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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.

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A Large Mutational Study in Pachyonychia Congenita

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Pachyonychia congenita (PC) is a rare autosomal dominant skin disorder characterized predominantly by nail dystrophy and painful palmoplantar keratoderma. Additional clinical features include oral leukokeratosis, follicular keratosis, and cysts (steatocysts and pilosebaceous cysts). PC is due to heterozygous mutations in one of four keratin genes, namely, *KRT6A*, *KRT6B*, *KRT16*, or *KRT17*. Here, we report genetic analysis of 90 new families with PC in which we identified mutations in *KRT6A*, *KRT6B*, *KRT16*, or *KRT17*, thereby confirming their clinical diagnosis. A total of 21 previously unreported and 22 known mutations were found. Approximately half of the kindreds had mutations in *KRT6A* (52%), 28% had mutations in *KRT16*, 17% in *KRT17*, and 3% of families had mutations in *KRT6B*. Most of the mutations were heterozygous missense or small in-frame insertion/deletion mutations occurring within one of the helix boundary motif regions of the keratin polypeptide. More unusual mutations included heterozygous splice site mutations, nonsense mutations, and a 1-bp insertion mutation, leading to a frameshift and premature termination codon. This study, together with previously reported mutations, identifies mutation hotspot codons that may be useful in the development of personalized medicine for PC.

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INTRODUCTION

Pachyonychia congenita (PC) is a rare genetic skin disorder that is associated with mutations in one of four keratin genes, *KRT6A*, *KRT6B*, *KRT16* or *KRT17* (Bowden *et al.*, 1995; McLean *et al.*, 1995; Smith *et al.*, 1998). The most striking feature of PC is the painful and debilitating plantar keratoderma (Figure 1). The mechanism underlying the plantar pain is poorly understood; however, the formation of blisters beneath the keratoderma is likely to be a major contributing factor. Palmar keratoderma is less frequent. Nail dystrophy presents in variable forms, from very minor or almost absent nail changes through to the classic hypertrophic nail dystrophy that gives the condition its name (Leachman *et al.*, 2005; Figure 1). Other epithelial structures can be affected, particularly the mucosae and the pilosebacesous unit. A more detailed description of the disorder is given in McLean *et al.* (2011).

Historically, PC has been subdivided into two subtypes, PC-1 (Jadassohn–Lewandowski type) or PC-2 (Jackson–Lawler type), on the basis of the clinical presentation alone (Jadassohn and Lewandowski, 1906; Jackson and Lawler, 1951). A combination of factors have led to the suggestion that PC should be reclassified. First, the advent of molecular genetics and the identification of the genes causing PC provide a rational means of classifying patients. Second, clinical analysis of the large case series collected by the International Pachyonychia Congenita Research Registry (IPCRR), fully linked to molecular genetic data, has shown that there is considerable phenotypic overlap between the historical PC-1 and PC-2 subtypes (Eliason *et al.*, 2011). Thus, a new molecular genetic classification has been adopted, fully supported by members of the International Pachyonychia Congenita Consortium, whereby the subtypes of PC refer to the mutated keratin gene (PC-6a for a patient carrying a K6a mutation, PC-6b, PC-16, PC-17, and so on). The designation 'PC-U' is used for cases where the causative gene is unknown (McLean *et al.*, 2011). The new classification will (a) help discourage publication of spurious case reports lacking molecular data (often with misdiagnosed cases or cases with coincidental findings unrelated to PC) and (b) allow better prognostic predictions and patient counseling, especially when referenced to the IPCRR clinical data set.

The 54 human keratins belong to the intermediate filament protein family that consists of at least six types; keratins make up the type I and type II intermediate filament proteins. A major function of keratins is to form structural cytoskeletal networks within epithelial cells that allow cells to withstand everyday stress and physical trauma. Keratins are expressed in pairs in tissue-specific and differentiation-specific patterns (Lane, 1993). The keratins associated with PC, K6a, and K16, K6b and K17 are predominantly expressed

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Abbreviations: IPCRR, International Pachyonychia Congenita Research Registry; K, keratin protein; KRT, keratin gene; PC, pachyonychia congenita

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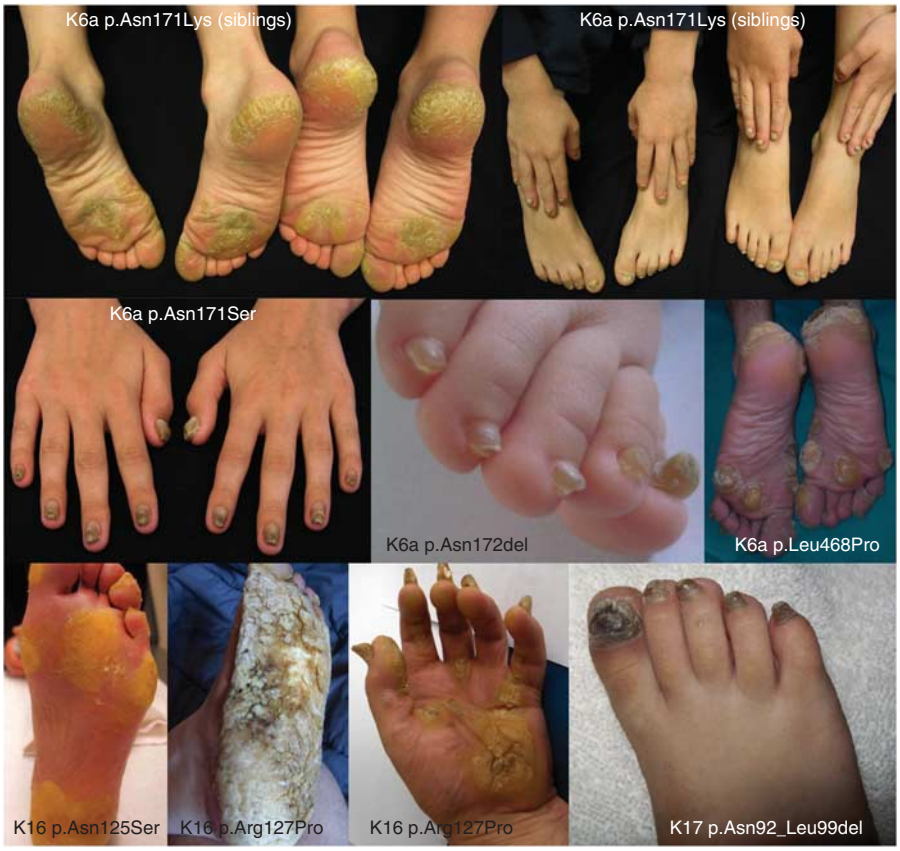

Figure 1. Clinical features of pachyonychia congenita (PC). Plantar keratoderma and nail dystrophy in PC patients with known keratin mutations. Mutations are annotated. Note the variation in severity and clinical appearance of these features.

in basal/suprabasal layers of palmoplantar skin, as well as in epidermal appendages and oral mucosa. In PC, these epithelial cell compartments are rendered fragile by the expression of dominant-negative mutant keratins. All cases of PC with a confirmed molecular diagnosis, including those in the literature, http://www.interfil.org (Szeverenyi *et al.*, 2008), and those in this study, are due to heterozygous dominant-negative mutations, inherited as an autosomal dominant trait. Although there are a small number of case reports of PC with recessive inheritance in the literature, there are no reports of recessive PC with a confirmed molecular diagnosis.

All keratins share a common protein structure consisting of a central α-helical rod domain of 310 amino acids subdivided into the 1A, 1B, 2A, and 2B domains. These domains are connected by non-helical linker regions, L1, L12, and L2. The rod domain is flanked by short regions of sequence homology (H1 and H2 regions), followed by the variable, non-helical head (V1) and tail domains (V2). At either end of the rod domain are the helix boundary motifs (the helix initiation motif and the helix termination motif). These highly conserved motifs are thought to be important in mediating end-to-end interactions during filament assembly. The majority of mutations in PC occur in these helix boundary motifs, emphasizing the critical importance of these sequences for correct keratin filament formation and the mechanical resilience of epithelial cells.

The IPCRR was established in 2004 by the patient advocacy group, Pachyonychia Congenita Project (http://www.pachyonychia.org). At the time of writing, 478 families are registered (928 individuals), 223 families have completed the detailed questionnaire (Eliason *et al.*, 2011) and 199 families have undergone genetic testing. Genetic testing results from some of these cases have been previously published (McLean *et al.*, 1995; Smith *et al.*, 1997, 2000, 2005; Liao *et al.*, 2007a; Oh Adib *et al.*, 2008; Cogulu *et al.*, 2009; Gruber *et al.*, 2009).

Here, we present the findings of 90 new families with mutations in *KRT6A*, *KRT6B*, *KRT16*, or *KRT17*. Within this case series, we identified 21 previously unreported mutations (22 families) and 22 known mutations (68 families). This mutation analysis study not only confirms the clinical diagnosis of these individuals but, together with previously reported mutations (http://www.interfil.org), also identifies mutation hotspot codons that may be useful in the development of future allele-specific therapies.

RESULTS

Clinical details

All individuals involved in this study were recruited through the IPCRR, an ongoing research program to identify PC patients worldwide. This research registry is approved by an institutional review board that complies with all principles of the Helsinki Accord (Western IRB Study no. 20040468). An

important part of this study was the detailed clinical consultations that were performed for all cases analyzed. This bank of data allows us now, and in the future as the number of cases analyzed increases, to identify any useful genotype–phenotype correlation for PC. The predominant clinical features of individuals involved in this study are summarized in Supplementary Table S1 online. Of the 90 families analyzed, 36 represent familial occurrence of PC, with many showing autosomal dominant inheritance through several generations; the remaining 54 cases represent spontaneous mutations.

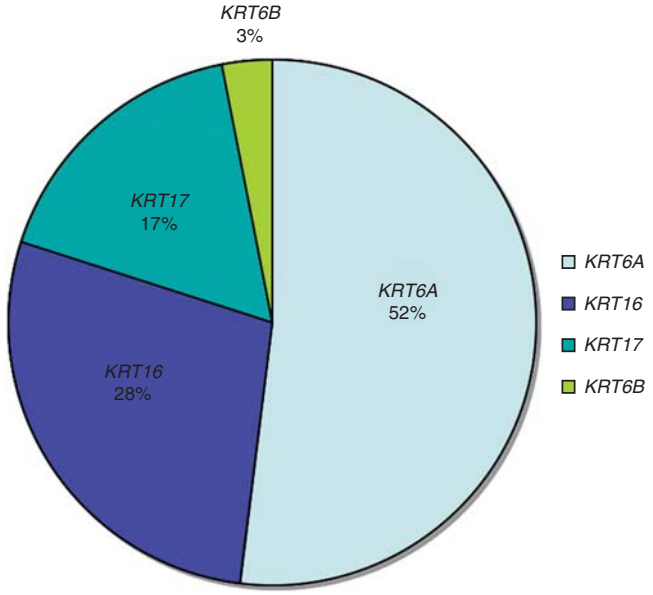

Figure 2. Mutational spectrum in pachyonychia congenita (PC). Pie chart showing percentage of families in this study with mutations in the four keratin genes, namely, *KRT6A*, *KRT6B*, *KRT16*, and *KRT17*.

Both common and rare dominant mutations cause PC

Within this large PC case series, pathogenic mutations were identified in the *KRT6A* gene in approximately half (52%) of the kindreds, whereas 28% had mutations in *KRT16*, 17% had defects in *KRT17*, and 3% had mutations in *KRT6B* (see Figure 2 and Supplementary Table S1 online). Mutations in *KRT6A* also account for ~50% of previously reported cases of PC (http://www.interfil.org), consistent with our finding here that this is the predominant PC gene. The majority of the mutations we identified in all four genes were heterozygous missense mutations occurring within one of the helix boundary motif regions. In addition, we found some small in-frame insertion/deletion mutations and, in particular, the common K6a p.N172del mutation was identified in 16 families.

An unusual V2 domain mutation in one PC family

A more unusual mutation identified was a 1-bp insertion in exon 9 of *KRT6A*, the last exon of this gene (K6a c.1511_1512insG). This insertion results in a frameshift and a premature stop codon just two amino acids upstream of the natural stop codon, whereby the last 60 amino acids of the K6a V2 domain are exchanged for a foreign peptide of 58 amino acids. Protein–protein BLAST (basic local alignment search tool) analysis showed that this mutant peptide sequence has no significant similarity to any human protein (data not shown; http://blast.ncbi.nlm.nih.gov/). Kyte–Doolittle hydrophilicity analysis revealed that the normal K6a V2 domain consists of alternating hydrophobic and hydrophilic sequences, followed by a short hydrophilic C terminus (Figure 3), consistent with the glycine-loop structure proposed by Steinert for keratin variable domains (Korge *et al.*, 1992). In contrast, the mutant V2 domain is almost completely hydrophilic (Figure 3). In terms of protein

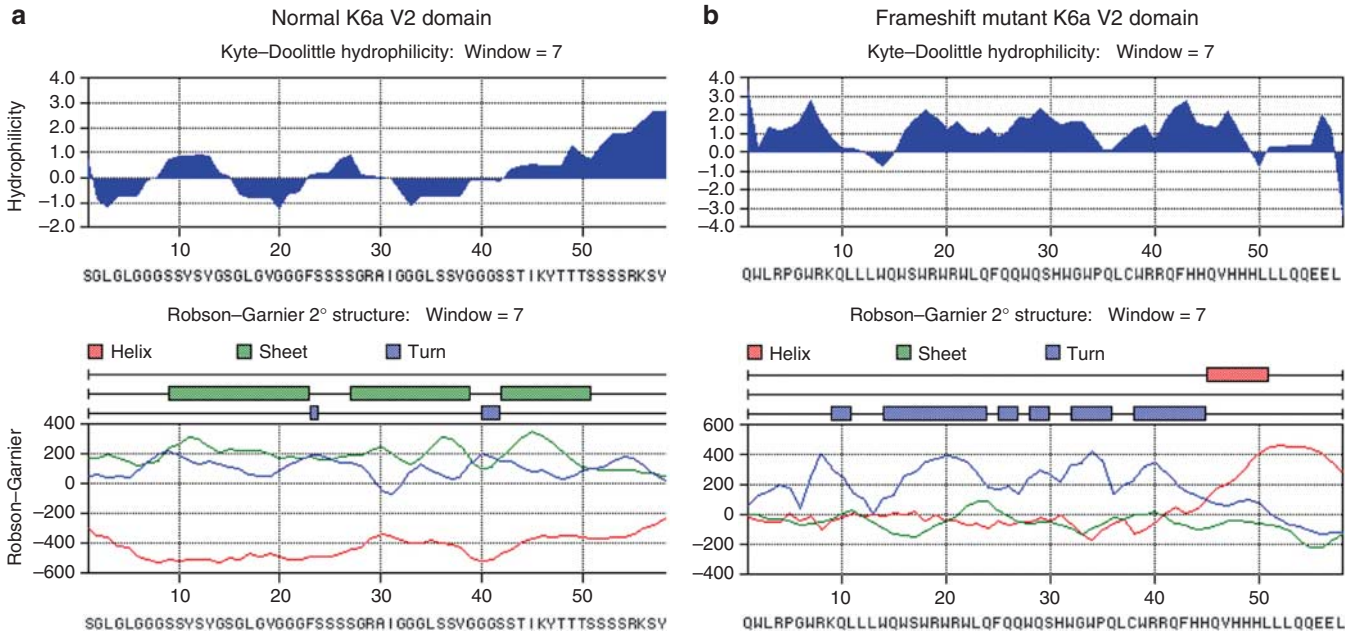

Figure 3. Kyte–Doolittle hydrophilicity analysis of normal and mutant K6a V2 domain. (**a**) The normal K6a V2 domain consists of alternating hydrophobic and hydrophilic sequences, followed by a short hydrophilic C terminus, whereas (**b**) the mutant V2 domain is almost completely hydrophilic.

secondary structure predicted by Robson–Garnier analysis, the normal K6a V2 domain is predicted to adopt three large areas of sheet conformations, separated by short regions predicted to adopt turn conformations (Figure 3). This contrasts with the mutant V2 domain, which is predicted to consist largely of turn conformation with one helical region near the C terminus (Figure 3). This *in silico* analysis underscores the fact that the mutant polypeptide is very different in both sequence and predicted secondary structure from the wild-type K6a tail domain, consistent with a dominant-negative gain-of-function mutation, as seen in other keratinizing disorders due to C-terminal frameshift mutations in K1 (Sprecher *et al.*, 2001, 2003; Richardson *et al.*, 2006) or K5 (Sprecher *et al.*, 2003). In the case of loricrin keratoderma, a similar C-terminal gain-of-function mutation has been shown to lead to creation of a new nuclear localization signal, which in turn leads to nuclear accumulation of mutant protein (Ishida-Yamamoto *et al.*, 2000). The mutant K6a polypeptide sequence generated here was analyzed for potential nuclear localization signal motifs (http://cubic.bioc.columbia.edu/services/predictNLS/), but none were found.

Splice site mutations identified in five kindreds

Interestingly we also detected four previously unreported splice site mutations (in five families) at the intron 1/exon 2 boundary of *KRT6A*. These are clearly inherited in an autosomal dominant manner. Unfortunately, we were unable to obtain mRNA from lesional skin that would allow analysis of the effects of these genomic mutations on RNA splicing. One possible predicted consequence of this type of mutation is skipping of exon 2; however, as this is an out-of-frame exon, its deletion would lead to a frameshift and premature termination codon. This is unlikely to create a dominant-negative mutant protein, because nonsense-mediated mRNA decay is predicted to occur, leading to loss of expression of this allele. In the case of *KRT5* and *KRT1*, both of which are type II keratin genes closely related to *KRT6A*, analogous mutations have been reported affecting the intron 1 splice sites (Rugg *et al.*, 1999; Terron-Kwiatkowski *et al.*, 2002). In both these genes, the mutation led to activation of an identical cryptic splice site in exon 1, producing a 66-nucleotide (22 amino acid) in-frame deletion. Given the strong sequence homology between these genes, it is probable that a similar mechanism will also occur with these *KRT6A* splice site mutations.

Nonsense mutations in a few PC families

In addition, two heterozygous nonsense mutations were also identified in PC cases. One of these was identified in the 2B domain of K6a, p.Gln435X, which is predicted to lead to expression of a truncated dominant-negative K6a protein, lacking the end of the rod domain and the tail domain. Because this mutation is close to the natural stop codon of K6a, it is likely to escape nonsense-mediated decay to some extent and, therefore, be expressed as mutant polypeptide (Frischmeyer and Dietz, 1999). Analogous mutations have been seen in K5 in dominantly inherited epidermolysis bullosa simplex (Muller *et al.*, 1999; Livingston *et al.*, 2001). The other nonsense mutation was found within the head domain of K16, p.Lys15X. Analogous premature termination codon mutations just downstream of the ATG codon have been reported in other dominant keratin disorders, including K5 in Dowling–Degos disease (Betz *et al.*, 2006; Liao *et al.*, 2007b) and in K14 in Naegeli syndrome (Lugassy *et al.*, 2006). Although it remains somewhat unclear whether these mutations act via haploinsufficiency or via expression of a dominant-negative mutant protein through use of an alternative initiation codon (McLean *et al.*, 2003), it is however clear that they exhibit dominant inheritance (Betz *et al.*, 2006; Lugassy *et al.*, 2006). Unfortunately, in the case of the two nonsense mutations identified here, it was not possible to obtain tissue to allow analysis of mRNA or protein.

A spectrum of keratin mutations cause PC

For each of the four genes associated with PC, it has been suggested that there are some codons that represent mutation hotspots, as well as several rare or even family-specific mutations. Our results confirm the previously identified mutation hotspots and also identify 21 previously unreported mutations. Table 1 summarizes the data from previous publications (http://www.interfil.org) together with the data from this large case series.

DISCUSSION

The mutation results from this PC case series of 90 families, together with those from at least 131 previously reported cases (http://www.interfil.org), provides a large data set for analysis in terms of where mutations occur within the keratin protein, the most common mutations, the types of mutations found, and allows for preliminary genotype–phenotype

Table 1. Summary of mutations identified in this study and previous publications

Gene	Number of different mutations	Number of recurrent mutations	Most common mutation site
KRT6A	39	13	K6a p.Asn171—as missense or deletion mutation (K6a p.Asn172del), this codon is mutated in 46% of those with *KRT6A* mutations, of which p.Asn172del accounts for 30% of all *KRT6A* mutations
KRT16	19	8	K16 p.Leu132Pro in 23% of families with *KRT16* mutations
KRT17	22	8	K17 p.Asn92Ser in 36% of families with *KRT17* mutations
KRT6B	4	1	K6b p.Glu472Lys in 71% of families with *KRT6B* mutations

correlation. All cases with confirmed PC have a mutation in one of the four keratin genes associated with this disease, *KRT6A*, *KRT6B*, *KRT16*, and *KRT17*. In the course of running the international mutation screening service for PC, in concert with the IPCRR, we have received a small number of samples from isolated cases or families in which no mutation was found in these four keratin genes. In these cases, careful review of the clinical phenotype by the International Pachyonychia Congenita Consortium clinicians often has often led to correction of the diagnosis, confirmed by analysis of other keratin genes or non-keratin genes. For example, a few cases, in which there is alopecia in addition to nail dystrophy, have turned out to carry heterozygous connexin-30 mutations (Lamartine *et al.*, 2000; Smith *et al.*, 2002; van Steensel *et al.*, 2003). Thus, Clouston syndrome should be considered in the differential diagnosis for PC. Similarly, a few families presenting with painful but very limited, circumscribed focal plantar keratoderma, with minimal or absent nail changes, have recently been shown to have mutations in the gene encoding K6c (*KRT6C*; Wilson *et al.*, 2010). Taking these families into account, we have a small number of families (<5%) in which careful clinical evaluation is consistent with a diagnosis of PC and in whom we cannot detect a mutation in any exon or splice sites of the four PC keratin genes. Genetic linkage analysis in at least one such family has yielded statistically significant linkage to the vicinity of a keratin gene cluster (FJD Smith, unpublished data), and so we conclude that there are either intronic or genomic deletion/rearrangement mutations that are missed by conventional PCR analysis, or that at least one other keratin gene or a nearby related gene can lead to a PC-like phenotype in a minority of cases. Sequence analysis of these cases is ongoing in the laboratory.

All PC causative mutations found to date are heterozygous changes that exhibit autosomal dominant inheritance with a proven, or very probable, dominant-negative pathomechanism. It is important to note that no recessive cases of PC have been confirmed at the molecular level, despite a few case reports appearing in the literature, in which, for example, recessive inheritance may have been suggested by coincidental consanguinity. It is therefore important that case reports of already characterized genetic diseases be backed up by molecular analysis, otherwise the literature may become misleading. The recurrence risk of a sporadic case of a dominant disorder is very low (involving only the risk of gonadal mosaicism) which is difficult to estimate with certainty. In the dominant disorder achondroplasia OMIM no. 100800, this is of the order of one in a few hundred (Mettler and Fraser, 2000); however, in epidermolysis bullosa simplex, this has been estimated at 2–5% (Pfendner *et al.*, 2005). In contrast, the recurrence risk for a recessive condition is as high as 25%. Thus, there are important genetic counseling implications in the correct assignation of inheritance patterns. So far, we have confirmed gonadal mosaicism in only one PC family out of 199 analyzed from the IPCRR.

The majority of the mutations causing PC are located in one of the helix boundary motifs of the mutated protein, and most of the causative variants are missense or small in-frame insertion–deletion mutations. Less common types identified include splice site and premature termination codon mutations. There are a number of mutation 'hotspot' codons for each of the keratin genes associated with PC as well as mutations that appear to be family specific (Table 1). The most commonly mutated codon is K6a p.Asn171, either as a missense mutation (e.g. p.Asn171Lys and p.Asn171Ser) or as a deletion mutation (designated as p.Asn172del using the Human Genome Variation Society guidelines; http://www.hgvs.org). Approximately half of the families with identified *KRT6A* mutations have a mutation at this site; the most common PC mutation is the p.Asn172del mutation that, to date, has been found in 32 out of 221 PC families (14%) with known mutations (http://www.interfil.org and this study).

Despite the increased number of genotyped individuals and families, there is no apparent correlation between the clinical features observed and the protein domain harboring the mutation in PC. Nevertheless, there are two mutations in *KRT16* in which the actual amino acid substitution appears to correlate with the severity of the clinical phenotype (see Fu *et al.*, 2011). There are also some differences in clinical features depending on the gene involved, such as the presence of natal teeth in many, but not all, individuals with *KRT17* mutations (Eliason *et al.*, 2011). In PC, there can also be variation in clinical severity between mutations in the same gene and even between individuals with the same mutation. Polymorphisms, copy number variation, environmental factors, lifestyle, and patient care may account for some of this clinical variation. Therefore, an important conclusion of this large study of a keratin disorder is that PC can really be considered as a spectrum of phenotypes ranging from very mild to more severe, in which (a) the particular gene involved appears to have a moderate influence on phenotype and (b) the specific mutation generally appears to have little influence on phenotype.

The detailed clinical information that is obtained by the IPCRR also identified several cases, which were not included in this study, that presented with both typical and atypical features of PC and that were unlikely due to a mutation in any of the four keratin genes, *KRT6A*, *KRT6B*, *KRT16*, or *KRT17*. These were analyzed for mutations in other candidate genes. For example, several families presented with varying degrees of alopecia in addition to some features typical of PC, which was suggestive of Clouston syndrome (Lamartine *et al.*, 2000; Smith *et al.*, 2002; van Steensel *et al.*, 2003) and mutations were subsequently identified in *GJB6* (which encodes connexin 30). Another candidate gene considered for individuals presenting with palmoplantar keratoderma was the third K6 gene (*KRT6C*). We have identified mutations in *KRT6C* in several families presenting with palmoplantar keratoderma, but with only mild/no nail changes (Wilson *et al.*, 2010).

Genetic analysis of individuals with PC not only confirms their clinical diagnosis but also aids in genetic counseling. The identification of mutations is especially important for the design of future mutation-specific and/or gene-specific therapies and, hence, the large, well-phenotyped, and fully genotyped case series we report here is an invaluable resource for future clinical trials.

MATERIALS AND METHODS

Clinical material

Genomic DNA was obtained with informed consent and ethical approval by an institutional review board that complies with all principles of the Helsinki Accord (Western IRB Study no. 20040468). Genomic DNA was extracted from peripheral blood lymphocytes using standard procedures or from saliva collected in an Oragene DNA sample collection kit (DNA Genotek, Ontario, Canada) and extracted according to the manufacturer's protocol.

Mutation detection

The coding regions of *KRT6A*, *KRT6B*, *KRT16*, and *KRT17* were amplified using primers specific to the respective functional genes to avoid amplification of *KRT6C* or pseudogenes (Supplementary Table S2 online). All primers were checked for single-nucleotide polymorphisms using Diagnostic SNPCheck (http://www.ngrl.org.uk/Manchester) and some were modified from our previous publications (Smith *et al.*, 2005) to increase specificity. For each gene, there are two primer sets for the mutation hotspot exons to overcome the potential problem of very rare or as yet unidentified single-nucleotide polymorphisms in primers designed to amplify these regions. Previously unreported mutations were excluded from at least 90 control DNA samples (180 chromosomes) by sequencing or restriction enzyme digests. For full-length genomic PCR reactions, Takara buffer and LA Taq polymerase (Lonza Biologics PLC, Slough, UK) were used and for the smaller PCR reactions and HotStarTaq DNA Polymerase and buffer system (Qiagen, Crawley, UK) were used according to the manufacturer's instructions. Specific PCR conditions for each primer set are available on request. PCR products were purified using QiaQuick PCR spin columns (Qiagen) or ExoSAP (using Exonuclease 1 and Shrimp Antartic Phosphatase, New England Biolabs, Hitchin, Herts, UK), and sequenced using internal primers on an ABI 3100 Automated DNA sequencing machine (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions.

Protein bioinformatics

Hydrophobicity (Kyte–Doolittle analysis) and protein secondary structure predictions (Robson–Garnier analysis) were performed using the Protein Analysis Toolkit function within the MacVector 9.0 software package (MacVector, Cary, NC).

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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