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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.

ORIGINAL ARTICLE

A novel missense mutation L468Q of keratin 6a in pachyonychia congenita type 1

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Abstract

Background Pachyonychia congenita is an autosomal dominant disorder that usually develops in early infancy. The major features of the syndrome are hypertrophic nail dystrophy, palmoplantar keratoderma and oral leukokeratosis, accompanied by other ectodermal defects, according to subtype.

Objective To analyse the K6a gene mutation in a sporadic Chinese patient with pachyonychia congenita type 1 (PC-1) and to explore the relationship between the genotype and phenotype of PC-1.

Methods Genomic DNA was extracted from peripheral blood of the patient with PC-1 and 100 unrelated controls. The whole coding region of K6a gene was amplified using long-range polymerase chain reaction (PCR); nested PCR was then used to amplify the mutation 'hot-spot' of the K6a gene. The PCR products were directly sequenced to detect the mutation.

Results A novel missense mutation L468Q in the helix 2B domain of the K6a polypeptide was identified in the patient but not in the healthy individuals from the family and 100 unrelated control individuals.

Conclusions We describe this mutation for the first time, and provide further evidence that the helix boundary motif sequences of K6a are a mutation 'hot-spot'.

Introduction

Pachyonychia congenita (PC) is a rare, autosomal dominant keratin disorder that typically affects the nails and palmoplantar skin, and often the oral mucosa, tongue, larynx, teeth, and hair.¹ Symmetrically thickened, dystrophic fingernails and toenails are the defining characteristic of PC. Jadassohn–Lewandowsky type or PC-1 (OMIM no. 167200) presents with nail dystrophy accompanied by focal non-epidermolytic palmoplantar keratoderma, follicular keratosis and oral leukokeratosis.² Palmoplantar keratoderma and oral changes are major distinguishing features of PC-1. Jackson–Lawler type or PC-2 (OMIM no. 167210) is characterized by nail dystrophy associated with palmoplantar keratoderma, natal teeth, and pili torti.³ The presence of pilosebaceous cysts following puberty is the best indicator of PC-2; natal teeth are also

indicative of PC-2, but this feature is not fully penetrant and its absence does not preclude the PC-2 phenotype.⁴ Specifically, PC-1 has been shown to be caused by mutations in keratins K6a and K16. In contrast, PC-2 has been associated with mutations in K6b and K17. K6/K16 are coexpressed in a number of differentiated epithelial structures, including palmoplantar epidermis, mucosal epithelia, follicular keratinocytes and nail bed, whereas K17 is expressed in the pilosebaceous unit and basal appendageal keratinocytes.⁵ A delayed onset, or tarda subtype of both PC-1 and PC-2, has been described and mutations associated with PC-tarda have been found outside the helix boundary motifs of the K16 and K17 proteins.^{6,7}

Here, we report a novel missense mutation in the K6a gene in a sporadic case of Chinese origin with a typical clinical presentation of PC-1.

Clinical report

The proband was a 12-year-old Chinese girl who presented with thick nails present since birth, followed by gradual development of thick plantar keratoderma. She complained of hyperhidrosis of the hands and feet. There was no consanguinity in the family, and none of the other siblings or family members had similar lesions. Physical examination revealed typical symmetrical thickening of all fingernails and toenails with a yellow-greyish colour (fig. 1a). Yellowish thick plaques with fissures on both soles were also observed (fig. 1b). She also had follicular hyperkeratosis on the buttocks and thighs. There were no hair anomalies, natal teeth or pilosebaceous cysts diagnostic of PC-2.

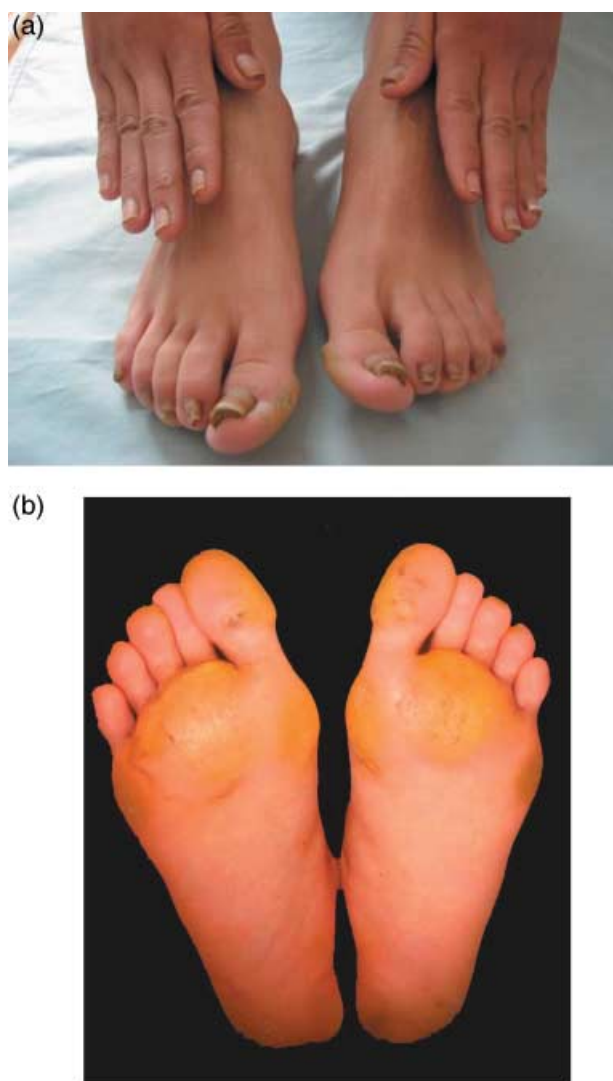


fig. 1 Clinical appearance of the patient studied. (a) Hypertrophic nail dystrophy of all fingernails and toenails. (b) Focal non-epidermolytic palmo-plantar keratoderma (FNEPPK), seen here on the soles, which often causes pain on walking.

Materials and methods

After informed consent, genomic DNA was extracted from peripheral blood by a kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. In addition, genomic DNAs from 100 healthy Chinese individuals were used as controls. This study was approved by Anhui Medical Institutional review board and was conducted according to the Declaration of Helsinki Principles.

In order to avoid pseudogene amplification, nested-primer PCR was used. First, a genomic DNA fragment of approximately 7 kb of the K6a gene was amplified using a primer pair specific to K6a, as previously described.⁸ Forward primer K6aP1: 5'CCA GCC TCT CAC ACT CTC CTC 3' and reverse primer K6aP2: 5'GAC CGA GAG CTA GCA GAC GC 3'. PCR were performed using a Genepha Long PCR kit (Bio Basic Inc., East Markham, ON, Canada). The final volume of PCR reaction was 50 µL. PCR reaction mixture consisted of 500 ng genomic DNA, 0.3 µmol/L of each primer, 1.4 mmol/L dNTPs, 1.75 mmol/L MgCl₂ and 0.75 units enzyme mix (containing thermostable Taq DNA polymerase and a proofreading polymerase). For amplification, the template was denatured for 2 min at 94 °C, followed by 35 cycles each comprising denaturation at 94 °C for 10 s, annealing at 60 °C for 30 s and extension at 68 °C for 10 min; the final extension was 68 °C for 10 min.

Then, oligonucleotide primers specific to exon 1 and 7 of the functional K6a gene were designed using Primer 3.0 (Table 1). Using PCR product as template, short fragments were amplified in standard ×10-PCR buffer containing 1.2 mmol/L MgCl₂. PCR conditions were similar for each primer pair, except that the annealing temperature varied: 94 °C for 5 min ×1; 94 °C for 45 s, 60–64 °C for 50 s, 72 °C for 50 s ×30; and 72 °C for 8 min ×1. The PCR products were analysed by 2% agarose gel electrophoresis.

After amplification, the products were purified using a QIAquick PCR Purification Kit (Qiagen Inc., Hilden, Germany) and directly sequenced on an ABI 377 automated sequencer. Primers K6a1F and K6a1R were used to sequence the helix initiation peptide region of K6a. Primers K6a7F and K6a7R were used to sequence the helix termination peptide region.

Table 1 Primers used for mutation detection

Primers (5'→3')	Exon	Size	Annealing
K6a1F: caagctcaccttcaggact	1	791 bp	60 °C
K6a1R: ctgggctgagtcctcttctc			
K6a7F: accatcaattctgggtctct	7	475 bp	64 °C
K6a7R: gtggaagagtcctatgggaaa			

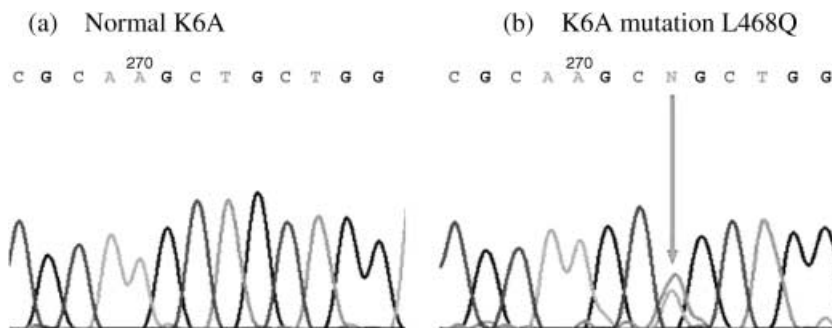
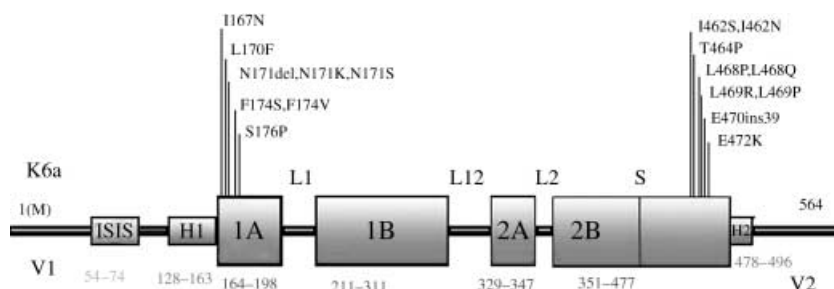


fig. 2 (a) Normal K6a exon 7 sequence (+ strand) corresponding to codons 1396–1408. (b) Same region as shown in (a) from the affected individual showing heterozygous missense mutation 1403T→A (arrow), predicting amino acid change L468Q.

fig. 3 Diagrammatic representation of K6a protein showing the domain organization and the location of reported mutations. The α -helical rod domain is subdivided into 1A, 1B, 2A and 2B segments by flexible linkers L1, L12 and L2. The rod domain is flanked by variable domains V1 and V2. The 'stutter' sequence is marked by S.



Results

A heterozygous missense mutation T→A at the 1403rd nucleotide acid position of the mRNA of the K6a gene was detected, which changed codon 468 from leucine (CTG) to glutamine (CAG). This mutation was not found in four healthy individuals of the PC-1 family and 100 unrelated controls, suggesting that L468Q is not a common polymorphism (fig. 2a,b). This particular defect has not been reported previously; Smith *et al.* have recently reported a different mutation of L468P in the same codon.⁹

Discussion

Keratins have a similar protein structure consisting of a highly conserved central helical rod domain, which is responsible for polymerization of these proteins to form keratin tonofilaments. This rod domain is divided into 1A, 1B, 2A and 2B domains, each consisting of heptad repeats. Connecting these are three non-helical linkers, L1, L12 and L2, which are thought to provide flexibility to the rod domain. The rod domain is flanked by variable domains V1 and V2. Within the 2B domain there is a 'stutter' sequence where the helix polarity is reversed¹⁰ (fig. 3) (<http://www.interfil.org>).

The highly conserved regions that delineate the start and end of the central helical rod domain are termed the helix boundary motifs, which are encoded by exons 1 and 6 in type I keratin genes and exons 1 and 7 in type II keratin genes. The helix boundary motifs are mutational hot

spots for all keratin disorders, and most PC mutations reported to date have occurred in these regions. Therefore, PC mutation analysis should first focus on these exons. The presence of pseudogenes hampers mutation detection study using genomic DNA samples. For K6 there are at least six highly homologous copies of the gene, four of which are expressed, encoding mRNAs which differ by only a few base pairs.¹¹ Therefore, we used long-range PCR to amplify all exons of the K6a gene as described previously.⁸ Nested-primer PCR was then used for amplification of the helix boundary motifs. We identified a novel missense mutation 1403T→A in exon 7 of the K6a gene. This mutation predicted the amino acid change leucine to glutamine (L468Q) in the helix termination motif of the K6a polypeptide.

To date, over 32 mutations in the K6a gene have been identified in independently ascertained families¹ (Table 2) (<http://www.interfil.org>). In keeping with the other keratins, the majority of these mutations are missense mutations, with a smaller number of inframe insertions or deletions. The most common mutational location is codon 171 of the K6a gene. Twelve out of 33 patients carried the mutation K6aN171del. The same mutation can cause different clinical features. Nail changes can be seen in all patients. It is one of the earliest manifestations of PC and individuals are frequently affected at or soon after birth. Oral leukokeratosis is usually present soon after birth and may be the earliest sign of PC. Development of the plantar keratoderma occurs later than the nail findings, and the severity is highly variable. Follicular keratoses often present in

Table 2 Summary of reported mutations of K6a in pachyonychia congenita-1

Ref. no.	Disease	Inheritance	Mutation	Domain
12	PC-1	Familial	K6a N171del	1A
8	PC-1	Familial	K6a N171K	1A
8	PC-1	Sporadic	K6a F174S	1A
13	PC-1	Sporadic	K6a N171del	1A
13	PC-1	Sporadic	K6a N171K	1A
4	PC-1	Sporadic	K6a F174V	1A
4	PC-1	Sporadic	K6a E472K	2B
4	PC-1	Sporadic	K6a L469R	2B
4	PC-1	Sporadic	K6a F174S	1A
4	PC-1	Sporadic	K6a N171del	1A
4	PC-1	Familial	K6a N171del	1A
4	PC-1	Sporadic	K6a N171del	1A
14	PC-1	Sporadic	K6a I462S	2B
15	PC-1	Sporadic	K6a T464P	2B
16	PC-1/PC-2	Sporadic	K6a N171del	1A
9	PC-1	Familial	K6a I167N	1A
9	PC-1	Familial	K6a L170F	1A
9	PC-1	Sporadic	K6a N171S	1A
9	PC-1	Familial	K6a S176P	1A
9	PC-1	Familial	K6a I462N	2B
9	PC-1	Familial	K6a L468P	2B
9	PC-1	Sporadic	K6a L469P	2B
9	PC-1	Sporadic	K6a E470ins39	2B
9	PC-1	Sporadic	K6a N171del	1A
9	PC-1	Sporadic	K6a N171del	1A
9	PC-1	Familial	K6a N171del	1A
9	PC-1	Familial	K6a N171del	1A
9	PC-1	Sporadic	K6a N171del	1A
9	PC-1	Familial	K6a N171del	1A
9	PC-1	Sporadic	K6a N171K	1A
9	PC-1	Sporadic	K6a F174S	1A
9	PC-1	Sporadic	K6a F174S	1A
Present study	PC-1	Sporadic	K6a L468Q	2B

childhood. Neither natal teeth nor pilosebaceous cysts are seen in PC-1. The correlation between the clinical syndrome (PC-1 or PC-2) and the mutated gene (K6a/K16 or K6b/K17) has generally been highly consistent. However, there has been a report¹⁶ that described a case with overlapping clinical features of PC-1 and PC-2. After mutation detection, K6a N171del was identified. The author thought K6a was expressed in the hair follicles in the oral epithelia. Therefore, it is possible that mutation of K6a can lead to pilosebaceous cysts, a key diagnostic feature that distinguishes PC-2 from PC-1. All previously reported mutations occurred in the helix boundary motifs of K6a. Mutations in these motifs are predicted to be highly disruptive to intermediate filament assembly. However, Connors *et al.*⁶ reported a PC-1 patient with late onset due to a missense mutation K354N located in the central 2B domain of K16. Recently, late-onset PC-2 was found to be

caused by a missense mutation N109D in the second half of the 1 A domain of K17.^{7,17} These mutations occurred in regions where analogous K14 mutations have been found in the milder variants of EBS.¹⁸ Further mutation analysis should confirm whether mutations in the helix boundary motifs produce more severe phenotype mutations in the non-helical domains, or in the mid-region of the α -helical rod domain cause a milder phenotype, and whether late-onset of nail dystrophy is due to the site of the mutation. It is too early to make conclusive genotype-phenotype correlations because of the small number of K6a mutations. As Munro recognized, phenotype and severity depend on genetic background and environmental factors as well as the underlying mutation.¹⁹

In conclusion, we report a Chinese patient with PC-1 and detected a novel mutation, L468Q, in the helix 2B domain of the K6a gene. The identification of K6a and K16 as the disease-causing gene and the ongoing recognition of different mutations may give insight into the still-unknown mechanism leading to PC-1, and facilitate prenatal diagnosis of PC-1.

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