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Mutation of a type II keratin gene (K6a) in pachyonychia congenita

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Pachyonychia congenita (PC) is a rare autosomal dominant condition characterized by multiple ectodermal abnormalities[1–3]. Patients with Jadassohn-Lewandowsky Syndrome (MIM #167200; PC-1) have nail defects (onchyogryposis), palmoplantar hyperkeratosis, follicular hyperkeratosis and oral leukokeratosis[4]. Those with the rarer Jackson-Lawler Syndrome (MIM #167210; PC-2) lack oral involvement but have natal teeth and cutaneous cysts[5]. Ultra-structural studies have identified abnormal keratin tonofilaments[6] and linkage to the keratin gene cluster on chromosome 17 has been found in PC families[7]. Keratins are the major structural proteins of the epidermis and associated appendages and the nail, hair follicle, palm, sole and tongue are the main sites of constitutive K6, K16 and K17 expression[8–10]. Furthermore, mutations in K16 and K17 have recently been identified in some PC patients[11]. Although we did not detect K16 or K17 mutations in PC families from Slovenia, we have found a heterozygous deletion in a K6 isoform (K6a) in the affected members of one family. This 3 bp deletion (AAC) in exon 1 of K6a removes a highly conserved asparagine residue (ΔN170) from position 8 of the 1A helical domain (ΔN8). This is the first K6a mutation to be described and this heterozygous K6a deletion is sufficient to explain the pathology observed in this PC-1 family.

Keratin mutations occur in several heritable skin disorders[12–16]. Pachyonychia congenita (PC) is a rare form of hereditary palmoplantar keratoderma[1,2] and is generally inherited in an autosomal dominant manner but autosomal recessive pedigrees do exist[17]. PC is frequent in Slovenian[18] and Croatian[19] families that originate from geographically isolated populations with a high probability of intermarriage and consanguinity. Similarity of the clinical, pathological and ultrastructural features of PC to other reported keratin disorders, together with its dominant mode of inheritance, suggested that PC may be caused by a keratin defect. Keratins expressed by human epidermis and its appendages in a body-site and differentiation-specific manner[8,20]. They are obligate heteropolymers characterized by a highly conserved α-helical central rod domain which is flanked by non-helical terminal sequences of variable size and composition[21]. Keratins are classified as type I (K9–K21) or type II (K1–K8) and are expressed in pairs[22]. K6 and K16 are constitutively expressed in palmar-plantar epidermis[20], hair follicle[9], nail[8], oral epithelia including tongue[10], oesophagus and trachea[13] but not in normal interfollicular epidermis. However, expression is rapidly induced in traumatized or cultured epidermal cells and sustained in epidermal disorders[23]. Keratin genes are localized in clusters on chromosomes 12 and 17 (refs 24,25) and recently a gene involved in PC has been linked to the type I keratin cluster[7]. As the location of the abnormalities in PC correlate with the expression characteristics of K6, K16 and K17, we screened DNA from patients with PC for mutations in these keratin genes.

Five members of a Slovenian family (A) with PC-1 were investigated (Fig. 1*a*). The grandfather (I/2), father (II/2) and daughter (III/2) are affected while the other daughter (III/1), grandmother (W/I), mother (W/II) and father's siblings are normal. Clinically, II/2 and III/2 are classic cases of PC-1 having thickened nails, palmoplantar keratoderma and leukokeratosis of the tongue (Fig. 1*b–d*). However, the grandfather (I/2) has only minor nail changes and mild keratoderma.

The helical encoding domains (1A and 2B) of K6a, K16a and K17a were amplified from genomic DNA samples. Direct sequencing showed that both helical encoding regions of K16a and K17a were normal in all family members as was the 2B helical encoding region of K6a (data not shown). However, a 3 bp deletion was identified in the 1A helical encoding region of K6a (exon 1) in the affected individuals examined (II/2, III/2) while unaffected family members were normal (Fig. 2*a*). The deletion causes a shift in the sequence of one allele making the sequencing gel difficult to read beyond the mutation. The sequence was read in the reverse direction with an intron specific primer (HK6p17R) and again clear sequence was only observed up to the deletion (data not shown). No restriction fragment length polymorphism was created and the nature of the mutation made allele specific (AS) PCR or mismatch AS-PCR difficult. However, a single PCR reaction with DNA from the affected individuals was sequenced with primers specific to either the normal (HK6p15N) or mutant (HK6p16M) allele.

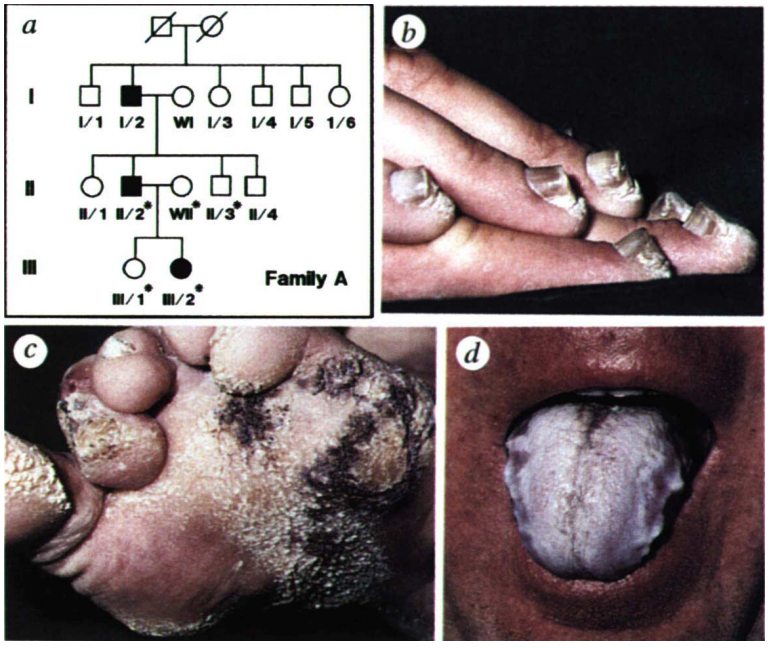

Fig. 1 Pedigree (*a*) and clinical photographs (*b–d*) of Family A. DNA was obtained from five family members (*) including two that are affected with type I pachyonychia congenita. This family showed the three typical phenotypic changes of this disease — extremely thickened nails (*b*), insular palmoplantar keratoderma (*c*) and leucoplakia (leukokeratosis) of the tongue (*d*).

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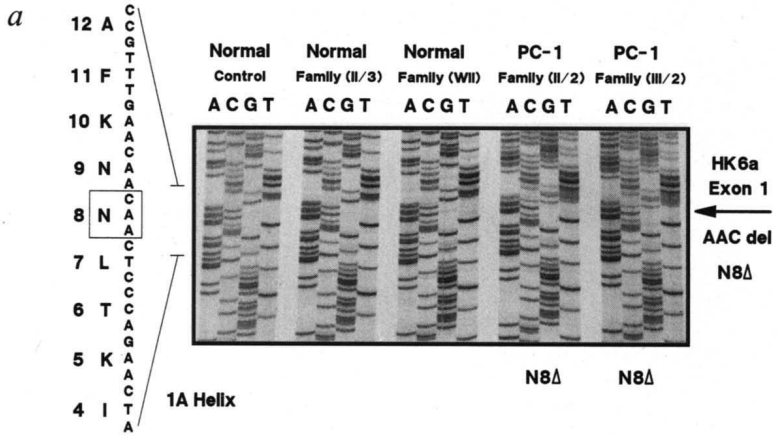

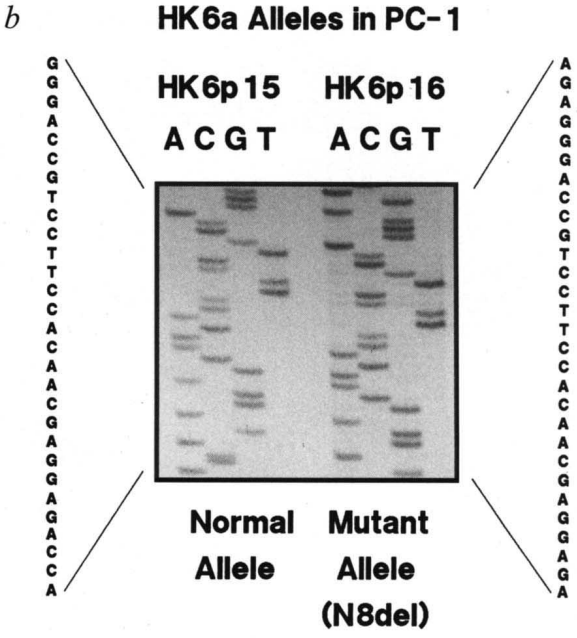

Fig. 2 Sequence of exon 1 of the K6a gene covering the region that encodes the 1A helix. *a*, Sequences from oligo HK6p14 (left to right: normal unrelated control, unaffected family members (II/3 and WII) and affected family members (II/2 and III/2). The sequences are identical in all samples up to the deletion point (arrow), then the PC-1 samples have two superimposed sequences (one from each allele). The first abnormal base is C→G, making the PC-1 samples AACAAG rather than AACAAC. *b*, Sequencing the same PCR reaction with allele specific primers eliminates this 'double-sequence' and the mutant allele is clearly 3 bp shorter than the normal allele.

Clear sequence was obtained for each allele beyond the deletion and the sequences showed a 3 bp shift when run side by side on a 6% sequencing gel (Fig. 2*b*). In DNA from normal controls and unaffected family members, the primer to the mutant allele (HK6p16M) sequenced with poor efficiency.

The deletion of a codon (AAC) from the 1A helical encoding region of K6a removes one of two adjacent asparagine residues (N170/N171) that are highly conserved in type II keratins (Fig. 3*a*,*b*). As the codon is perfectly repeated in the normal gene (AACAAC) it cannot be ascertained which of these has been removed but any disruption of this highly conserved sequence is likely to have a deleterious effect on the protein structure. Our data shows that one K6a allele has been mutated in PC patients while no alterations were found in normal family members or control DNA from 25 unrelated normal individuals.

Deletion mutations in keratin genes are not common and this is the first identified in the coding sequences of the 1A helix. So far only one other keratin deletion mutation has been reported[26], a ΔE375 in the 2B helical sequences of K14 in an epidermolysis bullosa patient. The residue deleted in K6a clearly represents a hot-spot for disease and alterations of the same asparagine have been identified in other keratins (K1, K9, K10, K17) associated with various heritable skin disorders[11,12,14,16]. This deletion results in a lateral shift of the key residues of the 1A helix initiation site, a region of the keratin molecule that is critical for normal function and this alteration is predicted to have a dramatic effect on filament assembly and stability.

These keratins (K6, K16, K17) are particularly interesting because they are not encoded by single copy genes. While some copies are non-functional pseudogenes, at least two functional cDNAs have been identified for each keratin. Tissue-specific expression of the individual isoforms has not yet been described but mutations in different isoforms are likely to give rise to a different phenotype. As there are

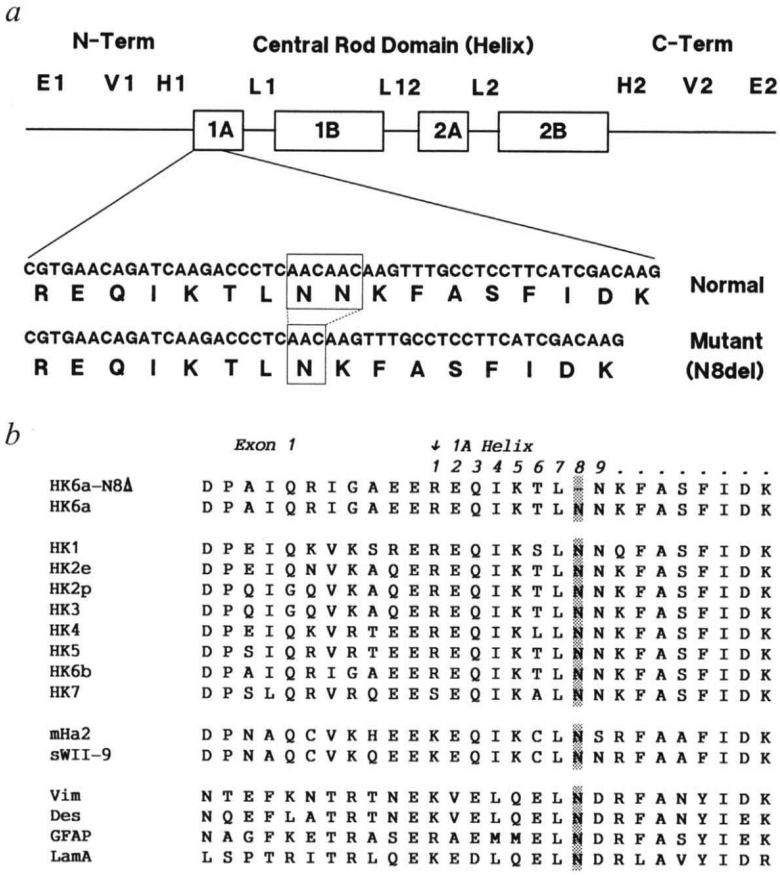

Fig. 3 *a*, Schematic of K6a protein structure showing the four α-helical domains (1A, 1B, 2A, 2B) separated by linkers (L1, L12, L2). The 3 bp deletion (AAC) in the mutant allele of the K6a gene and the location of the deleted codon (ΔN8) in the 1A helical domain of the encoded protein are shown. *b*, Amino acid sequence of the beginning of the 1A helical domain in several type II keratins (HK1-HK7), hair keratins (mHa2, sWII-9) and other IF-proteins (Vim, vimentin; Des, desmin; GFAP, glial fibrillary acidic protein; LamA, lamin A). The asparagines (N8/N9) are highly conserved and removal of either would be expected to disrupt α-helical formation and subunit interactions severely.

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at least four distinct types of PC, several types of palmoplantar keratoderma and other related disorders such as Papillon Lefèvre syndrome, steatocystoma multiplex and hydradenitis suppurativa, then mutations in different K6, K16 and K17 isoforms may explain the pathology underlying several heritable disorders. It is clear from this and other studies[11] that K6a and K16a mutations are involved in PC-1 and that K17a mutations are involved in PC-2. However, more families are now required to ascertain if mutations in K6a, K16a and K17a can completely account for all PC phenotypes and other related genodermatoses or whether other K6 and K16 isoforms or indeed other genes are involved.

Methods

Genomic DNA isolation. Blood (5–10 mls) was obtained from family members and normal volunteers, and genomic DNA prepared by standard procedures.

Keratin gene amplification (PCR). The 1A and 2B helical encoding regions of K6a, K16a and K17a were amplified by PCR. Each 20 µl reaction contained 200 ng genomic DNA, 2 µl 10× PCR buffer (500 mM KCl, 100 mM Tris HCl, 1% Triton X-100, 15 mM $MgCl_2$, pH 9), 120 ng of each oligonucleotide primer, 2 µl dNTP mix (2 mM stock) and 0.5 U of *Taq* polymerase. All reactions were initiated by a 'hot-start' procedure (7 min at 95 °C prior to adding *Taq* polymerase) and then run for 30 cycles (94 °C for 30 s, 60 °C for 50 s and 72 °C for 3 min) followed by 15 min extension (72 °C) and a cooling cycle to 4 °C. PCR of the 1A region of K6a was achieved by making an oligonucleotide to a region of K6b (exon 1)[27] conserved in type II keratins (HK6pJ1: 5'–AGATCAAGAACCTCAACAAC–3'), as the sequence of K6a in this region is unknown, and then amplifying genomic DNA together with an oligonucleotide to exon 2 of K6a (HK6pJ2R: 5'–AGGTCCTCCACCAGGTCCTGCATG–3')[28]. These PCR fragments were cloned, sequenced and a K6a isoform identified. A K6a specific primer (HK6p13R) was then made to the determined intron 1 sequence. A primer (HK6p14) was made to another region of K6b (exon 1)[27] conserved in type II keratins and used together with a K6a primer (HK6p4R, 3' non-coding) to amplify K6a cDNA from psoriatic epidermis. HK6p14 and HK6p13R were used to amplify a K6a exon 1/intron 1/exon 2 fragment from genomic DNA. The products were analysed to obtain the sequence of K6a exon 1 and a large part of intron 1. Finally, two primers were used to amplify exon 1 of K6a (sense oligo: HK6p14, 5'–CTGGGGGGCTCCAAGAGGATCTCC–3' and antisense oligo: HK6p13R, 5'–CGCACCTGAAAGAGAGACAA-GATG–3'). The 2B region of K6a was amplified with two primers determined from published sequences[28] (sense oligo: HK6p7, 5'–GATGGCCCTCAAGGATGC–3' and antisense oligo: HK6p4R, 5'–TCACTTGTGCTTTCATGGA-TACTGCCT–3').

PCR analysis and sequencing. An aliquot (4 µl) of each PCR amplified keratin gene fragment was analysed on 1.5% agarose gels, stained with ethidium bromide and photographed. The remainder (16 µl) was sequenced directly without further purification (Amersham kit #70170). Briefly, 5 µl of each PCR reaction was treated with Shrimp alkaline phosphatase (2 U) and exonuclease I (10 U) for 15 min at 37 °C followed by denaturation at 80 °C for 15 min. The amplified fragment of K6a (exon 1) was primed in each direction with specific oligonucleotides to sequences determined as detailed above (sense: HK6p11, 5'–CCAAGAGGTCAC-TGTCAACCAGAG–3' and antisense: HK6p17R, 5'–CTGCACTCTCTGCAGAGC–3') and sequenced by the dideoxy chain termination method. The oligo nucleotides used for PCR amplification (HK6p14 and HK6p13R) were also used for sequencing. After sequencing with ^{35}S-dATP (Amersham, >3,000 Ci mole^{-1}), the samples were analysed on both 6% and 8% sequencing gels (BRL). The gels were dried and exposed to X-ray film (Kodak XAR-2) for 1–3 days at RT.

Allele specific sequencing. Exon 1 and intron 1 of HK6a were PCR amplified from genomic DNA of PC-1 patients (II/2, III/2). The sequence was unreadable beyond the deletion site when a common primer was used (same in both directions). Thus, two aliquots of the same PCR reaction were sequenced separately (Amersham kit #70170) with allele-specific primers (normal allele: HK6p15N, 5'–CAGATCAAGACCCTCAACAACAAG–3' and mutant allele: HK6p16M, 5'–CAGATCAAGACCCTCAACAAGTTT–3'). The reactions were run side by side on a 6% sequencing gel, dried onto Whatman paper and exposed to X-ray film.

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