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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.
Novel proline substitution mutations in keratin 16 in two cases of pachyonychia congenita type 1

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Summary

Pachyonychia congenita (PC) is a group of inherited ectodermal dysplasias, the characteristic phenotype being hypertrophic nail dystrophy. Two main clinical subtypes, PC-1 and PC-2, are inherited as autosomal dominant disorders, but other less well characterized clinical forms also exist. The PC-1 phenotype may be distinguished by the absence of the epidermal cysts found in PC-2, and it has been shown to be caused by mutations in either keratin K16 or its expression partner, the K6a isoform of K6. Mutations in K16 have also been shown to cause a milder related phenotype, focal non-epidermolytic palmoplantar keratoderma. Recently, we have developed a long-range polymerase chain reaction (PCR) strategy which allows specific amplification of the entire functional K16 gene (KRT16A), without amplification of the two K16 pseudogenes (ϕKRT16B and ϕKRT16C), enabling mutation analysis based on genomic DNA. Here, using this methodology, we describe novel mutations R127P and Q122P in the helix 1A domain of K16 in two families presenting with PC-1. Both mutations were excluded from 50 normal unrelated individuals by restriction enzyme analysis of K16 PCR fragments. In one family, ultrastructural analysis was performed, revealing distinctive tonofilament abnormalities. Specifically, keratin filament bundles were greatly condensed, but did not form the dense amorphous aggregates seen in a number of other keratin disorders. In the second kindred, autosomal dominant cataract was present in some but not all members affected by PC. As the cataract phenotype did not fully cosegregate with the K16 mutation, and given that K16 is not expressed in the lens, these two phenotypes may be coincidental.

Key words: cataract, ectodermal dysplasia, genodermatosis, intermediate filaments, nail dystrophy

In recent years, through the application of transgenics and the study of human keratin disorders, the keratin intermediate filament cytoskeleton has been shown to be the primary stress-bearing structure within epithelial cells. Structural failure of the keratin cytoskeleton due to mutation leads to cytolysis of the particular epithelial cells expressing that protein and this manifests itself as blistering and/or hyperkeratosis of the affected tissue. Keratins form heteropolymermic 10 nm filaments composed of specific pairs of type I and type II keratin protein subunits, which are coexpressed in a tissue-specific fashion. Mutations in either protein of a keratin expression pair can lead to very similar or identical disease phenotypes. Genetic defects have now been demonstrated in 17 distinct keratin genes causing human epithelial fragility disorders, recent additions being mutations in K3 and K12 in Meesmann's corneal dystrophy, the association of a K18 mutation in cryptogenic...
Mutation detection in PC has been hampered by the existence of multiple functional genes and/or pseudogenes for K6, K16 and K17. Recently, we have cloned and fully sequenced two K16-like pseudogenes and developed a long-range polymerase chain reaction (PCR) system to amplify the functional K16 gene specifically. Here, we have used this strategy to detect two novel K16 mutations in families with PC-1, including a family where some affected persons have cataract.

cirrhosis, and mutations in hair keratins hHb1 and hHb6 in monilethrix. Recently, we have reported a mutation in K6b in pachyonychia congenita (PC) type 2 (PC-2), revealing that this keratin is the expression partner of K17. Most keratin mutations affect the highly conserved helix boundary motifs which delineate the central α-helical rod domain and whose function is thought to be that of mediating molecular overlap interactions in the filament assembly process.

PC is a group of ectodermal dysplasias characterized by hypertrophic and subungual nail dystrophy in addition to certain additional ectodermal abnormalities, specific to each subtype. The PC-1 phenotype is associated with severe focal non-epidermolysis palmoplantar keratoderma (FNEPPK) and variable expression of oral leucokeratosis. This form of PC is known to be produced by mutations in either K6a or K16, keratins expressed in the affected tissues. In contrast, the PC-2 variant is associated with multiple pilosebaceous cysts, which are the major feature distinguishing PC-1 and PC-2. This type of PC is caused by mutations in K17 or its recently discovered expression partner, K6b. In addition, PC-2 is characterized by variable expression of the following features: mild FNEPPK, pilo torti and natal teeth. Mutations in K17 have also been reported in a number of cases presenting with the related phenotype, steatocystoma multiplex. It is likely that certain K6b mutations can cause this disorder, although none has been reported to date. More complex clinical classifications of PC have been proposed, including the suggestion that cases with ocular lesions and cataract constitute a distinct type.
Subjects, materials and methods

We studied two caucasian pedigrees from the Netherlands and Hungary (Fig. 1), in whom affected members exhibited classical features of PC (Fig. 2). Genomic DNA was extracted from whole blood by standard methods. For mutation detection, using genomic DNA as template, primers specific for the functional K16 gene, K16sp2 (5’ AGG CCT CCT GGA TCA TGG GA 3’) and K16sp3 (5’ GGA TGT GCC AGA TGC TTC GT 3’) were used in High Fidelity PCR buffer (Boehringer, Lewes, U.K.) containing 15 mmol/L MgCl₂ and 4% dimethylsulphoxide. A ‘hot start’ was performed with 1 U AmpliTaq™ polymerase (Perkin-Elmer, Norwalk, CT, U.S.A.) and the following conditions were used: 94 °C for 5 min × 1; 94 °C for 30 s, 58 °C for 45 s, 72 °C for 2 min × 35; and 72 °C for 5 min × 1. This fragment is 2.5 kb in size and contains exons 1–7 of the KRT16A gene. Mutations were detected by direct sequencing of the 1A domain region using forward primer K16sp2 (above) and reverse primer K16sp990 (5’ GTC TTT GAT CTC ACT GGG CC 3’). Both mutations were confirmed in affected family members and were excluded from 50 normal unrelated individuals by restriction digestion of specific KRT16A PCR products.

Mutation R127P creates a new BsaI restriction site. A 506-bp fragment specific for the KRT16A gene was amplified using primers K16sp2 (above) in combination with K16sp6 (5’ TAC ACC AAA GTT ACC CAC CTT GTT 3’) in standard PCR buffer containing 1.5 mmol/L MgCl₂ and 4% dimethylsulphoxide. Reactions were subjected to a ‘hot start’ with 1 U AmpliTaq polymerase and the following conditions were used: 94 °C 5 min × 1; 94 °C for 30 s, 60 °C for 1 min, 72 °C for 1 min 30 s × 35; and 72 °C for 5 min × 1. PCR products (20 μL) were diluted 50% in BsaI buffer and digested overnight at 60 °C. Digests were analysed on 3% Seakem agarose gels. Cutting of an additional BsaI site was observed in PCR products derived from the proband in family A.

Mutation Q122P does not alter any known restriction site. Consequently, a mismatch primer was designed which, in combination with the mutation, produces an EagI site. PCR was first performed with primers K16sp2 and K16sp6 (above), followed by reamplification using primers K16Q122P (5’ TGG GCA GTG AGA AGG TGA CCC GCC 3’, mismatch bases underlined) and K16sp6 (above) in standard PCR buffer containing 1.5 mmol/L MgCl₂ and 4% dimethylsulphoxide. The resultant fragment is 209 bp and the following conditions were used: 94 °C for 5 min × 1; 94 °C for 30 s, 55 °C for 45 s, 72 °C for 45 s × 30; and 72 °C for 5 min × 1. Secondary PCR products (20 μL) were diluted 50% in EagI buffer and were digested overnight with 5 U EagI at 37 °C and analysed as above. Cutting of the single EagI site was only observed in PCR products derived from affected persons in family B.

Results

Clinical features

In family A, of Dutch origin, the proband is the daughter of clinically unaffected parents (Fig. 1), therefore, representing a sporadic case. The proband showed thickened nails which were evident in the early neonatal period (Fig. 2a). From the age of 1 year, blisters and circumscribed hyperkeratotic lesions developed at pressure sites on the palms and especially on the soles of the feet (Fig. 2b). At 4 years of age, the pachyonychia and palmar hyperkeratosis had become more severe. Leucoplakia of the buccal mucosa was not observed.

A 4-mm punch biopsy was taken from the border of a plantar blister from the proband. Light microscopy of a toluidine blue-stained semithin section showed both intracellular and intercellular oedema and vacuolization in the upper layers of the stratum spinosum, while the basal layer was intact (Fig. 3a). Orthokeratosis was seen in a number of sections. Electron microscopic examination (Fig. 3b) showed perinuclear vacuolization and condensation of tonofilament bundles in the suprabasal keratinocytes. At higher magnification (Fig. 3c) tonofilaments were seen to be somewhat aggregated and often limited to the peripheral areas of the cytoplasm.

Family B is of Hungarian origin, and several individuals were affected in multiple generations with male-to-male transmission, typical of the autosomal dominant inheritance observed in PC kindreds (Fig. 1). A father and son in family B affected by PC also suffered from cataract of a juvenile type, as reported previously.27 The affected members of either family showed no evidence of pilosebaceous cysts or the other distinguishing ectodermal features of PC-2, and were therefore diagnosed as having PC-1.

K16 mutation analysis and confirmation

The functional K16 gene, KRT16A, was amplified from genomic DNA derived from affected members of both

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families using the specific long-range PCR conditions recently described. In family A, heterozygous missense mutation 380G→C was identified in the proband (Fig. 4), predicting the amino acid substitution R127P, which affects the 10th residue of the helix 1A domain of the K16 polypeptide. The codon numbers used here are derived from the recently corrected sequences. The analogous proline mutation has been found in K10 causing bullous congenital ichthyosiform erythroderma (BCIE) and in K14 causing the Dowling–Meara form of epidermolysis bullosa simplex (EBS–DM). This mutation creates a new recognition site for the restriction enzyme BsaII. Therefore, the mutation was confirmed in the proband and excluded from 50 normal unrelated individuals by BsaII digestion of specific KRT16 PCR fragments (data not shown).

In the proband of family B, heterozygous missense mutation 365A→C was detected (Fig. 4), predicting the amino acid substitution Q122P, which affects the fifth residue of the 1A domain of K16. This pathogenic mutation has not been reported previously in any type I keratin gene. This mutation does not alter any known
Discussion

In this study, using a recently developed long-range PCR strategy to amplify the entire K16 rod domain from genomic DNA without pseudogene contamination, we have identified two new PC-1 mutations, R127P and Q122P in the 1A domain of K16. Both mutations were excluded from a population of 100 normal chromosomes by restriction enzyme analysis. This brings the total number of mutations reported in K16 to six. Among these cases, four presented with the PC-1 phenotype, and had mutations L132P, ΔS130I, Q122P and R127P. The other two reported K16 mutations produced FNEPPK either with minimal nail changes, such as widening of the onychocorneal band and/or splinter haemorrhages, or no nail changes at all. These FNEPPK families had mutations N125S and R127C.

It is too early to make conclusive genotype–phenotype correlations based on the small number of K16 mutations known to cause the PC-1 or FNEPPK phenotypes. However, it is tempting to speculate that proline mutations in the α-helical 1A domain of K16 are more likely to result in the PC-1, rather than the FNEPPK phenotype. This would be consistent with the fact that proline, due to its chemical structure, is strongly detrimental to α-helical tertiary structures within proteins, compared with other amino acid substitutions. Previous in vitro mutagenesis experiments have shown that proline substitutions are particularly disruptive to the assembly and function of intermediate filaments, especially those occurring in the helix boundary motif sequences. Small in-frame deletion mutations in the 1A domain of K16 and its expression partner, K6a, have also been found in PC-1 cases. Such mutations would be predicted to disrupt the heptad repeat pattern necessary for the coiled-coil α-helical conformation of this domain, and therefore might be predicted to lead to severe phenotypes. However, when one considers the FNEPPK mutations previously reported in the 1A domain, N125S and R127C, it is less easy to explain the milder phenotype purely in molecular terms. The analogous mutations cause severe disease phenotypes in a number of other keratin diseases, e.g. the equivalent R125C mutation in K14 causes EBS–DM. Analysis of larger numbers of PC-1 and FNEPPK cases should confirm or dispel these early genotype-phenotype predictions.

We have demonstrated that there are distinctive ultrastructural changes in plantar keratinocytes from a

restriction site and so a mismatch primer was used which, in combination with the mutation, produces an EagI site. The mutation was confirmed in affected individuals from family B and was excluded from 50 unaffected unrelated individuals by EagI digestion of specific nested PCR fragments derived from the KRT16A gene (not shown).
PC-1 patient carrying K16 mutation R127P (Fig. 3), compared with other keratin diseases. Specifically, keratin filament bundles were greatly condensed, but did not form the dense amorphous aggregates seen in a number of other keratin disorders, including EB5–DM, BCIE, epidermolytic palmoplantar keratoderma and ichthyosis bullosa of Siemens. The types of mutations observed in these other disorders are very similar to those described here. In the case of the R127P mutation in family A, the identical mutation has been described in K14 (R125P) causing EB5–DM and K10 (R156P) in BCIE. In the latter two cases, the more typical amorphous keratin clumps were seen.

One explanation for the different action of K16 mutations might involve the large number of keratins expressed in palmoplantar epidermis, which could dilute the mutant protein and ameliorate its detrimental effect. However, gross filament aggregation results from mutations of other keratins expressed in the same tissue, including K1, K10 and K9, and therefore this cannot fully explain the observed differences. However, there might be less K16 expressed in this tissue relative to the other keratins, again diluting the mutant polypeptide. The relative abundance of keratins in ridged epidermis at either the protein or mRNA level is currently unknown. A further hypothesis is that K16 may behave differently from the other keratins of ridged epidermis in terms of its interactions with the other filament systems present. There is in vitro biochemical evidence for specificity of keratin pairing. Therefore, K16 might not be fully incorporated into the K1/K9/K10 system or may form an independent cytoskeleton with K6 isoforms present. Furthermore, it has been shown that K16, unlike a number of other type I keratins, forms unstable tetramers in vitro and inappropriate expression of this protein in basal keratinocytes of transgenic mice delays the development of the skin. Overexpression of human K16 in the skin of transgenic mice produces ultrastructural changes similar to those seen here (Fig. 3) and in earlier PC-1 studies. In view of this evidence, the ultrastructural differences between PC-1 and other keratin disorders may reflect functional variations between K16 and other type I keratins.

In one of the kindreds analysed here, family B carrying K16 mutation Q122P, the PC-1 phenotype is accompanied by cataract in two affected persons. Initially, we speculated that both phenotypes in this family might be caused by a mutation in a keratin or related protein which is expressed in the epithelial cells of the lens, as well as the epithelia affected by PC-1. However, as these phenotypes do not fully cosegregate in family B here (Fig. 1), it seems likely that the cataract phenotype is caused by a mutation in a separate gene. Furthermore, in view of the fact that the mutation underlying PC in this family is in K16, a protein which is not expressed in the lens, it seems more likely that the two phenotypes are coincident and that the cataract defect lies in another gene.

In conclusion, we report two novel K16 mutations in PC-1 families. Ultrastructural analysis in one family showed striking tonofilament aggregation which was, however, distinct from that seen in other keratin disorders. In the other family, it was concluded that the previously reported cataract association was unrelated to the identified K16 mutation.

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