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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.
A mutation detection strategy for the human keratin 6A gene and novel missense mutations in two cases of pachyonychia congenita type 1

Smith FJD, McKenna KE, Irvine AD, Bingham EA, Coleman CM, Uitto J, McLean WHI. A mutation detection strategy for the human keratin 6A gene and novel missense mutations in two cases of pachyonychia congenita type 1.


Abstract: Pachyonychia congenita type 1 (PC-1) is an autosomal dominant ectodermal dysplasia characterized by hypertrophic nail dystrophy, focal non-epidermolytic palmoplantar keratoderma and variable features of oral leukokeratosis and follicular keratosis. Previously, we have shown that this disease can be caused by mutations in type I keratin K16 and one mutation has been reported in its type II keratin expression partner, K6a. Mutation analysis for K6a has been hampered by the presence of multiple copies of the K6 gene in the human genome, of which some are expressed and others are pseudogenes. Here, we describe a mutation detection strategy where the entire KRT6A gene, ~7 kb, is specifically amplified by long-range PCR. Using this technique, we have detected two novel mutations in the 1A domain of the K6a polypeptide, N171K and F174S. Mutations were confirmed in the affected individuals and were excluded from 50 unaffected unrelated individuals by restriction enzyme analysis of KRT6A PCR products. Additionally, mutation N171K was confirmed by RT-PCR in mRNA derived from lesional palmoplantar epidermis of an affected individual, confirming the specificity of the genomic PCR for the functional K6a gene. This, together with a similar strategy which we have developed for the K16 gene, provide a robust system for mutation detection and prenatal diagnosis for patients with PC-1.

Key words: keratin – pachyonychia – K6 – genodermatosis – intermediate filaments

Dr W. H. Irwin McLean, Epithelial Genetics Group, Department of Molecular and Cellular Pathology, Ninewells Hospital and Medical School, Dundee DD1 9SY, UK
Tel.: +44 1382 425518
Fax: +44 1382 425519
E-mail: w.mclean@hgp.mrc.ac.uk
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Introduction

Keratins are the intermediate filament proteins expressed in epithelial cells (1). Type I and type II keratins form heterodimers through coiled-coil association of their α-helical rod domains, which further polymerize to form 10 nm filaments (2). The precise nature of this higher-order polymerization remains unknown. However, it is clear from many experimental observations that two short sequences, the helix boundary motifs, which flank the rod domain are critical for molecular overlap interactions in the construction of mature keratin filaments (3–6). The function of intermediate filaments was unclear until the early 1990s when transgenic experiments (7) and subsequent studies of human keratin diseases (8–10) revealed that epithelial cells whose keratin cytoskeleton is compromised by mutation are fragile and lyse under mild mechanical trauma (11). This cell fragility leads to clinical phenotypes characterized by blistering and/or hyperkeratosis of the epithelial tissues expressing the mutant keratin (12, 13). One such epithelial fragility disorder is pachyonychia congenita (PC), a group of ectodermal dysplasias...
whose common characteristic is hypertrophic nail dystrophy (14).

Two main clinical subtypes of PC are recognized by Gorlin et al. (14), the Jadassohn–Lewandowsky type (PC-1; MIM#167200) first described in 1906 (15) and the Jackson–Lawler type (PC-2; MIM#167210), identified in the 1950s (16). Both variants are characterized by nail dystrophy, focal non-epidermolytic palmoplantar keratoderma (FNEPPK) and variable features of hoarseness, angular cheliosis, oral leukokeratosis and follicular keratosis. Multiple sebaceomas, which appear at puberty, show complete penetrance in individuals with PC-2, making this the key distinguishing feature of the two subtypes. Natale teeth and pili torti also appear to be uniquely associated with PC-2, although these show incomplete penetrance. Following a report of genetic linkage between PC and markers close to the type I keratin cluster on 17q (17), we showed that PC-1 and the closely related phenotype of FNEPPK without nail involvement can be caused by mutations in keratin K16, a type I keratin expressed in the tissues affected by the disease (18). Similarly, we have shown that mutations in K17 cause PC-2 and the related phenotype of sebaceoma multiplex (18–20). Another group later reported the complementary type II keratin mutation in K6a in a single PC-1 family (21). Recently, we reported the first mutation in K6b in a PC-2 family, demonstrating both genetic heterogeneity in PC-2 and that K6b and K17 are co-expressed in differentiated epithelial structures (22).

The existence of multiple copies of all 4 genes known to be involved in PC exist as multiple copies in the human genome has hampered mutation analysis (23–26). In particular, K6a analysis is made difficult by the presence of at least 6 copies of this gene, at least 2 of which are expressed (25). Recently, we cloned the functional K16 gene and 2 K16 pseudogenes, allowing development of a mutation detection strategy based on specific long-range PCR (26). Here, we describe a similar strategy for the K6a gene (KRT6A) which we have used to identify 2 novel mutations in PC-1 families, 1 of which was confirmed by RT-PCR, demonstrating the specificity of the genomic PCR for the functional K6a gene.

Materials and methods

DNA sequence analysis

DNA alignments were performed using the Intelligenetics Geneworks version 2.5 program (Oxford Molecular Systems). Oligonucleotide primers were designed using Oligo version 4.0 (National Biosciences Inc.).

Mutation detection

Poly(A)° mRNA extracted from frozen 3 mm punch biopsy (available from proband of family 1 only) using the QuickPrep Micro mRNA Purification Kit (Pharmacia) was reverse transcribed using oligo(dT18) and AMV-RT enzyme (Promega). A 1761 bp cDNA fragment was amplified using primers K6A.P1 (+strand 5’CCA GCC TCT CAC ACT CTC TCT 3’) and K6A.P2 (−strand 5’GAC CGA GAG CTA GCA GAG GC 3’) in standard 10× PCR buffer (1.5 mM MgCl2) containing 4% DMSO. Reactions were subjected to a “hot start” with 1U AmpliTaq polymerase (Perkin-Elmer). Amplification conditions were (94°C 5 min) ×1; (94°C 30 s, 60°C 1 min, 72°C 2 min) ×35; (72°C 5 min) ×1. PCR was performed using a Hybaid Omnigene thermocycler.

Genomic DNA was extracted from whole blood by standard methods. A genomic DNA fragment of approximately 7.0 kb was amplified using K6A.P1 and K6A.P2 (above) in High Fidelity buffer (Boehringer–Mannheim) containing 1.5 mM MgCl2 and 4% DMSO. Reactions were subjected to a “hot start” with 1U High Fidelity thermostable DNA polymerase mix (Boehringer–Mannheim). PCR conditions were (94°C 5 min) ×1; (94°C 30 s, 60°C 1.5 min, 72°C 2.5 min) ×35; (72°C 5 min) ×1.

PCR products were purified using QIA quick PCR purification kit (Qiagen) and directly sequenced on an ABI 377 automated sequencer using primers K6A.P7 (+strand 5’GAG CAG CGT GGG GAG ATG GC 3’) and K6A.P2 (above) to sequence the helix termination peptide region of K6A. Primer HK6.P14 (21) was used to sequence the helix initiation peptide region.

Mutation confirmation

As the N171K mutation does not alter any restriction enzyme site, a mismatch primer was designed to create a new Dral restriction enzyme site in combination with the mutation. Genomic DNA was first amplified with primers HK6P14 and HK6P13R (21) in buffer E (PCR optimizer kit, In- vitrogen) without DMSO. Reactions were “hot started” with 1 U AmpliTaq. The PCR conditions were (94°C 5 min) ×1; (94°C 30 s, 63°C 1 min, 72°C 2 min) ×38; (72°C 5 min) ×1. PCR products were diluted 1/1000 and a second round of amplification was performed using forward primer K6A.P10 (+strand, 5’GAG CGT GAA CAG ATC AAG ACC TTT AA 3’, mismatch bases underlined) and K6A.P11 (−strand 5’GCG CAC TCC AGA GAT CCC AT 3’) in standard PCR buffer containing 1.5 mM MgCl2 and 4% DMSO.
K6a mutations in pachyonychia congenita type 1

is a 15-year-old female and has had features of pachyonychia congenita since birth. All fingernails and toenails are characteristically thickened (Fig. 2a & c). She has marked focal plantar keratoderma (Fig. 2b) over pressure points with blistering of her feet during the summer months or following prolonged walking. Oral leukokeratosis is present affecting the sides of the tongue in addition to the buccal mucosa (Fig. 2d). Follicular keratosis was also present on the extensor aspect of her elbows, knees and lower legs. Hair and teeth are normal. Her mother and brother are also similarly affected, however, her brother has more severe oral involvement.

Case report, family 2. This patient, a sporadic case, presented to medical attention aged 4 years. There was marked subungual hyperkeratosis of all twenty nails and keratosis pilaris affecting the lateral aspect of the upper arms and the cheeks. The patient did not have any evidence of oral leukokeratosis or palmoplantar keratoderma.

Specific RT-PCR and genomic PCR for K6a

By alignment of the multiple K6 sequences, PCR primer sequences were designed to specifically amplify the K6a cDNA without contamination from the other isoforms. RT-PCR was performed using cDNA derived from primary keratinocyte cultures and palmoplantar biopsy material. Direct automated sequencing of PCR products generated under the optimized conditions described revealed that this reaction produces only the published K6a cDNA sequence (25). Similarly, re-optimization of these primers for long-range genomic PCR produced a 7 kb fragment. Sequence analysis revealed a single genomic sequence with exons correspond-

Figure 2. Clinical features of pachyonychia type 1 in the proband of family 1. Characteristic hypertrophic nail dystrophy seen on the fingernails (a) and toenails (c); focal non-epidermolytic keratoderma is evident on the pressure points of the soles (b); and oral leukokeratosis, which is not fully penetrant in the disease, is however seen here on the lingual mucosa (d).
ing to the published K6a cDNA sequence interrupted by 8 introns at the predicted locations specific for type II keratins (1), bounded by canonical splice sites (data not shown).

Detection and confirmation of novel K6a mutations in PC-1

To test the reliability of the long-range genomic PCR in detecting bona fide K6a mutations, snap-frozen plantar biopsy material was obtained from the proband in family 1 (Fig. 1). The full length K6a cDNA was amplified by RT-PCR using mRNA extracted from this tissue and was subjected to direct sequencing in the regions encoding the helix boundary motifs, mutational hotspots in keratin genes (12). A heterozygous missense mutation 513C→A was detected in the proband, resulting in the predicted amino acid change N171K (Fig. 3a & b). The full-length K6a gene (KRT6A) was amplified from the patient’s genomic DNA by long-range PCR as above. Direct sequencing of this PCR fragment revealed the identical N171K mutation in exon 1 of the KRT6A gene. In addition, exon 1 of the KRT6A gene was amplified from the patient’s DNA using the primers reported previously (21). This PCR reaction had not previously been confirmed at the mRNA level to be specific for the K6a isoform, which is particularly important when dealing with multiple copy genes. Again, direct sequencing revealed the same mutation, N171K, confirming specificity of these primers.

Mutation N171K does not alter any known restriction enzyme site and so a mismatch primer was designed which in combination with the mutation creates a DraI recognition site. DraI digestion of K6a-specific genomic PCR products derived from the affected persons in family 1 revealed an additional band confirming the mutation (Fig. 3d).

![Image of figure 3 showing the detection and confirmation of K6a mutations in PC-1 families.](image)

Figure 3. Detection and confirmation of K6a mutations in PC-1 families. (a-c) Direct automated sequencing of specific K6a PCR products in the region of exon 1 encoding the helix initiation motif, reverse strand shown in all cases. (a) Sequence derived from a normal individual. (b) DNA sequence derived from the proband in family 1 showing heterozygous missense mutation 513C→A predicting the amino acid change N171K. (c) Sequence derived from the proband in family 2 showing heterozygous missense mutation 521T→C, which predicts amino acid change F174S. (d) Confirmation of mutation N171K in family 1 by DraI digestion of K6a PCR products. Lane 1, molecular weight markers; lanes 2 & 3, digest derived from the proband in family 1 and her affected brother respectively; lanes 4–8, digests derived from normal unrelated individuals. (e) Confirmation of mutation F174S by BglII digestion of K6a PCR products. Lane 1, molecular weight markers; lane 2, digest derived from the proband in family 2; lanes 3–7, digests derived from normal unrelated individuals.
This assay was also used to exclude the mutation from 50 normal unrelated individuals.

In the proband of family 2, a heterozygous missense mutation 521T→C predicting the amino acid change F174S was identified by direct sequencing of full-length K6a genomic PCR products (Fig. 3a & c). As above, this mutation does not alter any known restriction enzyme site and so a mismatch primer was designed to create a new BglII site in combination with the mutation. BglII digestion of K6a-specific PCR products derived from the proband in family 2 revealed an additional cut band which was not observed in normal controls (Fig. 3e). This assay was used to exclude the mutation from 50 unaffected unrelated persons.

Discussion

Mutation detection for all exons of the KRT6A gene

Four keratins have now been identified in which mutations cause pachyonychia congenita, namely K6a and K16 in PC-1 (18, 21, 26); K6b and K17 in PC-2 (18-20, 22). Mutation detection for the genes encoding these proteins had been made difficult by the presence of multiple pseudogenes in the case of K16 (23–26) and K17 (24). In the case of the K6 isoforms, the situation is further complicated by the existence of 6 or more genes, some expressed and some pseudogenes, the coding sequences of which only differ by a few isolated base pairs (25). One specific PCR reaction has been reported for exon 1 of the KRT6A gene, which encodes K6a, however this PCR and the single mutation reported was not confirmed at the mRNA level (21). Here, we describe primers and conditions for long-range PCR amplification of all exons of the KRT6A gene which we have used to identify two novel mutations N171K and F174S in PC-1 kindreds. In one case, frozen biopsy material was available from plantar epidermis, a tissue where K6a is constitutively expressed, allowing confirmation of mutation N171K by RT-PCR specific for the K6a cDNA. In addition to the mutation, this cDNA sequence was found to possess the small number of sequence differences diagnostic for K6a (25). We also confirmed this mutation using the previously reported exon 1 genomic primers (21), showing that this PCR is also specific for K6a. Having established the reliability of the genomic PCR to identify K6a mutations, we analyzed a second PC-1 family which was found to carry mutation F174S. Neither mutation has been previously reported in any keratin. Both mutations were excluded from 50 unaffected individuals and therefore are unlikely to be polymorphisms in the normal population. We conclude therefore that we have achieved our goal of developing a mutation detection strategy for all exons of the K6a gene. Reliable mutation detection based genomic DNA rather than cDNA sources also opens up the possibility of prenatal diagnosis for PC-1 in the future.

Novel PC-1 mutations in K6a

Mutations in either K6a or K16, keratins which are co-expressed in the differentiated keratinocytes of the nail bed, palmoplantar epidermis and in mucosal tissues (27), result in PC-1: a disease characterized by fragility and subsequent hypertrophy of these specific epithelia. Here, we describe 2 new mutations in K6a, confirming that defects in this gene result in the PC-1. Only 1 K6a mutation in a Slovenian PC-1 kindred has been published previously, a 3 bp deletion causing loss of one amino acid, ΔN171 (21). The PC-1 phenotype in that family was not dissimilar to that seen in the 2 families seen here carrying missense mutations N171K and F174S in the same highly conserved region of the K6a polypeptide. There is a considerable body of evidence that this region of the keratin rod domain is involved in end-to-end interactions between dimers in the construction of keratin filaments (3–6). Furthermore, mutations in these regions in a total of 17 keratin genes have been associated with the most severe epithelial fragility phenotypes (12, 22, 28–30), so that the K6a mutations described here are predicted to be highly pathogenic. In the case of K16, the expression partner of K6a, there have been reports of certain mutations producing the milder phenotype of FNEPPK without nail changes or oral involvement (26–31). We hypothesize that some mutations in K6a might also produce this phenotype, perhaps mutations located outside of the helix boundary motifs analogous to those seen in the mild variants of EBS. The availability of genomic PCR for all regions of the K6a gene will allow mutation analysis in FNEPPK kindreds to test this hypothesis, in addition to routine molecular analysis of K6a defects in further PC-1 cases.

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