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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.
Cloning of Multiple Keratin 16 Genes Facilitates Prenatal Diagnosis of Pachyonychia Congenita Type 1

Frances J. D. Smith1†, Victor A. McKusick2, Karl Nielsen3, Ellen Pfendner3, Joumi Uitto4 and W. H. Irvin McLean1†

1Epithelial Genetics Group, Department of Dermatology and Cutaneous Biology, Jefferson Medical College, 233 South 10th Street, Philadelphia, PA 19107, U.S.A.
2Center for Medical Genetics, Johns Hopkins University School of Medicine and Johns Hopkins Hospital, Baltimore, MD, U.S.A.
3De.RA Molecular Diagnostics Laboratory, Department of Dermatology and Cutaneous Biology, Jefferson Medical College, 233 South 10th Street, Philadelphia, PA 19107, U.S.A.
4Department of Dermatology and Cutaneous Biology, Jefferson Institute for Molecular Medicine, 233 South 10th Street, Philadelphia, PA 19107, U.S.A.

Pachyonychia congenita type 1 (PC-1) is an autosomal dominant ectodermal dysplasia characterized by severe nail dystrophy, focal non-epidermolytic palmoplantar keratoderma (FNEPPK) and oral lesions. We have previously shown that mutations in keratin K16 cause fragility of specific epithelia resulting in phenotypes of PC-1 or FNEPPK alone. These earlier analyses employed an RT-PCR approach to avoid amplification of K16-like pseudogenes. Here, we have cloned the K16 gene (KRT16A) and two homologous pseudogenes (KRT16B and KRT16C), allowing development of a genomic mutation detection strategy based on a long-range PCR, which is specific for the functional K16 gene. We report a novel heterozygous 3 bp deletion mutation (388del3) in K16 in a sporadic case of PC-1. The mutation was detected in genomic DNA and confirmed at the mRNA level by RT-PCR, showing that our genomic PCR system is reliable for K16 mutation detection. Using this system, we carried out the first prenatal diagnosis for PC-1 using CVS material, correctly predicting a normal fetus. This work will greatly improve K16 mutation analysis and allow predictive testing for PC-1 and the related phenotype of FNEPPK. Copyright © 1999 John Wiley & Sons, Ltd.

KEY WORDS: pachyonychia congenita; K16; mutation; intermediate filaments; keratoderma; genodermatosis

INTRODUCTION

Mutations in human keratins produce fragility and/or hyperproliferation of specific epithelial structures (Corden and McLean, 1996; Irvine and McLean, 1999). Keratins form obligate heterodimers consisting of a type I and a type II keratin, which are usually co-expressed in specific pairs (Lane, 1993). The helix boundary motifs, located at the start and end of the rod domain, have been implicated in mediating critical overlap interactions in filament assembly (Geisler et al., 1993; Steinert et al., 1993a,b). In the 17 keratins which are now linked to human genetic disorders, most dominant mutations occur in these motifs (Corden and McLean, 1996; Irvine et al., 1997; Ku et al., 1997; Smith et al., 1998; Winter et al., 1997a,b).

Pachyonychia congenita is a group of autosomal dominant ectodermal dysplasias whose most prominent phenotype is hypertrophic nail dystrophy (Stevens et al., 1996). Gorlin et al. (1976) recognized two main clinical variants: type 1 (PC-1) with oral leukokeratosis (Jadassohn and Lewandowsky, 1906); and type 2 (PC-2), with multiple pilosebaceous cysts (Jackson and Lawler, 1951). Focal non-epidermolytic palmoplantar keratoderma (FNEPPK) is a feature of both variants but is typically more severe in PC-1. 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: the tissues affected in PC-1 (Lane, 1993; Lane et al., 1985; Purkis et al., 1990). Previously, we have shown that missense mutations in K16 cause either PC-1 (McLean et al., 1995) or FNEPPK with insignificant nail changes (Shamsheer et al., 1995). Mutations in K6a have also been found to produce PC-1 (Bowden et al., 1995; Smith et al., 1999) and are predicted to cause FNEPPK. In PC-2, the FNEPPK is less severe but there are numerous other epithelial defects. Mutations in keratin K17 and K6b have been shown to produce PC-2 (Covello et al., 1998b; McLean et al., 1995; Smith et al., 1997, 1998).

The population incidences of pachyonychia congenita and FNEPPK have not been determined. However, we recently reported a point prevalence in
keratoderma in infancy; hand involvement was noted at about six years. At the age of nine, all 10 toenails were removed. He has suffered life-long hyperhidrosis of the palms and soles. Hyperkeratosis of the knees, elbows and buttocks and follicular keratosis of the thighs were noted. He did not have oral leukoplasia and teeth were not present at birth. His parents and two siblings were clinically normal.

Sample collection

DNA samples were obtained from peripheral blood of the patient and healthy controls. CVS DNA was obtained at 10 weeks of gestation and multiple dissected villi were analysed. mRNA was obtained using plantar biopsy material from the proband, a tissue which constitutively expresses K16. Normal human epidermal keratinocyte cultures were used as a source of control mRNA. Poly (A) mRNA was extracted from a snap-frozen 3 mm skin biopsy using the Quick- Prep Micro mRNA Purification Kit (Pharmacia, St Albans, U.K.). mRNA was incubated for 1 hour at 37°C with 10 U RNase-free DNase I (Boehringer- Mannheim, Indianapolis, IN, U.S.A.) and phenol- chloroform extracted before reverse transcription using oligo(dT) and AMV reverse transcriptase (Promega, Madison, WI, U.S.A.).

Sequencing of the K16 mRNA

For characterization of the normal K16 sequence, near full-length cDNA was amplified using sense primer K16f484 (5' TCG CTG ACC TCC CTC CTT 3') and antisense primer K16r1933 (5' AGG CAG CTC AGT TCT AGG AGC 3') derived from the previously published genomic sequence (Rosenberg et al., 1988). PCR was performed in high fidelity PCR buffer with 1.5 mM MgCl₂ and 4 per cent dimethylsulphoxide. Amplification conditions were 94°C for 5 minutes × 1; 94°C for 30 seconds, 55°C for 1 minute, 72°C for 2 minutes × 30; and 72°C for 5 minutes × 1. A hot start was performed with 1 U AmpliTaq polymerase (Perkin- Elmer). PCR products were purified using QIAquick PCR purification kit (Qiagen, Valencia, CA, U.S.A.) and directly sequenced with forward and reverse primers on an ABI 377 automated sequencer, according to the manufacturer’s recommendations. The 5' and 3' UTR sequences were obtained by rapid amplification of cDNA ends (RACE), using the Marathon RACE system (Clontech, Palo Alto, CA, U.S.A.).

Sequencing of the K16 gene and pseudogenes

For characterization of the multiple K16 genomic sequences, a 3 kb genomic DNA fragment encompassing most of the gene was amplified using primer K16f484 and K16r1933 using the conditions described above. Genomic PCR fragments were cloned into the

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Northern Ireland of 4.4 per 100 000 for a closely related keratin disorder, epidermolytic palmpplantar keratoderma (EPPK), caused by mutations in K9 (Covello et al., 1998a). Only one keratin gene is involved in EPPK, whereas four are known to produce PC and/or FNEPPK (Irvine and McLean, 1999). Therefore, the combined prevalence of these conditions is likely to be higher than that of EPPK. Individuals affected by either form of PC and/or FNEPPK find walking painful and we have observed a high demand for prenatal testing from affected families.

Here, we have cloned and fully sequenced the K16 mRNA, its corresponding gene, KRT16A, and two K16-like pseudogenes, ψKRT16B and ψKRT16C. This has allowed us to develop a mutation detection strategy for K16 based on genomic DNA. We demonstrate the reliability of this system by identifying a novel mutation in genomic DNA, which was confirmed using RT-PCR. Using this robust system, we report the first prenatal testing for a K16 mutation in PC-1.

MATERIALS AND METHODS

Patient and case history

In family A, the proband is a 36-year-old man with 20 nail pachyonychia and severe, disabling FNEPPK, covering almost the entire surface of the soles (Fig. 1) and also affecting the palms in a linear distribution. The involvement of the feet began with blisters and

Fig. 1—Clinical characteristics of PC-1 in the proband in family A. (a) All nails were affected by hypertrophic nail dystrophy, seen here on the hands. Toenails had been removed in childhood. (b) The proband suffers from particularly severe and debilitating focal non-epidermolytic palmpplantar keratoderma (FNEPPK), seen here on the soles.

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KRT16A genomic organization

![Diagram of KRT16A genomic organization]

Fig. 2.—Structure of the gene encoding type I keratin K16 (KRT16A). Two pseudogenes were identified, whose structural organization was identical to the functional gene. Priming sites used for PCR amplification of the functional gene are shown. Lower case indicates bases which are identical in both pseudogenes but which differ in the functional gene and facilitate specific amplification.

pCR2.1 vector (Invitrogen Carlsbad, CA, U.S.A.) and several independent clones were sequenced with the amplification primers and additional internal primers on an ABI 377 automated sequencer.

Mutation detection and confirmation

For mutation detection using cDNA as template, a 1031 bp fragment of the K16 mRNA was amplified from cDNA using sense primer K16f800 (5' CTG GCT TTG GTC GTG TT 3') and antisense primer K16r831 (5' AGA CTC CGG GGG AAG AAT A 3') using the conditions above. This fragment was sequenced in the forward and reverse directions using the amplification primers. To allow sequencing of the normal and mutant alleles, PCR products were cloned into pCR2.1 (Invitrogen).

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 TGG GCC AGA TGC TTG CT 3') were used in high fidelity PCR buffer (Boehringer-Mannheim) containing 1.5 mM MgCl₂ and 4 per cent dimethylsulphoxide. A hot start was performed with 1 U AmpliTaq polymerase (Perkin-Elmer) and the following conditions were used: 94°C for 5 minutes × 1; 94°C for 30 seconds, 58°C for 45 seconds, 72°C for 2 minutes × 35; and 72°C for 5 minutes × 1. These conditions were found to be critical for specific amplification.

To confirm K16 mutation 388del3, a 140 bp fragment of genomic DNA was amplified using sense primer K16f851 (5' AGA AGG TGA CCA TGC AGA AC 3') and 32P-labelled antisense primer K16r990 (5' GTC TTT GAT CTC ACT GGG CC 3') in standard PCR buffer (1.5 mM MgCl₂) containing 4 per cent dimethylsulphoxide. PCR conditions were 94°C for 5 minutes × 1; 94°C for 30 seconds, 55°C for 45 seconds, 72°C for 45 seconds × 30; and 72°C for 5 minutes × 1. PCR products were analysed on standard 6 per cent sequencing gels and visualized by autoradiography.

RESULTS

Cloning of the K16 mRNA, functional gene and two pseudogenes

There have been three previous conflicting reports of the K16 coding sequence (Paladini et al., 1995; RayChaudhury et al., 1986; Rosenberg et al., 1988). In our previous studies of K16 mutations in PC-1 and FNEPPK (McLean et al., 1995; Shamsheer et al., 1995), we found that PCR primers designed to amplify only the functional K16 gene produced more than one K16-like sequence, so that mutation detection was only possible using mRNA extracted from the patients' palmpoplantar skin or cultured keratinocytes. To resolve this conflict, we undertook independent characterization of the K16 mRNA sequence by an RT-PCR strategy. The mRNA sequence obtained was found to be essentially identical to the one reported most recently (Paladini et al., 1995). Using RACE PCR, we also determined the 5' and 3' UTR sequences which were absent from the recent published sequence (Paladini et al., 1995). The cDNA sequence with the predicted amino acid translation has been submitted to Genbank, accession number AF061812.

To resolve the problems encountered in PCR amplification of the K16 gene using genomic DNA, we sequenced multiple K16 genomic clones generated by long-range PCR. This revealed three species of clone: one type whose exons were identical to our cDNA sequence and was therefore assumed to be the functional gene (designated KRT16A); and two distinct types of clone with closed reading frames which were
assumed to be pseudogenes (designated \( \psi KRT16 \)B and \( \psi KRT16 \)C). These genomic sequences have been deposited in Genbank, accession numbers AF061809, AF061810 and AF061811. The pseudogene sequences are each about 95 percent identical to the functional gene and differ from each other by more than 1 percent. The intron–exon organization of the \( KRT16A \) gene is shown in Fig. 2. The genomic organization of the pseudogenes was essentially identical to that of the functional gene (Fig. 2).

**Mutation detection strategy for K16**

Using the small areas of sequence specific to \( KRT16A \), we were able to develop primers and conditions which amplify most of the functional gene (a 2.5 kb genomic fragment), without contamination from the pseudogenes, \( \psi KRT16 \)A and \( \psi KRT16 \)C. The locations of these specific priming sites, aligned to the corresponding regions of the pseudogenes, are shown in Fig. 2. This genomic PCR fragment covers exons 1 and 6, which encode the helix boundary motifs of the K16 polypeptide, the sites where essentially all keratin mutations occur (Corden and McLean, 1996; Irvine and McLean, 1999). Direct sequencing with the amplification primers covers these two mutation hotspots of \( KRT16A \).

Mutation detection in the proband of family A was carried out by direct sequencing of the specific genomic PCR fragment. In parallel, cDNA derived from the patient's plantar epidermis was analysed by RT-PCR and direct sequencing. Both these approaches revealed overlapping sequencing ladders in exon 1, predicting the same 3 bp deletion, as shown in Fig. 3(a–c). Cloning of the RT-PCR products from the proband allowed sequencing of the mutant allele, which showed conclusively that the mutation was a 3 bp deletion (Fig. 3(d)).

To further confirm the mutation as pathogenic, we were able to resolve the deleted allele on DNA sequencing gels and, by this means, exclude the deletion from 50 normal, unrelated individuals (Fig. 3(e)).

**Prenatal testing for K16 mutation**

For prenatal analysis, CVS DNA was obtained at 10 weeks of gestation and multiple strands were analysed. The specific genomic PCR described above was used to amplify the full-length \( KRT16A \), which was directly sequenced with the forward amplification primer. A normal K16 sequence was obtained and a normal child was predicted (Fig. 3(f)). Additionally, the CVS

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**Fig. 3**—K16 mutation detection and confirmation. (a) K16 genomic sequence derived from a normal individual by direct sequencing of PCR products. Coding sequence base numbers 382–396, encompassing exons 126–132 are shown. (b) The equivalent K16 sequence as shown in (a), derived from the proband's genomic DNA. The arrow indicates the start of sequence overlap due to the heterozygous 3 bp deletion, 388del3. (c) Equivalent K16 sequence as shown in (a), derived from cDNA prepared from a plantar skin biopsy from the proband. The arrow indicates the start of sequence overlap due to the deletion. Identification of the mutation in cDNA and genomic DNA in parallel confirms the specificity of the genomic PCR for the functional K16 gene. (d) The equivalent K16 sequence as shown in (a), derived from the mutant allele cloned into pCR2.1 vector. This clarifies that the mutation is a 3 bp deletion: removal of a CCT repeat, as indicated. (e) Confirmation of mutation 388del3 and exclusion from normal control individuals. A 140 bp PCR fragment spanning the mutation was amplified from genomic DNA from the proband and fifty unaffected, unrelated individuals (five of which are shown here). Radioactively-labeled PCR products were separated on 6 percent sequencing gels and detected by autoradiography. Note that the mutant band is much weaker than the normal one because the PCR used here amplifies the functional K16 gene and its pseudogenes. (f) Prenatal testing for the K16 mutation 388del3 by direct sequencing of genomic PCR products derived from CVS DNA. The equivalent region of K16 sequence as in (a) is shown and no trace of the deletion mutation (b and c) can be seen, indicative of a normal fetus. Subsequently, a healthy baby girl was born, confirming the accuracy of the test.
sequence was found to be heterozygous for a single-nucleotide polymorphism, 220G→A. This predicts the amino acid substitution G69D in the variable V1 domain of the K16 protein (McLean and Lane, 1995). The affected father was similarly heterozygous, however, maternal DNA was homozygous for the G variant. This base change was not detected in either pseudogene sequence. Therefore, the presence of the A allele in the CVS sample was indicative of insignificant maternal contamination (data not shown). Subsequently, a baby girl was delivered without skin blistering, keratoderma or any other sign of PC-1. At the time of writing, she is eight months of age and remains free of PC-1, which is normally noted at birth or in the first few weeks of life.

DISCUSSION

PC-1 can be caused by mutations in either keratin K6a (Bowden et al., 1995; Smith et al., 1999) or its expression partner K16 (McLean et al., 1995). Mutations in K16 can also produce the related phenotype, FNEPPK, and it is likely that K6a mutations also occur in this disorder (Shamsher et al., 1995). Previously, mutation detection and prenatal diagnosis involving the K16 gene were made difficult by differences between the published sequences (Raychaudhury et al., 1986; Rosenberg et al., 1988) and the presence of undefined pseudogenes (McLean et al., 1995). Similarly, detection of K6a mutations was hampered by the presence of several functional and non-functional K6-like genes in the human genome (Takahashi et al., 1995). Recently, we reported a mutation detection system for K6a, based on specific long-range PCR amplification of the entire K6a gene and confirmed novel mutations identified in genomic DNA by RT-PCR analysis of mRNA (Smith et al., 1999). Here, we describe a similar system for the K16 gene.

To resolve the problem of differing K16 sequences in the databank (Paladini et al., 1995; Raychaudhury et al., 1986; Rosenberg et al., 1988), we independently sequenced the K16 mRNA and characterized the missing 2' and 3' UTR sequences. The coding sequence was identical to that reported most recently (Paladini et al., 1995). By a PCR-based approach, we isolated three species of K16 genomic sequence: the functional gene KRT16A; and two highly homologous pseudogenes, ψKRT16B and ψKRT16C. A long-range PCR method was developed which amplifies only the functional gene, KRT16A. A novel 3 bp deletion mutation in a sporadic case of PC-1 was detected using the specific genomic PCR and the mutation was confirmed by sequencing of cDNA. Prenatal testing was carried out using CVS DNA, resulting in the correct prediction of an unaffected child. The PCR primers and conditions developed here will facilitate the detection of K16 mutations using genomic DNA, rather than mRNA, as well as prenatal testing in the future. This, together with our recently developed system for analysis of K6a mutations (Smith et al., 1999), provides a robust molecular diagnostic system for PC-1 and FNEPPK.

The K16 mutation described here, 388del3, is the first in-frame deletion mutation in the helix initiation motif of a type I keratin, although 3 bp deletions have been reported in the analogous motif of type II keratins K4 and K6a (Bowden et al., 1995; Rugg et al., 1995). This mutation results in the deletion of serine 130, (or serine 13 in the 1A domain), from the helix initiation motif of the K16 polypeptide, the site where the vast majority of dominant-negative mutations in type I keratins have been reported (Corden and McLean, 1996; Irvine and McLean, 1999). The phenotype observed in the proband was unusually severe, particularly the very debilitating keratoderma, which affected both hands and feet. This might be due to more deleterious effects of the 3 bp deletion mutation compared with the reported missense mutations in K16 (McLean et al., 1995; Shamsher et al., 1995). However, in many keratin disorders, the phenotype varies both within and between families (Covello et al., 1996; McLean et al., 1994; Smith et al., 1997). Thus, a larger number of mutations should be studied before drawing firm conclusions regarding genotype-phenotype correlation.

In conclusion, we report a reliable mutation detection system for the K16 gene, which we have applied by identifying a novel mutation and performing the first prenatal testing for PC-1.

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