Eur J Pediatr
DOI 10.1007/s00431-013-2236-8

ORIGINAL ARTICLE

Mutation p.Leu128Pro in the 1A domain of K16 causes pachyonychia congenita with focal palmoplantar keratoderma in a Chinese family

Limeng Dai · Jun Wu · Hong Guo · Yangming Huang · Kun Zhang · Dan Liu · Liyuan Fu · Yuanyuan Wu · Xingying Guan · Yun Bai · Qiong Liao

Received: 20 June 2013 / Revised: 25 November 2013 / Accepted: 2 December 2013

Abstract Pachyonychia congenita (PC), a rare autosomal dominant disorder characterized by hypertrophic nail dystrophy, is classified into two main clinical subtypes: PC-1 and PC-2. PC-1 is associated with mutations in the *KRT6A* or *KRT16* genes, whereas PC-2 is linked to *KRT6B* or *KRT17* mutations. Blood samples were collected from three generations of a new Chinese PC-1 family, including three PC patients and five unaffected family members. A novel missense mutation p.Leu128Pro (c.383T>C) was identified in a highly conserved helix motif in domain 1A of K16. The disease haplotype carried the mutation and cosegregated with the affection status. PolyPhen2 and SIFTS analysis rated the substitution as probably damaging; Swiss-Model analysis indicated that the structure of the mutant protein contained an unnormal α-helix. Overexpression of mutant protein in cultured cells led to abnormal cell morphology. *Conclusion*: The wider spectrum of *KRT16* mutations suggests that changes in codons 125, 127, and 132 are most commonly responsible for PC-1 and that proline substitution mutations at codons 127 or 128 may produce more severe disease. This study extends the *KRT16* mutation spectrum and adds new information on the clinical and genetic diversity of PC.

Keywords Genodermatosis · keratin 16 · mutation · pachyonychia congenita

Abbreviations

PC	Pachyonychia congenital
K16	Keratin 16 protein
KRT16	Keratin 16 gene
PPK	Palmoplantar keratoderma

Introduction

Pachyonychia congenita (PC) is a rare autosomal dominant disorder characterized by hypertrophic nail dystrophy. Historically, PC has been classified into two main clinical subtypes: PC-1(OMIM 167200) and PC-2 (OMIM 167210). PC-1 is linked to mutations in *KRT6A* or *KRT16*, whereas PC-2 is associated with mutations in *KRT6B* or *KRT17* [4, 11, 15]. Oral leukokeratosis, palmoplantar keratoderma (PPK), and follicular keratosis are also commonly observed in PC-1. Sebaceous cysts which normally develop around puberty are a useful distinguishing feature in PC-2. Recently, a more specific molecular genetic nomenclature has been adopted by the International Pachyonychia Congenita Consortium. In this system, PC-6a, PC-6b, PC-16, and PC-17 refer to cases with known mutations in the genes *KRT6A*, *KRT6B*, *KRT16*, and *KRT17*, respectively. PC-U designates cases of suspected PC, where either a mutation has not been found or has not been investigated [10, 18].

Keratin proteins share a similar structural motif consisting of a highly conserved central helical rod domain. Each keratin polypeptide possesses an amino acid sequence of 310 residues

Electronic supplementary material The online version of this article (doi:10.1007/s00431-013-2236-8) contains supplementary material, which is available to authorized users.

L. Dai · H. Guo · K. Zhang · D. Liu · L. Fu · Y. Wu · X. Guan · Y. Bai (✉)
Department of Medical Genetics, College of Basic Medical Science, Third Military Medical University, Chongqing, China
e-mail: baiyungene@gmail.com

J. Wu
Department of Dermatology, Xinqiao Hospital, Third Military Medical University, Chongqing, China

Y. Huang · Q. Liao (✉)
Department of Ophthalmology, Xinqiao Hospital, Third Military Medical University, Chongqing, China
e-mail: liaoq375@sohu.com

Published online: 20 December 2013

and an α-helical rod domain composed of four helical segments (1A, 1B, 2A, and 2B). These domains are connected by non-helical linker regions, L1, L12, and L2. The rod domain is flanked by head (N-terminal) and tail (C-terminal) domains of more flexible and more variable structure. K16, a type I intermediate filament protein, is constitutively expressed in a variety of epithelial tissues, including the tongue and the hair follicle, and in glabrous skin [3]. Upon stressful epithelial stimuli, such as wounding or chronic inflammation, K16 and its binding partner K6 are selectively induced in the suprabasal layers of the epidermis [12].

Mutations altering the coding sequence of *KRT16* are known to be responsible for many cases of PC. We report the identification of a novel proline substitution mutation p.Leu128Pro in the 1A domain of K16 that is associated with PC with focal PPK in a Chinese family.

Materials and methods

Patients

The proband (III2) was a 29-year-old Chinese woman of Chongqing province; the chief complaint was familial nail dystrophy and hyperkeratosis (Fig. 1). All her fingernails and toenails had been characteristically thickened since the age of 5 months. Focal hyperkeratotic plaques with the formation of blisters appeared subsequent to nail thickening. There was evidence of palmar lesions subsequent to mechanical trauma.

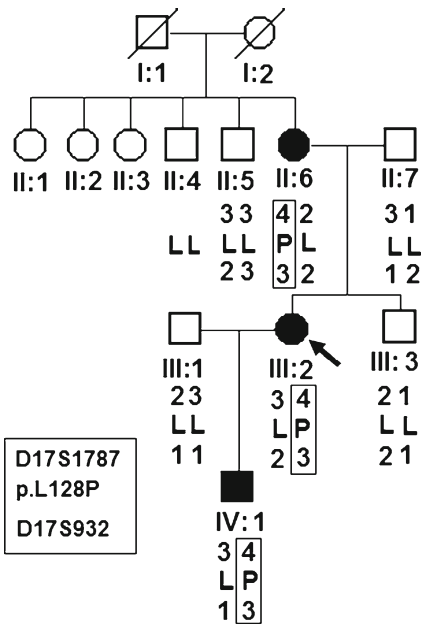

Fig. 1 Family pedigree of a three-generation Chinese PC-1 family. *Arrow* indicates proband (III-2). The disease shows autosomal dominant inheritance. The disease haplotype (shown in the *box*) carries the mutation Leu128Pro and cosegregates with the affection status

Hyperkeratotic lesions overspread the plantar surface of the proband and her mother (II6) at around 2 years of age; focal plantar keratoderma was observed in her 4-year-old son (IV1). Oral leukokeratosis was not found in affected family members. The photos of clinical data were shown on Fig. 2.

Sample collecting

The study was performed with the approval of the Ethics Committee of Third Military Medical University (Chongqing, China). With informed consent from family members, venous blood samples were collected from proband and other family members. One hundred blood samples from healthy persons provided controls.

Mutation detection and haplotype analysis

The coding regions of the *KRT6A* and *KRT16* were amplified by PCR and analyzed by direct sequencing. Two markers of microsatellite (D17s1787 and D17s932) near *KRT16* gene using the UCSC Genome Browser on Human Mar. 2009 Assembly (http://www.genome.ucsc.edu) were selected to refine the critical region of the disease locus.

Functional significance prediction and analysis

Three online programs, PolyPhen2 (http://genetics.bwh.harvard.edu/pph2), SIFT (http://sift.jcvi.org), and Swiss-Model (http://swissmodel.expasy.org), were used to predict the functional significance of mutations [2, 17]. Expression plasmids that expressed wild type K16 protein or Leu128Pro mutant as fusions with EGFP at the N-terminus were constructed. The mutant position was prepared using site-directed

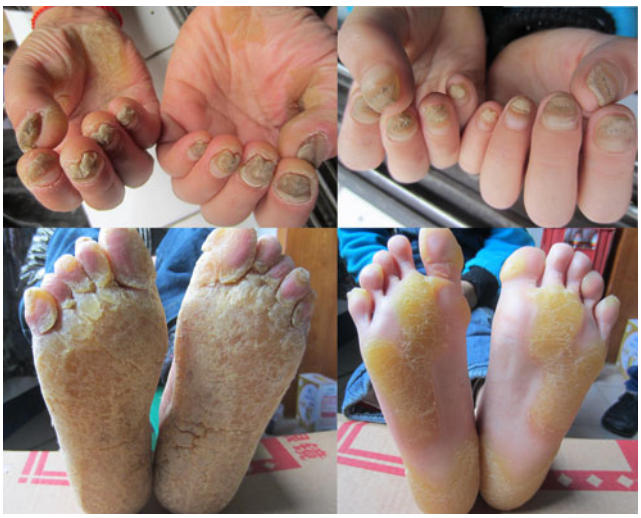

Fig. 2 Clinical features of family. Hypertrophic dystrophy of the fingernails in patients (*left* III-2, *right* IV-1). The proband (III2) and her son (IV1) suffered severe palmoplantar hyperkeratosis, particularly on the plantar surface

mutagenesis. Plasmids were transfected with lipofectamine2000 (Invitrogen) according to the manufacturer's instructions in 293FT cell line.

Results

Mutation detection and haplotype analysis

Sequencing analysis showed that proband carried a heterozygous mutation c.383T>C within exon 1 of *KRT16* gene; this mutation was also identified in her son and mother. No other sequence changes were observed in *KRT16* and *KRT6A* genes. No *KRT16* gene mutations were detected in five unaffected family members or in 100 healthy controls. The missense mutation causes a leucine to proline substitution at amino acid 128 of the K16 polypeptide (Fig. 3a). And the haplotype 4-P-3 of (D17s1787-L128P-D17s932) cosegregates with the affection status (Fig. 1).

A spectrum of *KRT16* gene mutations cause PC

Our results add new information and enlarge the spectrum of *KRT16* mutations. To date, 20 different variants of the *KRT16* gene have been reported in PC (http://www.interfil.org). Combined with our new case, four mutations underlie PC in the majority in families with *KRT16* mutations: c.395T>C (p.Leu132Pro), c.374A>G (p.Asn125Ser), c.379C>T (p.Arg127Cys), and c.380G>C (p.Arg127Pro), accounting for 20.7 %, 20.7 % 12.7 %, and 8.6 %, respectively, of PC associated with *KRT16* mutations. These data suggest that mutations in this region (121–133; Supplemental Fig. 1) of the protein underlie the majority of *KRT16*-associated PC cases and that this region may be particularly important for function.

Functional significance prediction and analysis

The p.Leu128Pro substitution was rated as probably damaging by PolyPhen2 with a score of 1.00 (sensitivity 0.00; specificity 1.00) and as damaging by SIFTS software with a score of 0.00 (median information 2.86). In addition, the mutant protein structure was mildly changed compared with wild type K16 by the prediction of Swiss-Model software and an unnormal α-helix appeared on the location of codon 128 (Fig. 3b). Mutant K16-EGFP fusion protein led to unmoral cell morphology compare with normal K16-EGFP protein in 293FT (Supplemental Fig. 2).

Discussion

PC-1 is due to mutations of the *KRT16* or *KRT6A* genes. To date, over 40 variants in *KRT6A/KRT16* have been identified in ~170 independently ascertained families with PC-1. It has been reported that most the known keratin mutations in patients with PC-1 are within the conserved helix boundary motifs of either the beginning of 1A or the end of the 2B domain. One mutation of *KRT6A* and three mutations of *KRT16* involve the head domain. For the tail domains of K6A and K16, only one *KRT6A* mutation has so far been identified.

According to the overall spectrum analysis of *KRT16* mutations, four missense mutations (c.395T>C, c.374A>G, c.379C>T, and c.380G>C) account for 20.7 %, 20.7 % 12.7 %, and 8.6 % of familial PC-1, respectively. In *KRT6A*, 31.3 % cases carry a deletion (c.514_516delAAC, p.Asn172del), whereas 16.4 % of cases harbor a missense mutation at codon 171 and 12.2 % at codon 174. Thus, in over 50 % of families with *KRT16*- or *KRT6A*-associated PC-1, the disorder can be ascribed to mutations at one of these

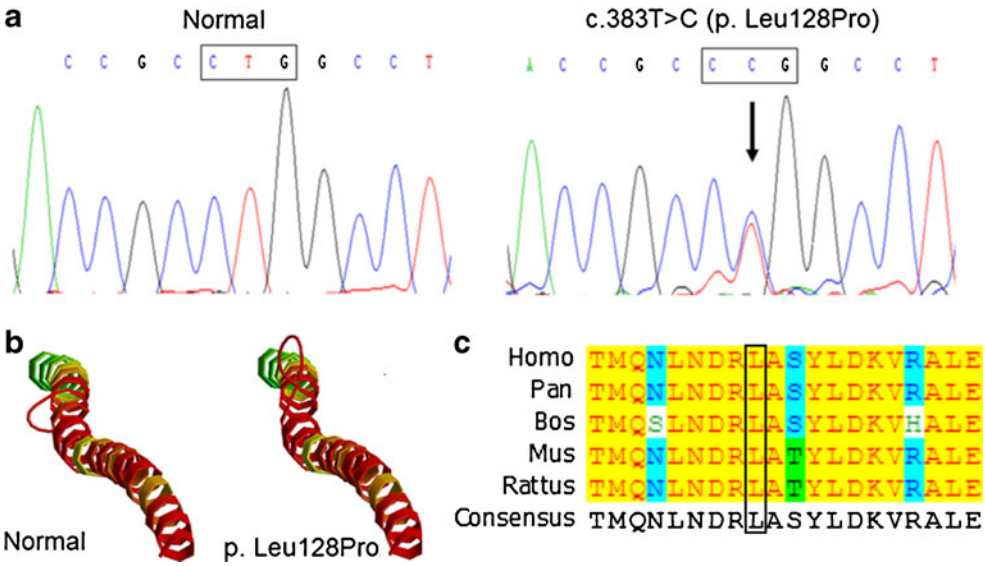

Fig. 3 **a** Mutation analysis of *KRT16* showing the heterozygous missense mutation, c.383T>C transition (p.Leu128Pro) in the proband. **b** The mutant protein changes compared with wild-K16 by Swiss-Model software, and unnormal α-helix appears on the location of mutant codon 128. **c** Partial amino acid sequences alignment among several species. The position of mutated amino acid of our cases was highly conserved indicated by the *black box*

specific sites. These data suggest that positions 125, 127, and 132 of K16 and positions 171, 172, and 174 of K6A may be of particular importance for protein function. Our study, together with previously reported data, is likely to be of utility in the development of personalized medicine for PC.

PC-associated mutations tend to cluster in specific sites within the coding sequences, but disease phenotype varies widely both among and within families. Correlation between the specific clinical features observed and the protein domain harboring the mutation has proved to be difficult. Clinical severity can differ between mutations in the same gene and even between individuals harboring the same mutation. For example, a different PC-associated mutation was reported to affect the identical codon to that reported here, and a c.383T>A (p.Leu128Gln) mutation was previously linked to familial PC-1 [9, 16]. However, in our study, family members harboring the p.Leu128Pro mutation develop PC with severe PPK and present phenotypic heterogeneity with both focal and diffuse hyperkeratosis (Fig. 2), which were not reported for patients harboring p.Leu128Gln. Furthermore, in previous studies, p.Arg127Pro mutations were reported to produce more severe disease than p.Arg127Cys mutations in terms of age of onset of symptoms, the extent of nail involvement, and impact upon daily quality of life [6]. These findings are consistent with the fact that proline, reflecting its atypical chemical structure, is more strongly detrimental to α-helical tertiary structures within proteins than are other amino acid substitutions [13]. We therefore speculate that proline substitutions at codons 127 or 128 cause a larger and more disruptive change in K16 protein structure and could thereby lead to more severe disease phenotypes. Functional prediction by Swiss-Model analysis and expression of mutant K16-EGFP fusion protein in culture cells supported it. Conversely, one would expect that mutations introducing more conservative substitutions at the same sites are associated with milder disease, although our analyses failed to reveal a direct correlation between phenotype and mutation type in *KRT6A*.

Overall, PC-1 may be considered as a spectrum of phenotypes, ranging from very mild to more severe, in which the particular gene involved appears to have a moderate influence on phenotype but the specific mutation type has only a small influence on phenotype [18].

Almost all human PC patients harboring *KRT16* mutations report PPK. However, many mutations in *KRT16* only elicit milder overall PC phenotypes, often diagnosed as FPPK, reflecting the limited and at times absent nail involvement [7–9]. A mouse model of deletion of *Krt16* gene (*Krt16*−/− or *Krt16*+/−) produced spontaneously arising PPK-like lesions [8]. The available data do not fully explain the genotype–phenotype correlations and mechanism of pathogenesis of PC-16. The molecular mechanisms underlying PPK pathogenesis in PC remain unclear; this symptom is currently thought to result from intermediate filament network disruption by dominantly acting mutations or loss-of-function of keratin due to haploinsufficiency in relevant keratins [5, 6]. Keratins, non-keratins, and/or environmental influences can all modulate PPK pathogenesis [14]. The heterogeneity may partly reflect individual lifestyles, occupation [1], and differences in PC management but may also reflect the specific type of mutation, in addition to environmental factors and other genetic factors—such as genetic polymorphisms linked to PC-associated genes and epigenetic regulation of gene expression.

In conclusion, we report a novel mutation p.Leu128Pro (c.383T>C) of K16 in a Chinese family with PC and PPK of heterogeneous phenotype. Proline substitution mutations at codons 127 or 128 of *KRT16* may be associated with more severe PC disease phenotype. Pathogenesis may reflect either dominant negative effects or, potentially, loss-of-function associated with the proline substitution. Our case adds new information regarding the involvement of *KRT16* gene mutations in the clinical and genetic diversity of PC and PPK.

Acknowledgments We thank the families for their cooperation and participation in this study.

Funding This work was supported by the National Natural Science Foundation of China (No.81171678 and No.81100068).

Conflict of interest None.

References

1. Akasaka E, Nakano H, Nakano A, Toyomaki Y, Takiyoshi N, Rokunohe D, Nishikawa Y, Korekawa A, Matsuzaki Y, Mitsuhashi Y, Sawamura D (2011) Diffuse and focal palmoplantar keratoderma can be caused by a keratin 6c mutation. Br J Dermatol 165(6):1290–1292. doi:10.1111/j.1365-2133.2011.10552.x
2. Arnold K, Bordoli L, Kopp J, Schwede T (2006) The SWISS-MODEL workspace: a web-based environment for protein structure homology modelling. Bioinformatics 22(2):195–201. doi:10.1093/bioinformatics/bti770
3. Bernot KM, Coulombe PA, McGowan KM (2002) Keratin 16 expression defines a subset of epithelial cells during skin morphogenesis and the hair cycle. J Invest Dermatol 119(5):1137–1149. doi:10.1046/j.1523-1747.2002.19518.x
4. Bowden PE, Haley JL, Kansky A, Rothnagel JA, Jones DO, Turner RJ (1995) Mutation of a type II keratin gene (K6a) in pachyonychia congenita. Nat Genet 10(3):363–365. doi:10.1038/ng0795-363
5. Cao LH, Luo Y, Wen W, Liu WL, Jiang L, Chen C, Ji CY, Zhang X (2011) A novel frameshift mutation in keratin 16 underlies pachyonychia congenita with focal palmoplantar keratoderma. Br J Dermatol 165(5):1145–1147. doi:10.1111/j.1365-2133.2011.10450.x
6. Fu T, Leachman SA, Wilson NJ, Smith FJ, Schwartz ME, Tang JY (2011) Genotype-phenotype correlations among pachyonychia congenita patients with K16 mutations. J Invest Dermatol 131(5):1025–1028. doi:10.1038/jid.2010.373
7. Leachman SA, Kaspar RL, Fleckman P, Florell SR, Smith FJ, McLean WH, Lunny DP, Milstone LM, van Steensel MA, Munro CS, O'Toole EA, Celebi JT, Kansky A, Lane EB (2005) Clinical and pathological features of pachyonychia congenita. J Investig Dermatol Symp Proc 10(1):3–17. doi:10.1111/j.1087-0024.2005.10202.x

8. Lessard JC, Coulombe PA (2012) Keratin 16-null mice develop palmoplantar keratoderma, a hallmark feature of pachyonychia congenita and related disorders. J Invest Dermatol 132(5):1384–1391. doi:10.1038/jid.2012.6
9. Liao H, Sayers JM, Wilson NJ, Irvine AD, Mellerio JE, Baselga E, Bayliss SJ, Uliana V, Fimiani M, Lane EB, McLean WH, Leachman SA, Smith FJ (2007) A spectrum of mutations in keratins K6a, K16 and K17 causing pachyonychia congenita. J Dermatol Sci 48(3):199–205. doi:10.1016/j.jdermsci.2007.07.003
10. McLean WH, Hansen CD, Eliason MJ, Smith FJ (2011) The phenotypic and molecular genetic features of pachyonychia congenita. J Invest Dermatol 131(5):1015–1017. doi:10.1038/jid.2011.59
11. McLean WH, Rugg EL, Lunny DP, Morley SM, Lane EB, Swensson O, Dopping-Hepenstal PJ, Griffiths WA, Eady RA, Higgins C et al (1995) Keratin 16 and keratin 17 mutations cause pachyonychia congenita. Nat Genet 9(3):273–278. doi:10.1038/ng0395-273
12. Paladini RD, Takahashi K, Bravo NS, Coulombe PA (1996) Onset of re-epithelialization after skin injury correlates with a reorganization of keratin filaments in wound edge keratinocytes: defining a potential role for keratin 16. J Cell Biol 132(3):381–397
13. Serrano L, Sancho J, Hirshberg M, Fersht AR (1992) Alpha-helix stability in proteins. I. Empirical correlations concerning substitution of side-chains at the N and C-caps and the replacement of alanine by Glycine or serine at solvent-exposed surfaces. J Mol Biol 227(2):544–559
14. Smith FJ, Fisher MP, Healy E, Rees JL, Bonifas JM, Epstein EH Jr, Tan EM, Uitto J, McLean WH (2000) Novel keratin 16 mutations and protein expression studies in pachyonychia congenita type 1 and focal palmoplantar keratoderma. Exp Dermatol 9(3):170–177
15. Smith FJ, Liao H, Cassidy AJ, Stewart A, Hamill KJ, Wood P, Joval I, van Steensel MA, Bjorck E, Callif-Daley F, Pals G, Collins P, Leachman SA, Munro CS, McLean WH (2005) The genetic basis of pachyonychia congenita. J Investig Dermatol Symp Proc 10(1):21–30. doi:10.1111/j.1087-0024.2005.10204.x
16. Terrinoni A, Smith FJ, Didona B, Canzona F, Paradisi M, Huber M, Hohl D, David A, Verloes A, Leigh IM, Munro CS, Melino G, McLean WH (2001) Novel and recurrent mutations in the genes encoding keratins K6a, K16 and K17 in 13 cases of pachyonychia congenita. J Invest Dermatol 117(6):1391–1396. doi:10.1046/j.0022-202x.2001.01565.x
17. Thusberg J, Olatubosun A, Vihinen M (2011) Performance of mutation pathogenicity prediction methods on missense variants. Hum Mutat 32(4):358–368. doi:10.1002/humu.21445
18. Wilson NJ, Leachman SA, Hansen CD, McMullan AC, Milstone LM, Schwartz ME, McLean WH, Hull PR, Smith FJ (2011) A large mutational study in pachyonychia congenita. J Invest Dermatol 131(5):1018–1024. doi:10.1038/jid.2011.20