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Keratin K6c Mutations Cause Focal Palmoplantar Keratoderma

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The palmoplantar keratodermas (PPKs) are a large group of clinically and genetically heterogeneous genodermatoses. The gene defects underlying many PPKs still need to be resolved to facilitate definitive molecular diagnosis and genetic counseling. Dominant-negative mutations in any of the four identified keratin genes, *KRT6A*, *KRT6B*, *KRT16*, or *KRT17*, cause pachyonychia congenita (PC), characterized by hypertrophic nail dystrophy and other ectodermal features. In PC, focal PPK (FPPK) is the most painful and debilitating phenotypic feature. Some families presenting with FPPK alone, or with minimal nail changes, carry mutations in *KRT16*; however, most FPPK families do not harbor mutations in any of these keratin genes. Here, we report three unrelated families who presented with familial FPPK with minor or absent nail changes. The four PC/FPPK-related keratin genes were excluded; however, mutational analysis of the recently identified *KRT6C* gene, encoding keratin K6c, showed heterozygous in-frame deletion mutations in all three kindreds. Affected members of Families 1 and 2 carried the same mutation, p.Asn172del. In Family 3, the mutation p.Ile462-Glu470del co-segregated with the disease. *KRT6C* was shown to be expressed in the plantar epidermis using reverse transcription-PCR, consistent with the phenotype observed in this tissue. These data expand the genetic testing repertoire for the PPKs.

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INTRODUCTION

The palmoplantar epidermis is a histologically and developmentally distinct region of the epidermis that is highly specialized to resist the high degree of mechanical trauma to which this tissue is subjected during everyday life (Swensson *et al.*, 1998). To this end, the palmoplantar epidermis expresses a very large number of structural reinforcement molecules including many keratins and associated proteins (McLean, 2003). The palmoplantar keratodermas (PPKs) are a large group of genodermatoses characterized by keratinocyte fragility, blistering, and thickening (hyperkeratosis) within the palmoplantar epidermis (Judge *et al.*, 2004; Itin and Fistarol, 2005). The OMIM clinical genetics database lists 59 distinct disorders that include PPK as a part of the phenotype, and there are a number of forms of PPK recognized by dermatologists that do readily fall into these categories (Stevens *et al.*, 1996; Judge *et al.*, 2004). The major subdivisions of the PPKs are diffuse, focal (including striate), and punctate, according to the pattern of hyperkeratotic lesions observed clinically.

Focal PPK (FPPK) is one of the cardinal features of pachyonychia congenita (PC; OMIM #167200, #167210), a rare autosomal-dominant keratin disorder caused by mutations in the differentiation-specific keratin genes *KRT6A* (Bowden *et al.*, 1995), *KRT6B* (Smith *et al.*, 1998), *KRT16*, or *KRT17* (McLean *et al.*, 1995; Liao *et al.*, 2007). Blisters occur under the hyperkeratotic lesions on the plantar surfaces, which are often extremely painful, resulting in restricted mobility (Leachman *et al.*, 2005). Palmar lesions are less prominent and tend to occur in response to occupational mechanical trauma. Other clinical characteristics of PC include hypertrophic nail dystrophy and other ectodermal features (Leachman *et al.*, 2005). FPPK can also present as a separate clinical entity with subtle or absent nail changes and, interestingly, mutations in *KRT16* have also been reported in some of these families (Shamsher *et al.*, 1995; Smith *et al.*, 2000, 2005; Liao *et al.*, 2007). This condition has been described previously as non-epidermolytic PPK (OMIM #600962); however, histological and ultrastructural evidence of epidermolysis has been reported in at least some patients with *KRT16* mutations (McLean *et al.*, 1995; Liao *et al.*, 2007); hence, this diagnostic distinction is somewhat ambiguous. In many FPPK families, no mutations

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Abbreviations: FPPK, focal PPK; PC, pachyonychia congenita; PPK, palmoplantar keratoderma

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can be detected in any of the four PC-related keratins (F.J.D. Smith and W.H.I. McLean, unpublished observations).

There are at least 54 functional keratin genes expressed in a tissue- and differentiation-specific manner that have a major structural role in the epidermis and its appendages (Schweizer *et al.*, 2006). Earlier, the existence of as many as six copies of nearly identical *KRT6* genes have been proposed in the human genome (Takahashi *et al.*, 1995). However, with the availability of finished genome sequence for the type II keratin gene cluster on chromosome 12q13.13, it was shown that there are only three tandemly repeated epidermal K6 isogenes, *KRT6A*, *KRT6B*, and *KRT6C*, encoding the proteins K6a, K6b, and K6c, respectively (Rogers *et al.*, 2005). The naming of these genes and proteins has recently been agreed by a nomenclature group representing the keratin field (Schweizer *et al.*, 2006). The previously reported epidermal K6-like genes are now believed to be polymorphic variants or more distantly related type II keratins of the hair follicle. The coding sequence of all the three authentic epidermal K6 isogenes is very similar, and there is a high degree of overlap in the tissue expression pattern of K6a and K6b but with some distinct differences (Smith *et al.*, 1998). There are no reports on the tissue expression range of K6c, and as the amino-acid sequence of all the three K6 proteins is almost identical, it has not been possible to generate isoform-specific antibodies. As even the upstream promoter regions of these duplicate genes are highly homologous, we predicted there might be some degree of overlap in the expression pattern of K6c with K6a and/or K6b, in which case *KRT6C* might also be a candidate gene for FPPK. Here, we present a study of three families with autosomal-dominant FPPK with very minor or absent nail changes for which we have identified heterozygous in-frame deletion mutations in the K6c gene.

RESULTS

Clinical features of focal keratoderma in three families

In Family 1, there were two individuals affected by plantar FPPK in two generations with male-to-male transmission, suggestive of autosomal-dominant inheritance. Neither patient had nail changes (Figures 1 and 2). Plantar blistering was a feature in both cases and a diagnosis of epidermolysis bullosa simplex was originally considered. In Family 2, there were six individuals affected by FPPK in four generations, again with autosomal-dominant inheritance (Figures 1 and 2). Affected persons had minor nail changes, specifically nail hypertrophy affecting only the fifth toe, but this did not resemble the classic presentation of PC and no other ectodermal findings consistent with PC were identified. In Family 3, there were nine affected individuals with FPPK (Supplementary Figure S1). The proband experienced blistering in the summer months alleviated by low-dose acitretin. She had subtle leukokeratosis along the buccal bite line and occasional nail splinter hemorrhages. Autosomal-dominant transmission was also evident in Family 3 (Figure 1).

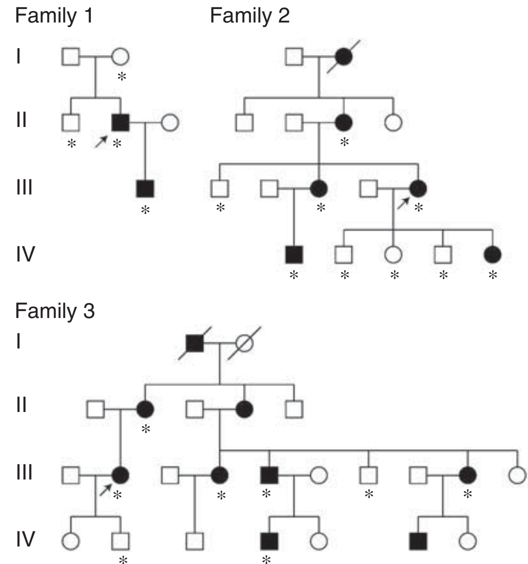

Figure 1. Pedigrees of Families 1–3 showing autosomal-dominant inheritance with male-to-male transmission evident in Families 1 and 3. Asterisk indicates the individuals whose DNA was studied here.

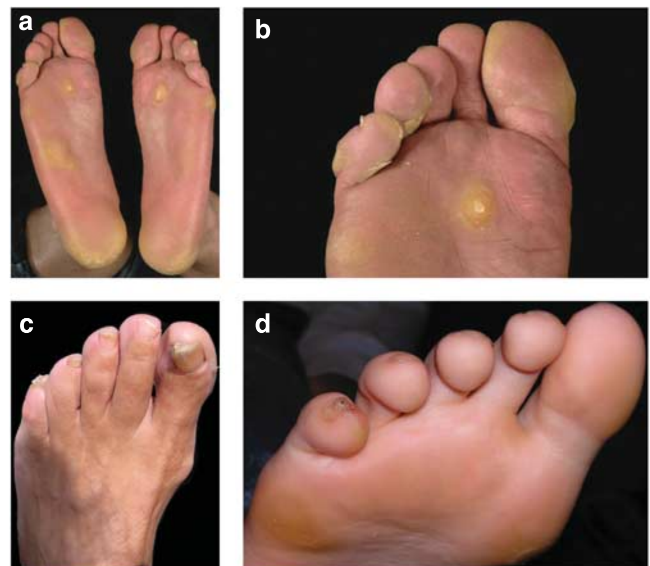

Figure 2. Clinical features. (**a** and **b**) FPPK on the soles of the feet of the proband from Family 1. (**c** and **d**) Minor nail changes and (**d**) FPPK on the soles of the feet of two affected individuals in Family 2. The clinical features of Family 3 are shown in Supplementary Figure S1.

K6c helix 1A defect in two unrelated FPPK families

Microsatellite markers within the type I keratin loci on chromosome 17 excluded the *KRT16* and *KRT17* genes in Families 2 and 3; however, markers within the type II keratin locus on chromosome 12 were consistent with linkage, suggesting the K6 genes as candidates (data not shown). Family 1 was too small for informative linkage analysis. Sequencing of all coding regions and splice sites of the *KRT6A* and *KRT6B* genes did not show any potentially pathogenic mutations in the three families. Reverse transcriptase-PCR with intron-spanning primers specific for the *KRT6C* transcript showed that this gene, similar to *KRT6A* and

KRT6B, is expressed in mRNA derived from the plantar epidermis (Supplementary Figure S2), suggesting that this might be an appropriate candidate gene for FPPK. Consistent with this hypothesis, mutational analysis of the *KRT6C* gene, encoding keratin K6c, showed mutations in all three families. Specifically, all affected members of Families 1 and 2 carried a heterozygous 3-bp deletion mutation (Figure 3), designated p.Asn172del (c.516_518delCAA), within the conserved N-terminal end of the helix 1A domain of the K6c polypeptide. The protein sequence motif is one of the two, at either ends of the keratin rod domain, known to be functionally critical in mediating molecular overlap interactions during the intermediate filament assembly (Steinert *et al.*, 1993; Herrmann *et al.*, 2000; Strelkov *et al.*, 2001). The mutation was absent in the unaffected members of both kindreds and, therefore, fully co-segregated with FPPK in these unrelated families. Owing to the high homology of the three K6 genes, the mutation was independently confirmed in a second, smaller PCR fragment spanning exon 1 using an alternative set of primers designed to be specific for *KRT6C*. A fluorescently labeled version of this short PCR was used to screen anonymous population control DNA samples to confirm the mutation. Interestingly, 3 of 335 population controls were found to be heterozygous carriers of the mutation p.Asn172-del mutation, which was confirmed by sequencing. We speculate that this K6c mutation may exist at a low level within the general population, but that the relatively mild plantar callus formation associated with this mutation does not always come to clinical attention. The ethical approval framework covering the use of our anonymous population control series did not allow for retrospective clinical evaluation of these mutation carriers.

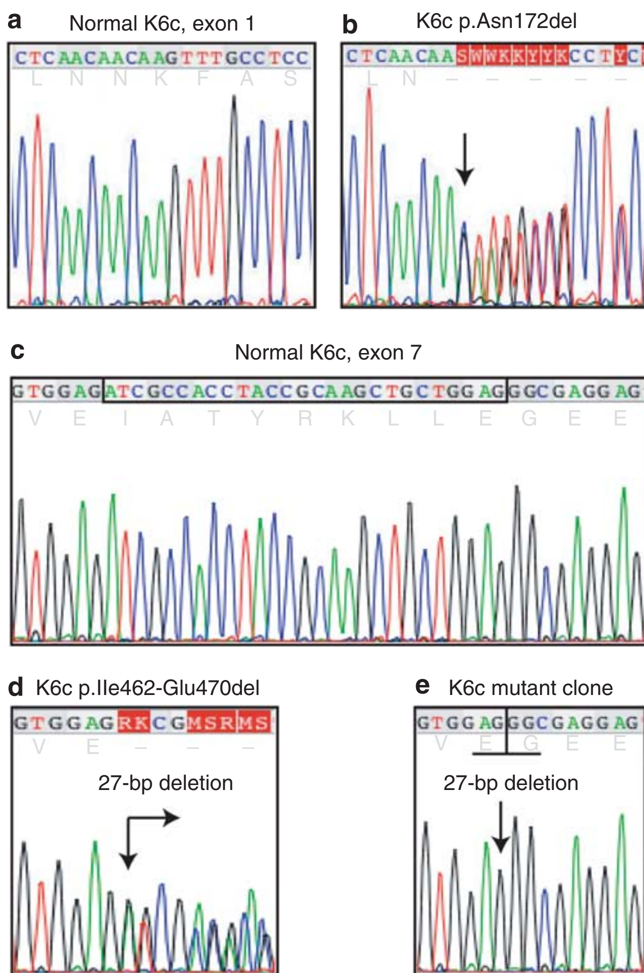

Figure 3. Mutational analysis of *KRT6C* in FPPK families. (**a**) DNA sequence of exon 1 of *KRT6C* derived from a normal control individual, showing codons 170–176. (**b**) The equivalent region shown in panel **a** from an affected individual in Family 2 showing the deletion mutation p.Asn172del (c.516_518delCAA). (**c**) DNA sequence of exon 7 of *KRT6C* derived from a normal control individual, showing codons 460–473. (**d**) Analogous region of *KRT6C* as shown in panel **c** derived from the proband in Family 3, showing a 27-bp, p.Ile462-Glu470del (c.1384-1410del27). (**e**) *KRT6C* exon 7 sequence from a clone derived from a PCR product from the proband in Family 3 to confirm the extent of the mutation p.Ile462-Glu470del. Arrows indicate position of mutation.

K6c helix 2B deletion in a third FPPK family

DNA sequencing of the *KRT6C* gene from the proband of the third family showed an in-frame heterozygous 27-bp deletion mutation within the 2B domain of K6c protein (Figure 3), designated as p.Ile462-Glu470del (c.1384-1410del27). This mutation causes an in-frame deletion of nine amino acids, leading to the loss of most of the highly conserved helix termination motif from the helix 2B domain of the K6c protein—a sequence known to be critically important for the keratin intermediate filament assembly. The mutation was confirmed by cloning a PCR fragment spanning exon 7; sequences from both normal and mutant clones were obtained. To exclude this mutation as a polymorphism, 354 population control samples as well as 2 unaffected and 6 affected family members were screened by either sequencing or analyzing small PCR fragments spanning exon 7 on high-resolution 3% agarose gels. Only the six affected individuals from Family 3 carried the deletion mutation, K6c p.Ile462-Glu470del.

DISCUSSION

In summary, we report the first mutations to be discovered in the third human K6 gene, *KRT6C*, in cases presenting with PPK and very minor or absent nail changes. The affected individuals in all three families have relatively mild, site-restricted keratoderma compared with the much more severe form seen in many cases of the related condition PC (Leachman *et al.*, 2005), although the affected family members, nevertheless, all experience a significant amount of pain and discomfort from their plantar keratoderma, requiring secondary medical care.

The p.Asn172del mutation is located within the helix initiation motif domain of K6c, a region well recognized in other keratins to be a 'hotspot' for pathological mutations (Szeverenyi *et al.*, 2008), as shown by the data collected within the Intermediate Filament Database. The analogous mutation to K6c p.Asn172-del has been reported in both *KRT6A* (Bowden *et al.*, 1995; Liao *et al.*, 2007) and *KRT6B* (Sharma and Stein, 2007) in cases presenting with typical PC and in patients with both severe FPPK and severe nail

dystrophy. Similarly, the equivalent mutation has been seen in another type II keratin gene, *KRT4*, in patients with white sponge nevus, an autosomal-dominant disorder of the mucosal epithelia (Rugg *et al.*, 1995).

We previously reported a large deletion mutation in *KRT16* in a family also presenting with PPK, in which most of the helix termination motif of the K16 protein was deleted (Smith *et al.*, 2000), which is very similar to the genetic lesion identified here in Family 3 involving K6c. One possible explanation for the milder phenotype observed in these cases is that mutant keratin lacking the highly conserved termination motif would be less able to interact in critical stages of the keratin filament assembly, and thus might be more likely to be excluded from the assembly process altogether (Steinert *et al.*, 1993; Herrmann *et al.*, 2000). This could result in a milder dominant-negative effect than that of a mutant keratin that carries a missense or a small deletion mutation within one of the highly conserved termination domains.

An alternative explanation for the milder phenotype in the FPPK families with mutations in *KRT6C* compared with those with mutations in *KRT6A* and *KRT6B* is that K6c may have a more restricted expression pattern. Although the number of cases studied is still too small to draw any real conclusions, this is perhaps the most likely explanation as the equivalent Asn172del mutation in *KRT6A* is the most common cause of the more severe PC/FPPK phenotype (Smith *et al.*, 1999; Terrinoni *et al.*, 2001; McLean *et al.*, 2005; Liao *et al.*, 2007). To date, little is known about the expression pattern of K6c and expression studies have been hampered by the inability to prepare K6 isoform-specific antibodies. The phenotype observed in our three families strongly implies overlap with *KRT6A* and *KRT6B* gene expression. The milder phenotype in the FPPK families with mutations in *KRT6C*, plus the fact that some individuals in the general population carry one of these mutations, p.Asn172del, implies that K6c is expressed at much lower levels in thick skin and nail than K6a and K6b. Thus, these *KRT6C* mutations may be a common genetic predisposing factor for the plantar callus formation. This could be addressed by future prospective studies of large populations that have been examined by dermatologists. In any case, the identification of K6c mutations expands the genetics of keratoderma and sheds light on the function of the keratin gene family, 22 of which are now associated with human epithelial disorders.

MATERIALS AND METHODS

Genetic linkage analysis

Microsatellite markers D17S800, D17S1861, D17S1868, and KRT12 (Corden *et al.*, 2000) within the type I keratin locus on chromosome 17 and the marker D12S368 within the type II keratin locus on chromosome 12 were used. Fluorescently labeled PCR fragments were genotyped by sizing on an ABI 3100 automated DNA sequencer (Foster City, CA). All PCRs were carried out according to the manufacturer's protocol for the ABI PRISM Linkage Mapping Set-HD5 (Foster City, CA). The study was carried out in accordance with the Declaration of Helsinki Principles and approved by local Research Ethics Committees. All subjects or the subjects' guardians gave written informed consent.

KRT6C-specific reverse transcriptase-PCR

mRNA was extracted from the plantar skin using the QuickPrep Micro mRNA Purification Kit (GE Healthcare UK Ltd., Little Chalfont, Bucks, UK) and reverse transcribed using AMV reverse transcriptase (Promega, Southampton, UK). cDNA was amplified using primers KRT6C-4F (forward: 5′-CAGCCTCCAACGCTCGC CA-3′) and KRT6C-2149R (reverse: 5′-GGGCGGGGGTTCACAA TACT-3′) using Expand High Fidelity PCR buffer (Roche Diagnostics Ltd., Burgess Hill, UK) containing 1.5 mM of $MgCl_2$, 4% DMSO, and 1 U of Expand High Fidelity Enzyme mix (Roche). The following PCR conditions were used: 94 °C for 5 minutes ×1, followed by 94 °C for 30 seconds, 54 °C for 1.5 minutes, and 72 °C for 3 minutes ×35 and 72 °C for 5 minutes ×1. This PCR amplifies a 2,165-bp fragment.

Specific full-length genomic *KRT6C* PCR

Primers KRT6C-FLF1 (forward: 5′-ATGGCTGGGGAATGGCTTT AG-3′) and KRT6C-FLR1 (reverse: 5′-GGGGCGGGGGTTCACAA TACT-3′) were used with PCR buffer containing 2.5 mM of $MgCl_2$ and Takara (Lonza Biologics PLC, Slough, UK) LA Taq polymerase. The following PCR conditions were used: 94 °C for 1 minute ×1, followed by 98 °C for 5 seconds and 68 °C for 15 minutes ×30 and 72 °C for 10 minutes ×1. The resultant 7,034 bp PCR products were purified using QiaQuick PCR spin columns (Qiagen House, Craloley, UK) or ExoSAP (using Exonuclease 1 and Shrimp Antarctic Phosphatase) and sequenced using internal primers on an ABI 3100 Automated DNA sequencer. The presence of several diagnostic sequence variations confirmed that this set of primers and conditions is specific for *KRT6C* gene and does not amplify *KRT6A* or *KRT6B*.

Specific *KRT6C* exon 1 PCR

Primers KRT6C-1L (forward: 5′-CCAGCCTCCAACGCTCGCCA-3′) and KRT6C-1R (reverse: 5′-AGGGCATGGCACTGGCTCAC-3′) were used in GeneAmp PCR Buffer (Applied Biosystems, Foster City, CA) containing 1.5 mM of $MgCl_2$, 4% DMSO, and 1 U AmpliTaq Gold (Applied Biosystems). The following PCR conditions were used: 94 °C for 12 minutes ×1, followed by 94 °C for 30 seconds, 58 °C for 45 seconds, and 72 °C for 1 minute ×30, and 72 °C for 5 minutes ×1. The resultant fragment was 603 bp. Population control samples were screened by DNA sequencing, using the above PCRs, or by fluorescent PCR using modified *KRT6C* exon 1 primers. The forward primer was labeled with 6-FAM, and the 'GTTTCTT' sequence was added to 5′-end of the reverse primer to improve adenylation of 3′ end of PCR product. PCR conditions were as mentioned above, products were diluted and analyzed on an ABI 3100 Automated DNA Sequencer.

Specific *KRT6C* exon 7 PCR

Primers KRT6C-e7F3 (forward: 5′-GTTACAGGACCCTGGTTCACTA-3′) and KRT6C-e7R3 (reverse: 5′-AGGAAGTCGCGTCAGTTACCT-3′) were used with Expand High Fidelity PCR buffer (Roche) containing 1.5 mM of $MgCl_2$, 4% DMSO and 1 U Expand High Fidelity Enzyme Mix (Roche). The following PCR conditions were used: 94 °C for 5 minutes ×1, followed by 94 °C for 30 seconds, 59 °C for 45 seconds, and 72 °C for 1 minute ×30 and 72 °C for 5 minutes ×1. The resultant PCR fragment was 330 bp in size.

Internet resources

Human Intermediate Filament Database (http://www.interfil.org)

Online Mendelian Inheritance in Man (OMIM) (http://www.ncbi.nlm.nih.gov/omim/)

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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