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We hope that making available the relevant information on Pachyonychia Congenita will be a means of furthering research to find effective therapies and a cure for PC.
Keratin 16 and keratin 17 mutations cause pachyonychia congenita


Pachyonychia congenita (PC) is a group of autosomal dominant disorders characterized by dystrophic nails and other ectodermal aberrations. A gene for Jackson-Lawler PC was recently mapped to the type I keratin cluster on 17q. Here, we show that a heterozygous nonsense mutation in the helix initiation motif of K17 (Asn92Asp) cosegregates with the disease in this kindred. We also show that Jadassohn-Lewandowsky PC is caused by a heterozygous nonsense mutation in the helix initiation peptide of K16 (Leu130Pro). The known expression patterns of these keratins in epidermal structures correlates with the specific abnormalities observed in each form of PC.

Mutations causing genodermatoses (genetic skin disorders) have been identified in six different keratin genes to date. Mutations in keratins K5 and K14, the proteins synthesized in basal cells of the epidermis, cause epidermolysis bullosa simplex (EBS)1,2. Dysfunction of the K5/K14 cytoskeleton leads to blister formation following rupture of basal cells in response to mild physical trauma. Similarly, in bullous congenital ichthyosiform erythroderma (BICE) mutations in the differentiation-specific keratins K1 and K10 lead to suprabasal cytolsis3,4 and epidermolysis hyperkeratosis. Keratin K9 is expressed exclusively in palm and sole skin5,6 and mutations in this gene lead to the disease epidermolytic palmoplantar keratoderma (EPPK)7,8. In ichthyosis bullosa of Siemens (IBS), mutations occur in K2e6,9, which is expressed in the upper spinous layers of the epidermis outwards10. This restricted expression pattern leads to the observed phenotype of mild epidermolytic ichthyosis. In all keratin disorders recognized so far, the presence of pronounced keratin aggregates visible by electron microscopy is correlated with mutations within the helix boundary peptides11, mutations occurring elsewhere in the protein structure are less disruptive of filament organization.

The phenotypes of PC suggests to us that these diseases may also be caused by keratin mutations. Here we describe the confirmation of that hypothesis, showing that two forms of this disease are caused by mutations in a critical domain of K16 (the Jadassohn-Lewandowsky form) and K17 (the Jackson-Lawler type). Keratins K16 and K17 are usually associated with hyperproliferation and show similar but non-identical tissue distribution patterns; the epithelial structures affected in these syndromes correlate well with the expression patterns of these cytoskeletal proteins.
oval or round darkly staining perinuclear inclusions.

By transmission electron microscopy, the darkly stained inclusions were seen on light microscopy appearing to correspond to densely aggregated keratin filament bundles at the ultrastructural level (Fig. 3b). These aggregates are not seen in suprabasal keratinocytes from normal individuals. Instead, these aggregates were arranged in a wavelike configuration. Aggregates were also located in the cell centres where the periphery of the cytoplasm contained sparse amounts of microtubules and short keratin filament bundles. The number of desmosomes in the spinous and granular layers was reduced (Fig. 3b). However, ultrastructural signs of cellular disruption or cell death were not observed. Moreover, granular cells showed reduced amounts of keratohyalin.

**Keratin mutation analysis in PC**

The linkage data was highly suggestive of a mutation in type I keratin in PC. Several type I keratins were considered as candidates for PC-1 and PC-2. Some type I keratins (K9, K10, K14) have known pathologies distinct from PC and consistent with their known expression patterns. Other keratins were excluded on the basis of expression patterns consistent with PC phenotypes. These were K12 (cortex-specific); K13 (non-cornifying mucosal epithelia); K15 (basal cells only); K18 and K20 (simple epithelia). K19 is expressed in simple epithelia and also in the hair follicle and nail bed. K19 was thus not excluded since it is not expressed in palmoplantar epidermis. Similarly, trichohyalin keratin genes (which also map to this locus) are expressed in hair and nail but not in palmoplantar epidermis. K11 is probably a derivative of K10 (ref. 24). The remaining known type I keratins, K16 and K17, were considered good candidates for causative mutations in PC since they are expressed in hair follicle, nail bed and palmoplantar skin.

Both K16 and K17 have pseudogenes, which create potential problems for amplifying these genes by PCR from genomic DNA. To circumvent this problem, we used mRNA obtained from cultured cells or frozen biopsy material; this was reverse transcribed and the cDNA encoding the entire rod domain of K16 and K17 was amplified by PCR. The resultant PCR products were gel purified and sequenced directly.

**K16 mutation in Jadassohn-Lewandowsky PC**

The affected member of family PC-1 was found to have a T to C transition at position 896 of the K16 mRNA, which predicts a leucine to proline mutation at codon 130 in the IA domain of K16 (Fig. 4a). This change was not seen in K16 sequence from a normal unrelated individual. The mutation creates a new restriction site for Msp I, which does not normally occur in this region of the K16 gene. A small PCR fragment was amplified from affected and unaffected DNA and digested with Msp I. The normal fragment was unaffected by Msp I but the mutant PCR fragment was digested.
...stained...aggregated...les at the 3b). These suprabasal normal...keratin...in a whorl-patterned..cells is mostly...filament...nuclear layers...keratin...darker and...darker...controls...filamentous...cortex...keratin...lanes...normal...individuals, excluding this change as a...common polymorphism. Furthermore, no evidence of...non-paternity was observed in the kindred using several...microsatellite probes, so the mutation is probably sporadic.

**K17 mutation in Jackson-Lawler PC**

In family PC-2, DNA sequences covering the K16 boundary...peptides were found to be identical to those previously...published. However, an A to G transition was found at...position 422 of the K17 genomic sequence, producing...an asparagine to aspartic acid mutation in codon 92 (Fig....fragmentation. This mutation is also located in the 1A domain,...highly conserved region where mutations associated with...severe phenotypes have been reported in other keratins. Like...the K16 mutation, this base change also creates a new...restriction enzyme site, in this instance TaqI. A small...fragment of K17 spanning the mutation was amplified...from cDNA derived from affected and unaffected...individuals. The mutant K17 fragment was found to cut...with TaqI, thus confirming the presence of the mutant...allele (not shown). A genomic screen was developed...based on this cDNA test using primers designed to amplify...only the functional K17 gene (K17A) and the two...reported pseudogenes (wK17B and wK17C). Lack...of pseudogene contamination in this PCR was confirmed...by TaqI digestion since both pseudogenes contain a TaqI site...close to the 5' end of the amplified fragment. The functional...gene has no TaqI sites in this PCR fragment. No pseudogene...bands were observed using the PCR conditions described,...although these were seen with lower annealing...temperatures (not shown).

This restriction fragment analysis was used to exclude...the mutation from 50 normal unrelated individuals (Fig....4d). The test also was applied to 37 members of the...kindred PC-2 for which samples were available. All affected...individuals produced the mutant genotype and all...unaffected family members were found to have the normal...genotype.

**Discussion**

The molecular pathology of pachyonychia congenita.
We have shown for the first time that two major subtypes...of pachyonychia congenita are caused by mutations in...
Fig. 4 K16 and K17 mutations in pachyonychia congenita. a, Excerpt from a DNA sequencing gel showing coding strand sequence encoding part of the 1A domain of K16 from a normal cDNA (left) and the proband from family PC-1 (right). Partial sequence and deduced amino acid translation is shown either side. The transition T896C is shown in the affected producing a Leu129Pro mutation. b, DNA sequence (-strand) of part of the 1A domain of K17 from normal cDNA (left) and the proband in family PC-2 (right). The A422G mutation in the affected individual produces the amino acid transition Asn92Asp. Both mutations occur in the critical helix initiation motif, c, RFLP screen for the K16 mutation. PCR fragments (191 bp) were amplified from genomic DNA, digested with MspI and analysed on a 4% NuSieve gel. Lane 1, DNA markers type VI (BCL); lanes 2–5, members of family PC-1, 1, 1, 1, and 2, respectively; lanes 6–12, eight unaffected unrelated individuals. Additional 95 bp/66 bp bands indicate mutation (+) in the affected individual II, lane 8. This RFLP test was used to exclude the K16 mutation from 100 normal unrelated chromosomes. d, RFLP screen for K17 mutation. 1% agarose gel showing TaqI digested K17 genomic PCR fragments (978 bp) from affected and unaffected individuals in generation III of family PC-2 as they appear in the pedigree (Fig. 1b). Lane 1, DNA markers type VI (BCL); lanes 2–18, individuals from family PC-2: III, III, III, III. Additional 605 bp and 373 bp bands indicate the mutation (+). The mutant genotype segregated completely with the mutation in 37 members of the kindred analysed and this PCR test was used to exclude the mutation from 100 normal unrelated chromosomes.

Like all the dominant keratin disorders described so far these mutations act in a dominant negative manner. The A137T mutation in the K16 gene was found in a sporadic case of Jadassohn-Lewandowsky PC, and a similar mutation was found in the K17 gene in a family with the Jackson-Lawler variant. These findings add two more keratins to the six already known to be involved in human disease.

The keratin mutations both occur in the helix initiation peptide, a highly conserved motif which marks the start of the α-helical rod domain. This is the most frequently mutated region in all keratins examined. The K16 mutation is a proline substitution and is predicted to be very detrimental to α-helical conformation. The K17 mutation changes a neutral asparagine residue to a negatively charged aspartic acid and is also likely to disrupt protein structure. Both residues show a high degree of evolutionary conservation, reflecting their functional importance. Asn 92 in K17 is completely conserved in all type I, II and III intermediate filaments. Leu 130 in K16 is conserved in all type I proteins and is replaced by isoleucine in all type II proteins and most type III proteins.

Mutations have been found in the helix initiation peptides of K14 causing EB5-DM8; K1 and K10 causing BCIE1,2,109; K9 causing EPPK15; and K2e causing IBS14,17.

Fig. 5 The type I keratin consensus domain structure showing the sequence of the 1A domain. The DNA and deduced amino acid sequence of the 1A domain of K16 and K17 are shown together with the pachyonychia congenita mutations described here. Note the high degree of conservation in this motif at both DNA and protein sequence levels between the two type I keratins, emphasizing the functional importance of this peptide. Despite their homology, the expression patterns of K16 and K17 are different as shown by the two distinct PC phenotypes.

Genotype-phenotype correlation in PC. Although K16 and K17 are very similar proteins in terms of molecular weight and protein sequence, they have slightly different tissue distributions, implying that they are functionally distinct. This tissue-specificity is emphasised by the phenotypic differences between K16 and K17 mutants.

The phenotypes of the two forms of pachyonychia described here are similar in that they both have the nail changes diagnostic of the condition. Nail dystrophy is
much worse on the the feet; on the hands, the nails of the thumb and the first two digits are generally more severely affected in both types. These observations suggest that physical trauma is a factor in production of the abnormal nails. Both K16 and K17 are expressed in the nail bed in the vicinity of the nail progenitor cells (D. de Berker and I.M. Leigh, manuscript in preparation). Our findings imply that traumatic damage of these cells due to a compromised keratin cytoskeleton leads to hypertrophic nail dystrophy. Direct histopathological and ultrastructural examination of the nail bed in PC is desirable to investigate how changes in nail bed keratinocytes lead to nail hypertrophy but is not clinically feasible without complete nail removal.

Focal plantar keratoderma is also a feature of both forms of PC studied. Although this is relatively mild in the K17 family, it is severe and debilitating in the K16 patient. This reflects the expression of these keratins in plantar epidermis, where K16 is abundant suprabasally and K17 is found basally in a restricted pattern. In contrast, keratin filament aggregation was observed in plantar keratinocytes from the patient carrying the K16 mutation and these suprabasal cells were seen to be abnormal (Fig. 3). Traumatic damage to these cells due to cytoskeletal dysfunction appears to lead to plantar hyperkeratosis analogous to that seen in BCIE and EPPK (due to mutation in K1/K10 or K9, respectively). Keratinocyte wound healing is associated with upregulation of inflammatory cytokines and keratinocyte growth factors, particularly TGFα, which may mediate secondary changes in keratinocyte growth and differentiation leading to the hyperkeratosis observed in PC, EPPK, IBS and BCIE.

In contrast, the two forms of PC differ in ways reflecting the differential expression of K16 and K17. K16 and K17 are type I (acidic) keratins associated with high epidermal turnover. Both are expressed in mucosal stratified squamous epithelia, with K17 in basal cells and K16 in suprabasal cells in the normal tissue. They are variably expressed in the hair follicle and nail bed. They are also both induced in damaged or stressed epidermis (E.B. Lane & A.A. Baker, unpublished observations). Similarly, both proteins are found in palmoplantar epidermis where K16 is abundant in suprabasal cells and K17 in a restricted expression pattern within basal cells (O. Swenson, manuscript in preparation). They are both found in sweat and mammary glands, although K17 is found in the basal cells and K16 is found in the luminal cells of the duct. Keratin 16 is expressed suprabasally in oral epithelium, where K17 is undocumented. Both proteins are found in specific regions of sebaceous glands.

The high turnover of oral keratinocytes and expression of large amounts of K16 presumably leads to the buccal and lingual leukokeratosis in the individual carrying the K16 mutation. There are however three main phenotypic changes in the Jackson-Lawler PC family which were not observed with K16 mutation: epidermal cysts, abnormal hair and nail teeth. The epidermal cysts are a major clinical problem, with affected individuals requiring frequent cyst excisions and in some cases, surgical excision. Histologically, these cysts are heterogeneous. Some derive from infundibular hyperkeratosis and others are steatoctycts (C.S. Munro, manuscript in preparation). These regions predominantly express K17 rather than K16. Folicular hyperkeratosis may also be responsible for moulding hair into the abnormal patterns observed. Natal

The function of K16 and K17. Like the epidermal keratins (K1, K2e, K5, K9, K10 and K14), in which mutations result in epidermal fragility with or without hyperkeratosis, mutations in K16 and K17 lead to keratinocyte damage and hyperproliferation in specific ectodermal structures. This appears as the variety of abnormal phenotypic features which make up pachyonychia congenita. We conclude that K16 and K17 play a significant role in maintaining resistance to physical trauma in specific ectodermal regions. How the K16 and K17 keratin filaments network is unknown. Until this is examined directly, the question as to why there are so many differentially expressed keratin genes remains unanswered.

**Methodology**

**Immunohistochemistry.** Cryostat sections were cut from a biopsy of a follicular keratin from the family of PC. Sections were stained with haematoxylin and eosin by standard methods. Serial sections were stained with monoclonal antibody E3 and anti-mouse IgG FITC conjugate (DAKO).

**Transmission electron microscopy.** Plantar skin specimens from the PC-1 proband were processed for EM in a standard fashion. Briefly, primary fixation was for 2 h at 4 °C in phosphate-buffered saline (PBS) containing 1% osmium tetroxide in distilled water for 2 h at 4 °C. After dehydration in a graded alcohol series, specimens were embedded in epoxy resin (Taab 812) via propylene oxide. Semithin sections (0.5 μm) and ultrathin (60–90 nm) were cut on a Reichert OMU-4 ultramicrotome. Semithin sections were stained with Richardson's stain for light microscopy. Ultrathin sections were collected on pinhole-covered copper grids and double-stained with uranyl acetate in ethanol and Reynolds' lead citrate. Sections were viewed using a JEOL 100 CX transmission electron microscope.

**Keratinocyte culture.** Cells were cultured according to the protocol of Reinwald and Greenwood from a plantar biopsy obtained from the affected individual in family PC-1.

**Mutation analysis.** Poly(A) mRNA was extracted using the QuickPrep Micro mRNA purification kit (Pharmacia) from cultured cells (probands, PC-1) or from 10–125 μm cryostat sections from an arm skin biopsy (probands, PC-2). mRNA was primed with oligo(dT) (12–18 base) in the presence of RNAin (Boehringer) and reverse transcribed. A 1,031 bp fragment encoding the entire rod domain and short flanking sequences of K16 was PCR amplified from cDNA using primers K16600 (5'-GGTTCTTGGTTGGTTCT-3', K161831 (5'-AGCACTGCAGAAAATGAT-3') and K161831 (5'-AGCACTGCAGAAAATGAT-3'). Approximately 300 ng of genomic DNA was used per 100 μl reaction containing 1.5 mM MgCl₂, 0.2 mM each dNTP, 5 μg/ml of each primer and 1 U Taq polymerase (Perkin Elmer Cetus). After an initial incubation at 94 °C for 5 min, PCR was performed for 35 cycles consisting of 94°C for 30 s, 55°C for 1 min, 72°C for 2 min on a DNA Thermal Cycler (Perkin Elmer Cetus).

The regions encoding the helix boundary peptides were sequenced using the primers above, end-labelled with [32P]P. PCR templates were purified on 1% LMP-agarose (Ultra pure grade, BRL) and approximately 50 fmol in molten gel sequenced using the BRL DyeCycle sequencing kit. The following program was used for cycle sequencing: 20 cycles consisting of 94°C 30 s, 60°C 30 s, 72°C 1 min.
followed by 10 cycles consisting of 94°C 30 s, 72°C 1 min. Sequencing ladders were resolved on standard 6% polyacrylamide denaturing gels. The K16 mutation was confirmed by sequencing the non-coding strand using primer K16990 (5'-GGT ACCG TCTAG AGTCTTG-3') and primer K17p4 (5'-GGT ACCG TCTAG AGTCTTG-3'). PCR conditions were as above except that 42 cycles were used. Amplification primers were used for sequencing and the K17 mutation was confirmed by sequencing the non-coding strand with primer K17p4 (5'-GGT ACCG TCTAG AGTCTTG-3').

Mutation screening by restriction enzyme analysis. The K16 mutation creates an MspI site. A 191 bp fragment of K16 message was PCR amplified from patient and control DNA using primers K16990 and K16990. Digested with MspI and analyzed on 4% NuSieve/TBE minigels. The K16 mutant allele digested to give bands of 95 bp and 96 bp and the control PCR fragment was not cut. The same PCR was used on genomic DNA. The K17 mutation creates a TaqI site. Initially PCR was carried out on normal and affected DNA using primers K17p4 and K17p4. TaqI digestion confirmed the presence of the mutation. Primers (5'-GGTC TAT AAA GGG CCG CCG-3', and 5'-GGTC CTTG GGC CCG CCG-3') were used. PCR conditions were as above except that 10% DMSO was added to the reaction and the annealing temperature was increased to 55°C. Digestion of the mutant allele gave bands of 373 bp and 269 bp visualized on agarose/TBE minigels.

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