCCTGTCTGCCCTCCTGGTGG
K1e2.R	CATGCTGCTTCATGATCTTAGC
T7.F	TAATACGACTCACTATAGGG

PCR Primers used in this study.

Doraville, GA). An aliquot was subjected to simultaneous bidirectional sequencing using IRD-700 and IRD-800 dye-labeled M13 oligonucleotide primers and the Dyenamic sequencing system (AP Biotech, Piscataway, NJ). Sequence reactions were analyzed on a LI-COR dual-dye DNA Analyzer.

To confirm the deletion of 24 bp from one allele of exon 7 of the patient's *KRT1* gene, the 390 bp DNA fragment was digested with the restriction endonuclease *StyI* and analyzed on 3% agarose (3 : 1 Nusieve, BioWhittaker). Only the expected pair of bands from a normal *KRT1* allele was seen in control DNA (208 bp, 182 bp), whereas an additional 158 bp band was observed in DNA from the affected patient (data not shown).

K1exon 1 5′ donor splice site mutation detection and confirmation Genomic DNA from the affected families and 50 unaffected local population controls was obtained from peripheral blood lymphocytes using the Nucleon DNA Extraction Kit (Amersham Pharmacia Biotech, Little Chalfont, Bucks, U.K.).

Two hundred nanograms of genomic DNA was used to amplify a 723 bp fragment of the *KRT1* gene, including exon 1 and the intron 1 5′ donor splice site, using 1 μM each of primers K1e1.F and K1e1.R (**Table I**), 0.25 mM of each deoxyribonucleoside triphosphate and 1 U *Taq* polymerase (Promega, Madison, WI) in 1 × NH_4 PCR Buffer (Bioline Ltd, London, U.K.) supplemented with 1.5 mM $MgCl_2$ and 4% dimethyl sulfoxide. Following DNA denaturation at 94°C for 5 min, PCR was performed for 35 cycles of 94°C, 30 s; 55°C, 45 s; and 72°C, 90 s; with a final 5 min extension at 72°C. PCR products were purified using QIAquick PCR purification method (Qiagen, Crawley, U.K.) prior to DNA sequencing on an automated ABI 377 DNA sequencer (Applied Biosystems, Perkin Elmer, Foster City, CA) using an internal primer, K1-H1 (**Table I**).

In this case the mutation was confirmed by denaturing high-pressure liquid chromatography (dHPLC). PCR with primers K1e1.F and K1e1.R was performed using HotStart *Taq* polymerase (Qiagen) as recommended by the manufacturer; otherwise conditions were as stated above. Following PCR, DNA fragments were subjected to denaturation at 95°C for 5 min and were renatured in 70 cycles of 95°C for 22 s cooling by 1°C per cycle to allow heteroduplex formation. dHPLC was carried out using the Wave System (Transgenomic, Crewe, U.K.) using 6–10 μl of each PCR product at 60°C melting temperature for the 723 bp K1e1F-R fragment sequence, as predicted with the WaveMaker program (Transgenomic). Any sample that showed a different elution profile compared with a normal *KRT1* control was sequenced.

Splicing assay system In order to demonstrate the effect of the *KRT1*-591 + 2T > A mutation at an mRNA level we developed an *in vitro* splicing assay system based on transfection of *KRT1* genomic DNA constructs into HaCaT cells (Boukamp *et al*, 1988). A 1466 bp fragment of *KRT1* gene, including part of exon 1, intron 1, and exon 2, which corresponds to H1, 1A, and part of L1 motifs of the K1 protein, was amplified from genomic DNA of the affected father in family 2. PCR was performed using 0.5 μM K1sp1 forward primer (which includes the initiation codon and Kozak sequence to enhance expression), and 1 μM K1e2.R reverse primer, 0.25 mM of each deoxyribonucleoside triphosphate and 0.5 U *Taq* polymerase (Promega) in 1 × NH_4 buffer (Bioline) with 1.5 mM $MgCl_2$ and 4% dimethyl sulfoxide. Following DNA denaturation at 94°C for 5 min, PCR was performed for 30 cycles of 94°C for 1 min, 59°C for 1 min 30 s, and 72°C for 2 min, with a final 5 min extension at 72°C. One microliter of the PCR product was cloned into eukaryotic expression vector pCR3.1 (Bidirectional Eukaryotic TA cloning kit, InVitrogen, Paisley, U.K.) and clones carrying the GT wild-type and GA mutant *KRT1* fragments were selected. Wild-type and mutant plasmids were isolated using Qiagen Plasmid Maxi Kit. The HaCaT keratinocyte cell line was transfected with GT wild-type and GA mutant plasmids using nonliposomal FuGENE-6 Reagent (Roche, Lewes, U.K.). mRNA from wild-type and mutant transfected cells was extracted 24 h after transfection using QuickPrep Micro mRNA Purification Kit (Amersham-Pharmacia). mRNA was treated with RNAse-free DNAse I (Promega) and phenol/chloroform extracted, prior to reverse transcription with oligo(dT) and AMV reverse transcriptase (Promega).

One microliter of each wild-type and mutant cDNA was amplified by nested reverse transcription–PCR; 0.5 μM T7.F and 1 μM K1e2.R primers were used in the first round of amplification (PCR performed for 42 cycles at 52°C annealing temperature, otherwise as stated above). One microliter of each 1 : 500 diluted PCR product was used in a second round of amplification using 0.5 μM exon 1 internal primer K1sp1 and 1 μM K1e2.R. PCR was performed as above for 30 cycles and an annealing temperature of 59°C. Nested PCR products were sequenced using primer K1sp1. PCR primers are listed in **Table I**.

RESULTS

Clinical description of families Two families were studied. In family 1 a single affected child was born to unaffected parents. At birth she was noted to have a symmetric diffuse PPK but there was no history of skin fragility or blistering, even in the neonatal period. Until 6 y of age, the phenotype consisted only of the PPK. When seen at 8 y of age, diffuse PPK with some superficial scale was noted (**Fig 1*a***). In addition, however, the patient showed fine scaling over the lateral and anterior neck, lower back, external ears, and axillae (**Fig 1*b***).

The second family comprised an affected father and an affected daughter. In each case, a mild diffuse PPK was observed at birth, there was no history of neonatal fragility or blistering. The father, now aged 34, had persistent mild diffuse PPK throughout life and mild flexural-limited scaling. His daughter has a similarly mild presentation, with involvement limited to the palms and soles, popliteal fossae, and axillae (**Fig 1*c,d***).

Identification of a novel deletion mutation in exon 7 of *KRT1* in family 1 Bidirectional sequence of *KRT1* exon 7 demonstrated a heterozygous in-frame deletion of 24 bp (**Fig 2**) that was confirmed by size analysis on agarose gel electrophoresis. This mutation, designated 1376del24, causes deletion of eight residues of the K1 protein midway between the helix 2B stutter sequence and the beginning of the helix termination motif (delA459-Q466).

Identification of a novel splice site mutation in *KRT1* and demonstration of a 22 amino acid deletion in the H1 and 1A domains of K1 in family 2 Sequencing of genomic DNA revealed a heterozygous T > A substitution at base position 591 + 2, this mutation disrupts the *KRT1* exon 1 donor splice site (**Fig 3**). With this type of splicing defect, it is desirable to determine the consequences at the mRNA level by reverse transcription–PCR of keratinocyte or epidermal-derived cDNA; however, biopsy material was not available, so in this case an *in vitro* splicing assay was developed. Partial genomic DNA constructs consisting of exons 1 and 2 of *KRT1*, with or without the splice site mutation were expressed in HaCaT cells. reverse transcription–PCR and direct sequencing of *KRT1* cDNA from cells transfected with the wild-type construct showed normal splicing of exon 1 to exon 2 (**Fig 3**). In contrast, direct sequencing of mutant reverse transcription–PCR products revealed a 66 bp deletion due to activation of an upstream cryptic donor splice site. This deletion results in removal of 22 amino acids from the H1 and 1A domains of the K1 polypeptide (delV175-K196); schematically represented in **Fig 3**.

Confirmation of K1 exon 1 splice site mutation and population screening The *KRT1* mutation 591 + 2T > A was confirmed using the Wave Transgenomic dHPLC System. Heteroduplexes formed by the presence of either GT or GA in the wild-type and mutant PCR fragments, respectively, were detected

in the affected father by dHPLC (**Fig 4*a***). Fifty DNA samples from an unaffected local population were screened to exclude this mutation by dHPLC. This mutation was not detected in 100 alleles examined by this technique (examples in **Fig 4*b–d***).

DISCUSSION

Mutations in K1 are now known to underlie a number of clinically distinct phenotypes, including classical BCIE/EHK, cyclic ichthyosis with EHK, striate PPK, diffuse nonepidermolytic PPK, and, in a single family, ichthyosis hystrix of Curth–Macklin. All mutations in K1 and associated phenotypes reported in peer-reviewed journals are listed in **Table II**. The subclassification of these phenotypes is not consistent throughout the literature; MIM numbers and entries can also lack clarity (McKusick, 2002). In a detailed review of 21 families with BCIE/EHK DiGiovania and Bale (1994) proposed a classification of the EHK phenotype into two broad categories based on the presence (PS) or absence (NPS) of palm and sole involvement. The PS group was further divided into PS-1 (largely limited involvement to palms and soles, hyperkeratosis surrounded by a red halo), and PS-2 (extensive involvement, with neonatal blistering). A single case did not fit easily into either of these categories and was classified as PS-3. The DiGiovania and Bale classification is useful and is applicable to most cases of EHK. To some extent it is possible to predict the molecular defect from the clinical phenotype: PS phenotypes are usually due to mutations in *KRT1*, and NPS phenotypes are usually due to mutations in *KRT10*. Despite this usefulness, the classification has not been widely adopted and many authors reporting mutations in K1 or K10 do not designate patients to either group. Here we have listed all mutations in K1 to date along with the associated clinical phenotype and PS classification where designated by the authors (**Table II**). It can be seen that the great majority of severe PS-2 cases is caused by point mutations in the helix initiation or termination motifs and that milder PS-1 cases are usually caused by point mutations outside of these sites. More unusual mutations such in the variable domains have been associated with distinct phenotypes (ichthyosis hystrix of Curth–Macklin and nonepidermolytic PPK) in single families. A single insertional mutation was associated with a mild PS-1 phenotype (Hatsell *et al*, 2001).

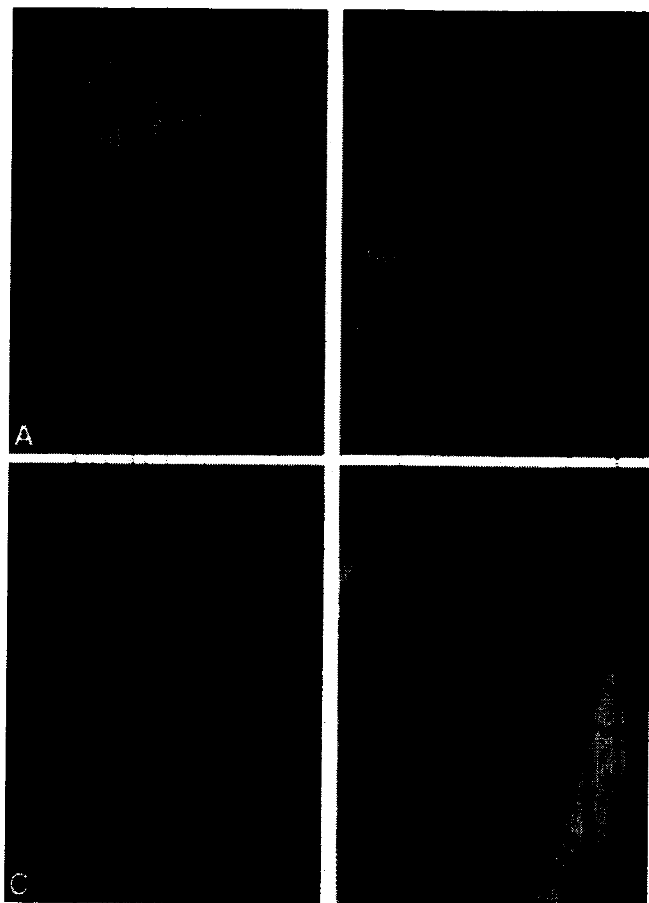

Figure 1. Clinical presentation. (a) Diffuse, fine PPK with some superficial scale and (b) mild ichthyosis in the affected child from Family 1. (c) Diffuse, well-circumscribed smooth PPK and mild, scaling limited to flexures in the affected child from Family 2.

Using the DiGiovania and Bale (1994) classification our second family would be classified as PS-1. The affected member of the first family is probably best classified as PS-1, although the later onset of mild ichthyosis is unusual. We report two novel mutations, both of which result in a deletion of part of the K1 polypeptide. Deletion mutations have been reported in other keratin genes, including a 24 bp deletion in the 2B domain of K16 causing a very mild focal nonepidermolytic PPK phenotype (Smith *et al*, 2000). In this case, expression of normal and mutant polypeptides in cultured epithelial cells revealed that the deleted keratin was less disruptive to the endogenous keratin network than "classic" keratin mis-sense mutations in the helix boundary motifs. It was postulated that loss of this critical protein motif that is known to be involved in end-to-end overlap interactions during filament assembly (Steinert *et al*, 1993) rendered the mutant protein less capable of exerting dominant-negative effects. A similar mechanism might explain the milder phenotype seen in patients here with deletions at either end of the K1 rod domain. More recently, a mild phenotype (PS-1 according to DiGiovania and Bale (1994) has been attributed to a splice site mutation in K1 that results in the insertion of 18 amino acids into the 2B domain (Hatsell *et al*, 2001) and in these families the phenotype was again limited to the palms and soles. In this case, it is possible that disruption of the alignment of heptad repeats within the 2B domain impairs the assembly competence of the mutant protein, thereby lessening dominant-negative effects. Alternatively, the inserted sequence, which is presumably unable to contribute to dimerization, may render mutant dimers more susceptible to degradation. Interestingly, a splice site mutation in K5, quite similar to the mutation in family 2 here, caused an in-

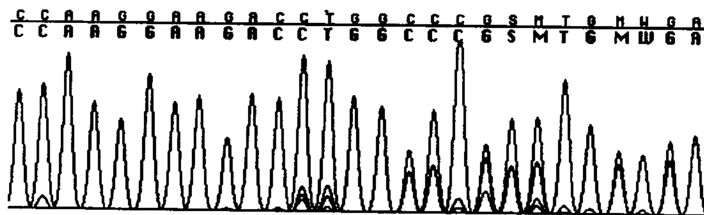

Figure 2. Identification of mutation 1376del24 in *KRT1*.Direct sequencing of **KRT1** exon 7 PCR products derived from the proband in Family 1 revealed a heterozygous deletion mutation 1376del24 which removes residues 459–466 from the K1 polypeptide in the region of the helix termination motif.

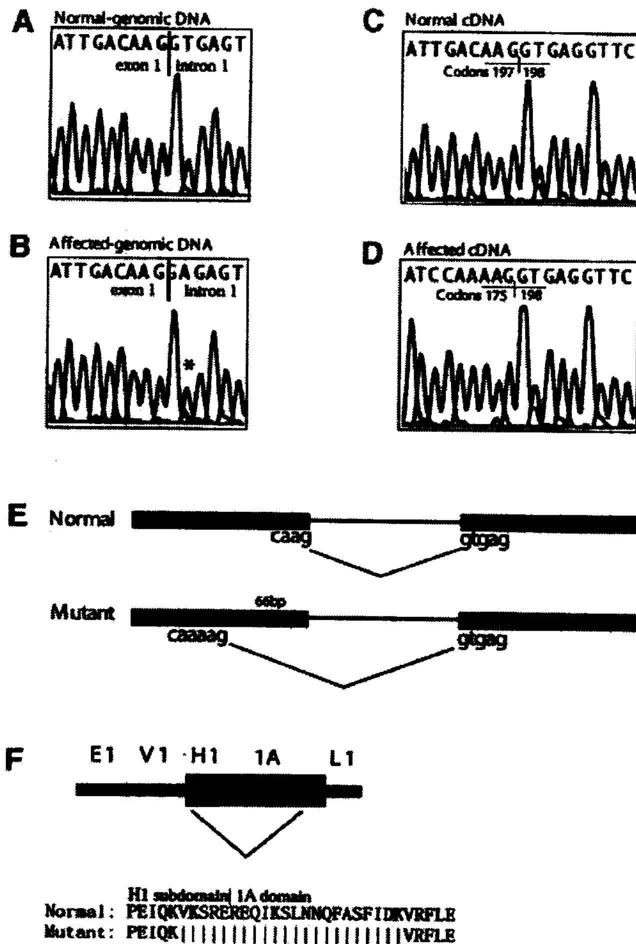

Figure 3. Identification of the 591 + 2T > A mutation in *KRT1* and demonstration of the effect on mRNA splicing and the resultant protein translation.(a & b) Direct sequencing of genomic PCR products from exon 1 of the *KRT1* gene. (a) Normal control sequence. (b) Sequence derived from the proband in Family 2 revealing a heterozygous transversion mutation in the 5′ donor splice site of exon 1, designated 591 + 2T > A (asterisk).(c & d) Direct sequencing of K1 reverse transcription–PCR products derived from HaCaT cells transfected with *KRT1* genomic expression constructs. (c) Normal splicing of exon 1 to exon 2 was observed with the wild-type construct. (d) In transfections of the 591 + 2T > A mutant construct, aberrant splicing was observed resulting in a 66-bp deletion(e). Schematic representation of normal and mutant splicing of exons 1 & 2 of the *KRT1* gene. As a consequence of this deletion, the last 5 amino acids are removed from the H1 subdomain and the first 17 residues are lost from the coil 1 A domain of the K1 polypeptide.(f). Position of the 22 amino acid deletion in the K1 polypeptide.

frame removal of the identical 22 amino acids in the H1 and 1A domains of K5. This K5 mutation, however, resulted in the severe Dowling–Meara form of epidermolysis bullosa simplex (Rugg *et al*, 1999) rather than a milder form of the disease analogous to the K1 phenotype observed here. Similarly, truncation of the K14 polypeptide immediately after the helix termination motif was found to produce a severe Dowling–Meara epidermolysis bullosa simplex phenotype (Muller *et al*, 1999). It should be remembered, however, that there are more keratins expressed suprabasally and, therefore, dilution of dominant negative effects may take place in the upper epidermis.

In conclusion, larger deletions that completely remove a keratin helix boundary motif might be expected to have a milder dominant-negative effect due to loss of these critical molecular

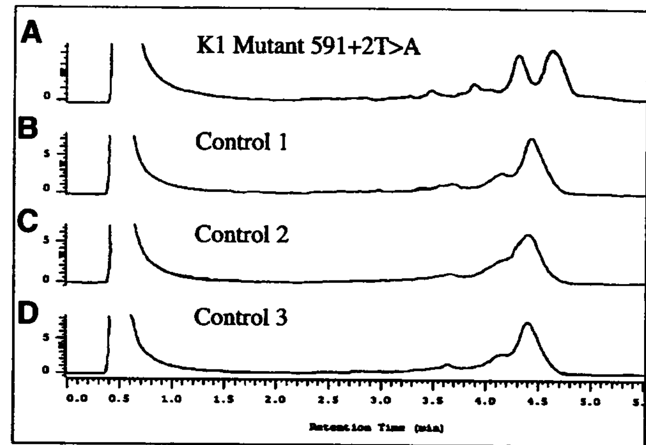

Figure 4. Confirmation and population screening of *KRT1* mutation 591 + 2T > A by dHPLC. Detection of the K1 splice site mutation 591 + 2T > A by dHPLC (Transgenomic Wave System) in the affected father from Family 2 (a)and exclusion from unaffected local population control DNAs (b–d).

overlap regions. Notwithstanding, the prediction of phenotype based on genotype is highly complex in keratin disorders. The precise nature of the mutation (mis-sense *vs* insertion/deletion and the affected protein domain) and as yet undefined genetic and environmental factors in the affected individual all contribute to the observed clinical phenotype.

We would like to thank the patients and their families for participation in this study. Thanks also to Linda Campbell, Molecular Genetics Laboratory, Ninewells University Hospitals NHS Trust for genomic DNA extraction and Andrew J. Cassidy, Molecular Genetics Analysis Facility, Department of Molecular and Cellular Pathology, Ninewells Medical School, Dundee, for DNA sequencing. W.H.I.M. is funded by a Wellcome Trust Senior Research Fellowship and A T was also supported by the Dystrophic Epidermolysis Bullosa Research Association (DEBRA) U.K. (to W.H.I.M.).

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Table II. Mutations reported in K1 in peer-reviewed journals

Mutation/ nucleotide	Amino acid	Domain	Neonatal blistering	Pattern of EHK	Diffuse PPK	MIM no.	Reference
221A>T	K74I	V1 (ISIS box)	No	Mild hyperkeratosis of nipple and umbilicus Mild involvement of knuckle pads, knees and elbows	Moderate to severe, extension along Achilles tendon	Previously 148400, incorporated into 144200	Kimonis *et al* (1994)
464T>G	V155G	H1	NK	Mild, truncal sparing [PS-1]	Yes, digital contractures	113800	Yang *et al* (1994)
464T>A	V155D	H1	Yes	Mild with flexural accentuation	Yes	113800	Whittock *et al* (2001)
482T>C	L161P	HI	Yes	Mild with flexural accentuation	Yes	113800	Chipev *et al* (1992)
536G>A	R179P	H1	NK	Moderate–severe, generalized [Designated PS-1/PS-2]	Yes	113800	Yang *et al* (1996)
591+2T>A	delV175–K196	H1/1A	No	Mild flexural hyperkeratosis	Yes		This report
556A>G	S186P	1A	Yes, severe	Severe, generalized	Yes	113800	McLean *et al* (1994)
563A>C	N188T	1A	Yes, severe	Severe, generalized	Yes	113800	Arin *et al* (1999)
563A>G	N188S	1A	NK	Severe, generalized [PS-2]	Yes, digital contractures	113800	Yang *et al* (1994)
563A>G	N188S	1A	Yes, severe	Severe, generalized	Yes	113800	McLean *et al* (1994)
563A>G	N188S	1A	Yes in patient with generalized disease	Nevoid BCIE/EH Severe in patient with germline mutation	Yes	600648	Nomura *et al* (2001)
577T>C	S193P	1A	NK	Severe, generalized [PS-2]	Yes, digital contractures	113800	Yang *et al* (1994)
641T>C	L214P	1A	NK	Severe, generalized	Yes	113800	Cserhalmi-Friedman *et al* (2000)
1019A>T	D340V	L12	Yes	Mild, generalized scaling [Designated PS-1]	Yes	113800	Kremer *et al* (1998)
IVS6+1G>A	I419ins18	2B	No	Mild hyperkeratosis of knees in one individual	Yes	NSN	Hatsell *et al* (2001)
1432G>T	E478D	2B	Red and scaling at birth	Mild, diffuse ichthyosis, flexural accentuation Flaccid blisters [PS-1]	Yes	113800	Yang *et al* (1999)
1436T>C	I479T	2B	Yes	Severe, generalized		113800	Arin *et al* (2000)
1445A>G	Y482C	2B	Yes	Severe, generalized	Yes	113800	Syder *et al* (1994)
1468G>C	E490Q	2B	Yes	Mainly flexural	Yes	113800	Rothnagel *et al* (1992)
1376del24	del459–466	2B	No	Mild generalized hyperkeratosis	Yes		This report
1436T>C	I479T	2B	Yes	Relatively mild CI-EH	Yes	NSN	Sybert *et al* (1999)
1435A>T	I479F	2B	Yes	Mild, flexural in some family members (CI-EH)	Yes	NSN	Sybert *et al* (1999)
1435A>T	I479F	2B	Yes	Epidermolytic hyperkeratosis with polycyclic psoriasiform plaques, similar to CI-EH	Yes	NSN	Michael *et al* (1999)
1628delG	V543fsX613	V2	No	None, limited to palms and soles	Striate PPK	NSN	Whittock *et al* (2002)
1609–1610delGGinsA	G537fsX613	V2	No	Ichthyosis hystrix of Curth–Macklin	Severe with pseudoainhum	146590	Sprecher *et al* (2001)

CI-EH, cyclic ichthyosis with epidermolytic hyperkeratosis; NSN, no specific number; NK, not known from published report; PS-1/PS-2 designation as from DiGiovania and Bale (1994). Note: Mutations in this table are classified according to GenBank accession no. 006121, in some cases this means that the specific assignment here is different from that reported in the original paper.

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