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15 March 2005

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Exp Dermatol 2000: 9: 170–177
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EXPERIMENTAL DERMATOLOGY
ISSN 0906-6705

Novel keratin 16 mutations and protein expression studies in pachyonychia congenita type 1 and focal palmoplantar keratoderma

Smith FJD, Fisher MP, Healy E, Rees JL, Bonifas JM, Epstein Jr EH, Tan EML, Uitto J, McLean WHI. Novel keratin 16 mutations and protein expression studies in pachyonychia congenita type 1 and focal palmoplantar keratoderma
Exp Dermatol 2000: 9: 170–177. © Munksgaard, 2000

Abstract: Pachyonychia congenita type 1 (PC-1) is an autosomal dominant ectodermal dysplasia characterized by nail dystrophy, focal non-epidermolytic palmoplantar keratoderma (FNEPPK) and oral lesions. We have previously shown that mutations in keratin 16 (K16) cause fragility of specific epithelia resulting in phenotypes of PC-1 or FNEPPK alone. Here, we report 2 novel mutations in K16 causing distinct phenotypes. A heterozygous missense mutation (L124R) was detected in a kindred with PC-1. In a family where mild FNEPPK was the only phenotype, a 23 bp deletion and a separate 1 bp deletion downstream were found in exon 6: [1244–1266del; 1270delG]. At the protein level, these mutations remove 8 residues and substitute 2 residues in the helix termination motif (HTM) of the K16 polypeptide. The HTM sequence is conserved in all known intermediate filament proteins and for convenience, this complex mutation was designated ΔHTM. Transient expression of K16 cDNAs carrying either the L124R or the ΔHTM mutation in epithelial cell line PtK2 produced aggregation of the keratin cytoskeleton. However, the aggregates observed with the ΔHTM mutation were morphologically different and appeared to be less disruptive to the endogenous cytoskeleton. Therefore, loss of the HTM sequence may render this mutant K16 less capable of contributing to filament assembly and decrease its dominant-negative effect, resulting in the milder FNEPPK phenotype.

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Key words: pachyonychia congenita – keratin – mutation – intermediate filaments – keratoderma – genodermatosis

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Accepted for publication 2 June 1999

Introduction

Keratins form the intermediate filament cytoskeleton of epithelial cells. In humans, different epithelial tissues express certain combinations of keratins according to tissue site and differentiation state (1). In recent years, transgenic experiments (2) and studies of human keratin diseases characterized by fragility of epithelial tissues, have demonstrated that the primary function of the keratin cytoskeleton is to resist traumatic damage to epithelial cells, reviewed in (3, 4). Mutation of a keratin can lead to a defective cytoskeleton, causing trauma-induced lysis of cells which differentially express that protein. Phenotypically, there is blistering or fragility of epithelial structures and hyperkeratosis of the affected and/or nearby epithelia.

Keratins share the domain organization of all other intermediate filament proteins, consisting of a central coiled-coil rod domain of about 310 amino acids, which mediates polymerization (5). The rod domain is flanked by globular V1 and V2 domains which vary widely between keratins and which are thought to contribute to the tissue-specific functions of a particular keratin cytoskeleton

(5, 6). Keratins form obligate heterodimers consisting of a type I and a type II keratin (7, 8), which are usually co-expressed in specific pairs (1). The process by which dimers assemble into 10 nm filaments remains obscure. However, two short peptides located at the start and end of the rod domain, termed the helix boundary motifs, show a remarkable degree of evolutionary conservation (5) and have been implicated in end-to-end interactions in the assembly process (9, 10). In the skin blistering disorder *epidermolysis bullosa simplex* (11), missense mutations in the helix boundary motifs of K5 or K14 produce aggregation of the cytoskeleton as seen by electron microscopy, resulting in severe disease phenotypes (12–14). In contrast, mutations elsewhere in the molecule produce no visible effect on the cytoskeleton and result in mild phenotypes (15–18). In other keratins, a total of 17 of which are now linked to human genetic disorders, most mutations have been confined to the helix boundary peptides, attesting to the structural importance of these sequences (3, 4, 19–24).

Pachyonychia congenita describes a collection of autosomal dominant ectodermal dysplasias with hypertrophic nail dystrophy (25). Gorlin recognized 2 main clinical variants (26): type 1 (PC-1) with oral leukokeratosis (27) and type 2 (PC-2), with multiple pilosebaceous cysts (28). Focal non-epidermolytic palmoplantar keratoderma (FNEPPK) is a feature of both types. The type I keratin K16 and type II keratin K6a are co-expressed in a number of differentiated epithelial structures, including palmoplantar epidermis, mucosa and nail bed, tissues affected in PC-1 (1, 29, 30). Previously, we have shown that missense mutations in the helix initiation peptide of K16 cause either PC-1 (31) or FNEPPK alone (32). Mutations in K6a has also been found to produce PC-1 (33, 34) and mutations in this gene might also cause FNEPPK, although none have been reported yet. In PC-2, the keratoderma is less severe but there are numerous other epithelial defects, including *pili torti* and natal teeth. Mutations in keratin K17 and K6b have been reported to produce PC-2 (23, 31, 35).

Recently, we cloned and sequenced the K16 mRNA, its corresponding gene, *KRT16A,* and 2 K16-like pseudogenes, ψ*KRT16B* and ψ*KRT16C*, facilitating development of a mutation detection strategy for K16 based on genomic DNA (36). Here, we demonstrate the reliability of this system by identifying 2 novel mutations in genomic DNA, which were confirmed at the mRNA level. One mutation deletes a K16 helix boundary motif and unexpectedly, results in a relatively mild phenotype. By expression of mutant cDNAs in cultured epithelial cells, we show that this deleted K16 may be less capable of disrupting keratin filaments.

Materials and methods

Mutation detection and confirmation

Genomic DNA was extracted from whole blood by standard methods from affected individuals and from 50 normal, unrelated individuals. For mutation detection using genomic DNA as template, primers specific for the functional K16 gene, K16sp2 (5′ AGG GCT CCT GCG GCA TCG GA 3′) and K16sp3 (5′ GGA TTG GCC AGA TGC TTG CT 3′) were used in High Fidelity PCR buffer (Boehringer) containing 1.5 mM $MgCl_2$, 4% dimethylsulfoxide. A hot start was performed with 1 U Amplitaq polymerase (Perkin-Elmer) and the following conditions were used: 94°C 5 min ×1; 94°C for 30 s, 58°C for 45 s, 72°C for 2 m ×35; and 72°C for 5 m ×1. The resultant 2.5 kb PCR fragments were directly sequenced with the amplification primers, using the ABI Prism system (Perkin-Elmer, Foster City, CA, USA). To allow sequencing of the normal and mutant alleles in the case of deletion mutation, PCR products were cloned into pCR2.1 (Invitrogen).

mRNA was derived from either plantar (family A) or palmar (family B) biopsy material and was reverse transcribed as described previously (31). For mutation detection using epidermal-derived cDNA as template, a 1031 bp fragment of the K16 mRNA was amplified from cDNA using sense primer K16f800 (5′ CTG GCT TTG GTG GTG GTT TT 3′) and antisense primer K16r1831 (5′ AGA CCT CGC GGG AAG AAT A 3′) using the conditions above. This fragment was sequenced using the amplification primers.

Mutation K16 L124R does not alter any known restriction site. A forward primer with a 2 bp mismatch was designed, which in combination with the mutation, creates a new *BsiE*I restriction enzyme site. A 143 bp fragment of genomic DNA was amplified using sense primer K16.C3 (5′ GTG AGA AGG TGA CCA TGC ACG ACC 3′, mismatch underlined) and [^{33}P]-labeled antisense primer K16r990 (5′ GTC TTT GAT CTC ACT GGG CC 3′) in standard 10× PCR buffer (1 mM $MgCl_2$) containing 4% dimethylsulfoxide. The following PCR conditions were used 94°C 5 m ×1; 94°C for 30 s, 56°C for 45 s, 72°C for 45 s ×30; and 72°C for 5 m ×1. Without purification, the PCR products were digested with 5U *BsiE*I at 60°C overnight. Digests were analyzed on a 6% sequencing gel and visualized by autoradiography.

To confirm the deletion mutation, a 213 bp fragment was amplified from genomic DNA using forward primer K16sp5 (5′ AGG AGT ACC AGA

TCT TGC TGG ATG 3′) and reverse primer K16sp3 (above) in standard PCR buffer containing 1.5 mM $MgCl_2$ and 4% dimethylsulfoxide. PCR conditions were 94°C for 5 min ×1; 94°C 30 s, 55°C 45 s, 72°C 45 s ×30; and 72°C 5 min ×1. PCR products were resolved on a 3% agarose gel. This PCR reaction is specific for the functional K16 gene under the conditions described.

Transient transfections of keratin 16 cDNAs

Full length wild-type K16 cDNA (1441 bp) was amplified using forward primer K16.CL (5′ CTC CCT CCT TGG CAC CAT GAC C 3′) and reverse primer K16.CR (5′ TTC TAG GAG CTC TGG CCC TGG C 3′). Standard PCR buffer containing 1 mM $MgCl_2$ and 4% DMSO was used and the amplification conditions were 94°C 5 m ×1; 94°C for 30 s, 60°C for 1 m, 72°C for 2 m ×30; 72°C for 5 m ×1. PCR products were cloned into eukaryotic expression vector pCR3.1 (Bidirectional Eukaryotic TA Cloning Kit, Invitrogen). Similarly, K16 cDNA constructs containing either mutation K16 L124R or K16 ΔHTM were made using cDNA prepared from biopsy material. Constructs were completely sequenced to exclude cloning artifacts. Plasmids were transfected into 50% confluent PtK2 cells using the calcium phosphate precipitation method. The cells were fixed 24 h and 48 h after transfection with methanol:acetone (1:1) and double-label immunofluorescence was performed. Transfected K16 was detected with a 1:500 dilution of rabbit polyclonal antisera against human K16 (a kind gift of Pierre Coulombe, Johns Hopkins University, Baltimore, MD, USA) and the endogenous K8 with neat supernatant monoclonal antibody LE41 to PtK2 K8 (a gift of Birgitte Lane, University of Dundee, Scotland). The secondary antibodies used were F3008 anti-mouse IgG FITC conjugate (Sigma) at 1:50 dilution and R156 anti-rabbit IgG TRITC conjugate (Dako) at 1:500 dilution. Fluorescently stained cells were observed and photographed using a Bio-Rad Confocal Laser Scanning microscope.

Results

Clinical features of PC-1 and FNEPPK

Two kindreds were studied, whose pedigrees are shown in Fig. 1. In both families, typical features of autosomal dominant inheritance were seen, including male-to-male transmission.

Case report, family A. The proband in family A was a 36-year-old male with gross subungual hyperkeratosis affecting all nails since the age of 4 years (Fig. 2a & b). There were hyperkeratotic areas predominantly on the pressure bearing areas

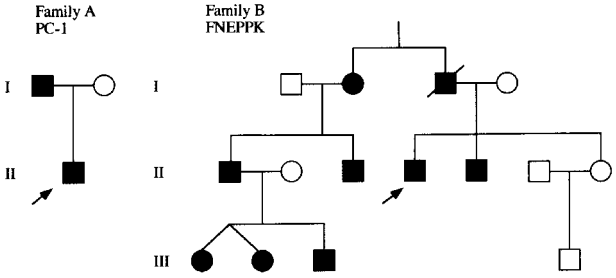

Figure 1. Pedigrees of kindreds studied. Affected persons in family A exhibited the classic pachyonychia congenita type 1 (PC-1) phenotype with hypertrophic nail dystrophy and severe focal palmoplantar keratoderma (FNEPPK). The affected persons in family B had mild FNEPPK as the only phenotype.

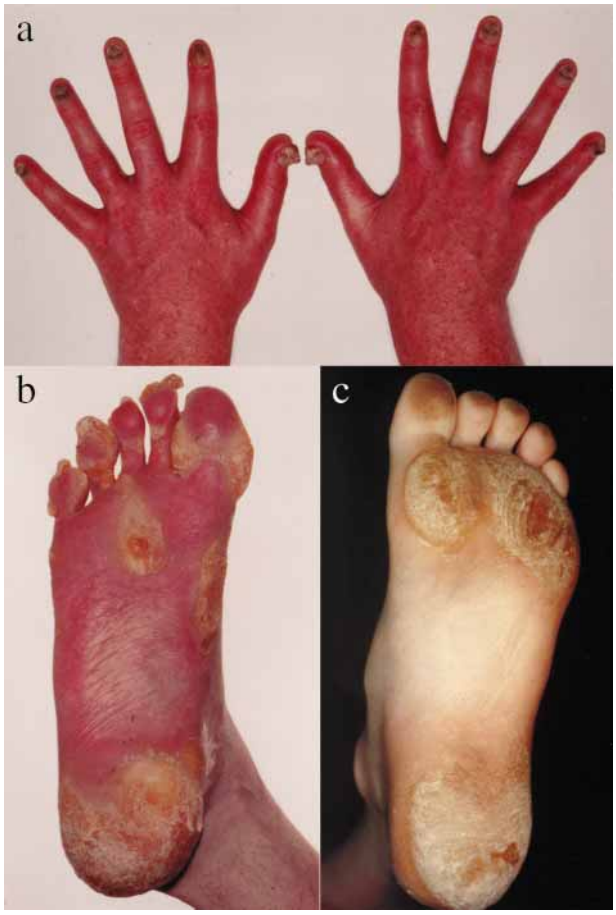

Figure 2. Clinical appearance of affected members of the 3 families studied. (a) Pachyonychia of the fingernails in the proband of family A. Affected persons in family A had 20 nail pachyonychia. (b) Severe focal non-epidermolytic palmoplantar keratoderma (FNEPPK) seen here in the proband of family A. Some focal palmar keratoderma was also seen in affected individuals in family A. (c) FNEPPK seen in the proband of family B. Affected persons in this kindred had no evidence of nail dystrophy and the keratoderma was less debilitating than in family A. No palmar involvement was seen in affected members of family B.

of both feet, and a few similar focal lesions on the palms. Follicular keratoses were present on the anterior aspect of both thighs, however, there were no oral lesions. The patient's father had similar nail and plantar lesions, but the patient's mother had been unaffected. Treatment with acitretin at doses of 25 mg and 50 mg daily over a period of several months did not result in visible improvement of the patient's hyperkeratosis, although there was a reduction in the pain in the plantar lesions on walking. Histological analysis of the keratoderma was not possible.

Case report, family B. The proband in family B presented with FNEPPK, confined to the weight bearing areas of the soles (Fig. 2c). There was no evidence of palmar keratoderma, nail changes or oral leukokeratosis observed in any of the 6 family members examined. Histological analysis of a plantar skin biopsy showed no signs of epidermolysis, consistent with the diagnosis of FNEPPK.

Identification of novel K16 mutations

Initially, mutation detection was performed using genomic DNA from the probands in families A and B. In family A, a heterozygous missense mutation 371T→G was identified, resulting in the predicted amino acid change L124R in the 1A domain of the K16 polypeptide (Fig. 3a & b). Mutation L124R does not alter any known restriction site. Consequently, a forward primer was designed with a 2 bp mismatch, which in combination with the mutation, creates a *BsiE*I restriction enzyme site. A 143 bp fragment of genomic DNA was amplified and digested with *BsiE*I. The digested product from the proband in family A showed an additional band which was not present in the 50 normal, unrelated individuals (Fig. 3c). The same mutation was also detected in cDNA derived from the proband's plantar epidermis (data not shown).

Genetic linkage analysis performed on family B excluded the type II keratin cluster on chromosome 12q (data not shown). Of the type I keratins, K16 was seen as the most likely candidate gene (32). Direct sequencing of K16 genomic PCR products from the proband (individual II_3, family B), revealed a complex deletion mutation in exon 6 by the presence of overlapping sequences (not shown). This PCR fragment was cloned into the pCR2.1 vector and several independent clones were sequenced to identify the exact mutation. Two species of clones were obtained, one with the wild type K16 sequence and the other with a 23 bp deletion mutation, together with a separate 1 bp deletion downstream (Fig. 4a & b). A 213 bp fragment was amplified using primers flanking the deletion mutation. Two bands were amplified from

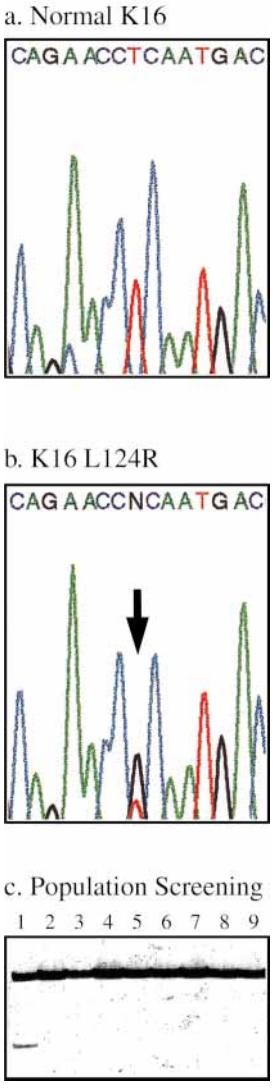

Figure 3. K16 mutation causing PC-1 in family A. (a) Normal sequence in the region of the K16 helix initiation motif. (b) Sequence derived from the proband in family A, showing heterozygous missense mutation 371T→G, leading to amino acid change L124R. (c) Confirmation of mutation L124R by *Bsi*EI digestion of [^{33}P]-labeled PCR products. Lane 1: *Bsi*EI digest derived from the proband in family A, showing cutting of the mutant allele by introduction of a *Bsi*EI site in one primer, which depends on the presence of the mutation. Lanes 2–9: digested PCR products derived from normal unrelated individuals.

the proband's DNA representing the normal allele and the smaller mutant allele with the 24 bp deletion (Fig. 4c). PCR products derived from 50 control samples revealed only a single band. The mutation was also confirmed in cDNA derived from palmar biopsy material from the proband in family B (not shown). Sequence peaks corresponding to both alleles appeared to be equally represented in the cDNA sequence, indicating that the mutant mRNA is stable. In terms of DNA, this

mutation is designated [1244–1266del; 1270delG] according to the guidelines issued by the Nomenclature Working Group (37). At the protein level, the mutations delete 8 residues and substitute a further 2 residues in the helix termination motif (HTM) of the K16 polypeptide. Specifically, the wild-type sequence EIATYRRLLEGEDA becomes EIGQDA in the mutant K16 polypeptide. For convenience, this mutation will subsequently be referred to as ΔHTM, since it deletes most of the HTM sequence.

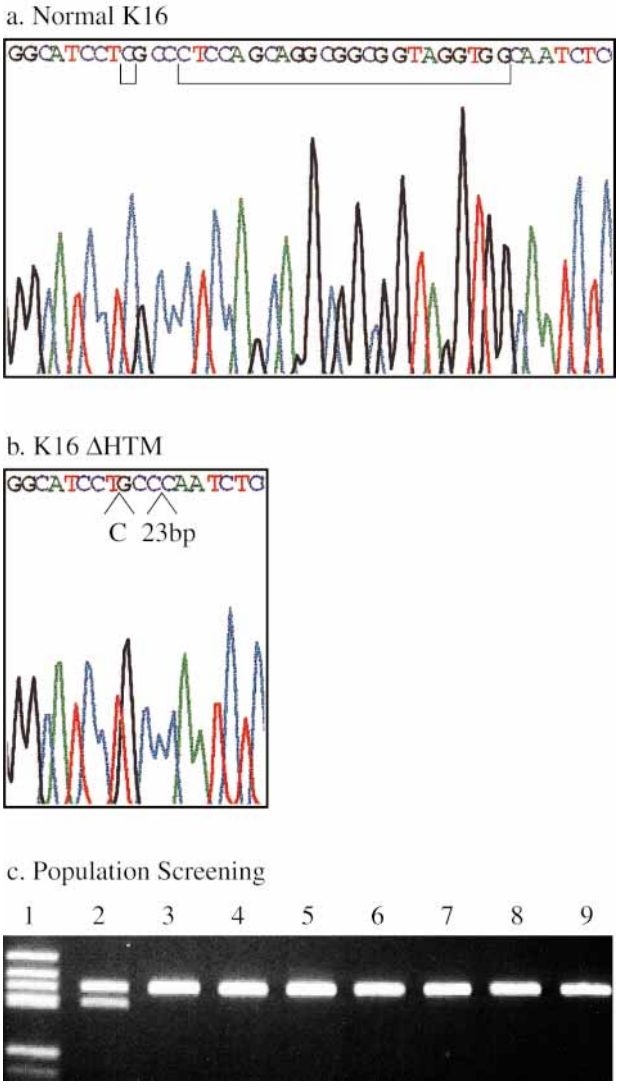

Figure 4. Complex deletion mutation in FNEPPK family B. (a) Normal sequence in the region of the K16 helix termination motif, derived from a cloned PCR fragment in pCR2.1 (antisense strand shown). (b) Sequence from a mutant clone in the same region shown in (a), derived from an affected person in FNEPPK family B (individual II_3), showing 1 bp and 23 bp deletions, which delete most of the highly conserved residues from the helix termination motif of K16 (mutation designated ΔHTM). (c) Confirmation of 24 bp deletion in family B by specific amplification of the helix termination motif region in exon 6 of the *KRT16A* gene. Lane 1, Molecular weight markers; lane 2, PCR products derived from affected individual II_3 in family B, showing normal and deleted alleles; lanes 3–9, PCR products derived from 8 normal, unrelated individuals.

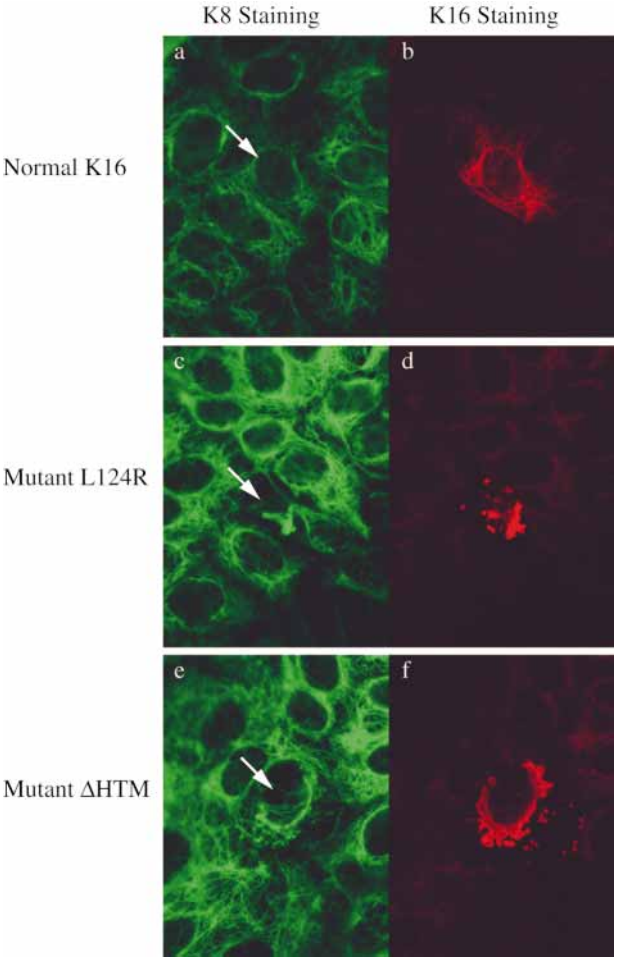

Figure 5. Transient expression of normal and mutant K16 cDNAs in transformed epithelial cell line PtK2, visualized by double-label immunofluorescence using antibodies LE41 against K8 (green FITC fluorescence, panels a, c & e) and rabbit-anti-human K16 polyclonal antiserum (red TRITC fluorescence, panels b, d & f). A representative field is shown for each construct. Results were consistent in more than 50 cells observed for each cDNA expressed. (a & b) Expression of normal K16 cDNA in PtK2 cells at 24 h post-transfection reveals fine, filamentous K16 staining in transfected cells (b), which co-localizes with the endogenous filament network, as seen by K8 staining (a, arrow indicates transfected cell). (c & d) Expression of K16 cDNA carrying mutation L124R in the helix initiation motif at 24 h post-transfection. In this case, the endogenous keratin cytoskeleton (c, arrow indicates transfected cell), is completely collapsed into dense aggregates by the expression of this mutant K16 cDNA (d). (e & f): Expression of the ΔHTM mutant cDNA produces aggregates which are much larger than those seen with the missense mutation (f). In this case, the K16 staining (f), does not fully co-localize with the endogenous K8 staining (e, arrow indicates transfected cell). Furthermore, some residual endogenous keratin is seen in filamentous form (e). This result implies that the ΔHTM mutation may render the mutant K16 protein less able to integrate into and disrupt the endogenous network.

Expression of mutant K16 cDNA constructs in epithelial cells

Since the phenotypes in the 2 families were quite different and the underlying K16 defects were also very different, we transiently expressed these mutant K16 cDNAs in epithelial cell line PtK2, to investigate the effect of each on a preformed keratin cytoskeleton. Full-length expression constructs in pCR3.1 were made for normal K16 and the 2 mutations, L124R and ΔHTM. At 24 h after transfection, PtK2 cells were fixed and stained for both K8, a marker for the endogenous keratin network, and for K16 by double-label immunofluorescence. More than 50 transfected cells were observed for each construct to confirm consistency of the observations, and typical results are shown (Fig. 5). Transfection of the normal K16 construct revealed a well defined keratin cytoskeleton which co-localized with the endogenous network (Fig. 5a & b). In contrast, transfection of the L124R construct produced dense K16-reactive aggregates and the endogenous network was completely collapsed (Fig. 5c & d). Similarly, the ΔHTM construct produced K16-reactive aggregates. However, these aggregates were consistently larger and there appeared to be less disruption of the endogenous filament network (Fig. 5e & f). Specifically, filamentous structures were still visible in transfected cells stained for the endogenous keratin, K8 (Fig. 5e). Similar results for all constructs were obtained at 48 h post-transfection (data not shown).

Discussion

Mutation detection for the K16 gene

Using a cDNA-based approach, we have previously shown that K16 mutations underlie the autosomal dominant ectodermal dysplasias pachyonychia congenita type 1 (PC-1) and focal non-epidermolytic palmoplantar keratoderma (FNEPPK) (31, 32). There have been conflicting reports of the K16 sequence in the literature (38–40). Recently, we independently cloned and sequenced the K16 cDNA (36). This was found to be identical to the most recently reported cDNA sequence (40). We also cloned the corresponding gene *KRT16A* and 2 species of K16 pseudogene, ψ*KRT16B* and ψ*KRT16C* (36). This information allowed us to develop primers and conditions for long-range PCR amplification of almost the entire *KRT16A* gene without pseudogene interference (36). We recently used this strategy to identify a novel 3 bp deletion in the 1A domain of K16 (388del3; S130del), for which we subsequently performed prenatal testing and correctly predicted a healthy baby (36). Here, we have used this methodology to identify 2 novel K16 mutations, L124R and ΔHTM, which were confirmed in cDNA. These data confirm that our long-range genomic PCR strategy is a robust method for detection of K16 mutations in PC-1 or FNEPPK, circumventing the RT-PCR method, which requires tissue samples obtained through an invasive biopsy procedure (31, 32).

An unusual K16 double deletion in FNEPPK

Previously, we reported mutations N125S and R127C in the helix initiation motif of K16 in 2 families presenting with FNEPPK (32). Here, we report an unusual mutation (ΔHTM) involving the helix termination motif of K16 in this disorder. In this case, a combination of a 23 bp deletion and a 1 bp deletion remove most of the helix termination sequence (Fig. 4). This sequence is the most highly conserved motif in keratins and all other intermediate filament proteins cloned to date (5). It is easily recognizable even in highly divergent species such as snail and nematode (41, 42). This high level of conservation implies an important functional role for this motif. Based on previous studies, where keratins carrying engineered deletion or truncation mutations similar to ΔHTM have been expressed in cultured cells (9, 43), or in transgenic animals (2), one might have anticipated that such a mutation in humans would have a severe phenotype. However, no palmar, oral mucosal or nail involvement is seen in this family and only plantar keratoderma was observed (Fig. 2). Thus, the only tissue involved is the one which undergoes the highest trauma in everyday life.

One possible explanation is that a keratin polypeptide lacking this sequence, which has been implicated in end-to-end interactions in keratin assembly (10, 44), might be less able to contribute to filament assembly and therefore have a milder dominant negative effect than a mutant keratin where the motif is present but carries a point mutation. Transient expression of K16 cDNAs carrying either the L124R or the ΔHTM mutation in PtK2 cells resulted in the formation of K16 aggregates (Fig. 5). In addition to morphological differences in the aggregates produced, the L124R mutation appeared to cause complete collapse of the endogenous filament network, whereas some residual filamentous keratin was seen in cells expressing the ΔHTM construct. These results imply that ΔHTM mutant K16 may be less capable of disrupting keratin filaments by dominant-negative interference, compared to the L124R mutation.

Despite the putative genotype–phenotype correlation reported here, it should be noted that very similar mutations in keratin disorders have been

reported to cause different phenotypes both within and between families. For example, the missense mutations N125S and R127C, which have been reported previously in K16 in 2 FNEPPK families (32), are rather similar to that detected in 1 of the PC-1 families here, L124R in family A. Furthermore, a mutation in K14 (R125C), which is equivalent to the R127C mutation in K16, results in the most severe Dowling–Meara form of EBS (45). However, this mutation has been associated with the milder FNEPPK phenotype in K16 (32). Similarly, the equivalent K17 mutation (R94C), has been identified in families with either pachyonychia congenita type 2 (PC-2) or kindreds with a mild steatocystoma phenotype (46). Overall, it may be that in some rare cases, such as the ΔHTM mutation seen here, genotype may correlate with disease severity. However, in the broader context there are other factors, probably both genetic and environmental, which modulate keratin disease phenotype. Since trauma is a factor in producing epithelial damage in keratinizing disorders, lifestyle and occupation are possible factors influencing the phenotype. However, the identity of the other modifiers remains unknown.

Acknowledgements

This paper is dedicated to the late Dr Jarek P. Fisher, who envisioned that the FNEPPK gene affecting his family would be one day be identified. We wish to thank Yijun Zhao, Department of Dermatology and Cutaneous Biology, Jefferson Medical College, Philadelphia for her help with the cell culture; Hans-Jürg Alder and his staff, Nucleic Acid Facility, Kimmel Cancer Center, Jefferson Medical College, Philadelphia, for DNA synthesis and sequencing. WHIM is a Wellcome Trust Senior Research Fellow and this work was funded by grants from The Dystrophic Epidermolysis Bullosa Research Associations (DebRA) of UK and America; and by the US Public Health Service, National Institutes of Health (grant PO1-AR38923).

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