Original Paper

Identification of a Novel Keratin 9 Missense Mutation in a Chinese Family with Epidermolytic Palmoplantar Keratoderma

Heng Xiao^{a,b,c} Yi Guo^d Junhui Yi^e Hong Xia^a Hongbo Xu^a Lamei Yuan^a
Pengzhi Hu^a Zhijian Yang^a Zhenghao He^a Hongwei Lu^a Hao Deng^{a,b}

^aCentre for Experimental Medicine, the Third Xiangya Hospital, Central South University, Changsha, Hunan, ^bDepartment of Neurology, the Third Xiangya Hospital, Central South University, Changsha, Hunan, ^cDepartment of Pathology, the Third Xiangya Hospital, Central South University, Changsha, Hunan, ^dDepartment of Medical Information, Information Security and Big Data Research Institute, Central South University, Changsha, Hunan, ^eDepartment of Ophthalmology, the Third Xiangya Hospital, Central South University, Changsha, Hunan, China

Key Words

Epidermolytic palmoplantar keratoderma • *KRT9* gene • Exome sequencing • Genetic counseling • Keratin filament network • Dominant negative effect

Abstract

Background/Aims: Epidermolytic palmoplantar keratoderma (EPPK) is an autosomal dominant genodermatosis. It is characterized by diffuse yellow keratoses on the palmoplantar epidermis, with an erythematous border. The keratin 9 gene (*KRT9*) and less frequently the keratin 1 gene (*KRT1*) are responsible for EPPK. This study aims to identify and analyse genetic defects responsible for EPPK in a Han Chinese pedigree. **Methods:** A four-generation Han Chinese pedigree containing five individuals affected with EPPK was recruited. Exome sequencing, Sanger sequencing, and bioinformatics tools were conducted to identify the mutation in this pedigree. HaCaT cells were transfected with either wild-type or mutated *KRT9*. Confocal laser immunofluorescence assay, imaging processing, and statistical analysis were performed to evaluate wild-type and mutant *KRT9* groups. **Results:** A novel heterozygous c.1369C>T transition (p.Leu457Phe) in exon 6 of the *KRT9* gene was identified in four patients. It co-segregated with the disorder in the family. Functional analysis showed that withdrawal of the filament network from the cell periphery and particle formation were present in about 10% of Leu457Phe-transfected HaCaT cells, while approximately 3% of cells transfected with wild-type *KRT9* showed this phenotype. The particles in mutant group were larger than that in wild-type group (P -value < 0.05). **Conclusion:** The variant may be the disease-causing missense mutation and produce dominant negative effects by interrupting keratin network formation. This study indicates the pathogenic role of the *KRT9* gene mutation in this pedigree with EPPK, and may be helpful in genetic counseling, prenatal diagnosis and gene-targeted therapies of EPPK.

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H. Xiao and Y. Guo contributed equally to this work.

Hao Deng
and Hongwei LuCentre for Experimental Medicine, the Third Xiangya Hospital, Central South University,
138 Tongzipo Road, Changsha, Hunan (China)
Tel. 86-731-88618372, E-Mail hdeng008@yahoo.com, hwlv2226@163.com

Introduction

Epidermolytic palmoplantar keratoderma (EPPK, OMIM 144200) is a highly penetrant autosomal dominant genodermatosis [1, 2], which was first described by Vörner in 1901 [3]. It has a prevalence of 1.0 to 4.4 per 100,000 without sex predilection [4-6]. The typical clinical feature of EPPK is diffuse yellow keratoses over the entire epidermis of palms and soles, with a well-demarcated erythematous border [7, 8]. Other features are knuckle pads on the digital joints, clubbing of the nails [1], camptodactyly [9], digital mutilation [10], hyperhidrosis, increased sensitivity of the palms and soles to mechanical trauma [1, 11], and decreased heat sensitivity [12]. It usually manifests shortly after birth [7]. Rarely, EPPK has been reported to be associated with woolly hair and heart diseases [13], or with breast and ovarian tumour in a large pedigree [14].

Histologically, palmoplantar epidermis presents varying degrees of dyskeratosis and epidermolytic hyperkeratosis, including pronounced perinuclear vacuolization of the keratinocytes and huge keratohyalin granules located in the upper stratum spinosum [15-17]. Ultrastructural analysis reveals abnormal perinuclear tonofilament clumping and the presence of large and distorted keratohyalin granules [6].

Keratins are a multigene family that are generally expressed in specific type I (acidic)/type II (neutral or basic) keratin pairs [18, 19]. In 1991, a gene for EPPK was mapped to the region of the type I keratin gene cluster at chromosome 17q12-q21 with linkage analysis in a large pedigree from Northwest Germany [15]. The keratin 9 gene (*KRT9*, OMIM 607606), one of the type I keratin genes, was proposed as the candidate gene for EPPK due to its exclusive expression in epidermal keratinocytes of the palms and soles [15, 20, 21]. Subsequently, *KRT9* gene mutations were reported as a causative genetic factor for EPPK [7]. To date, at least 28 mutations in the *KRT9* gene have been described in the Human Intermediate Filament Database (HIFD) and the Human Gene Mutation Database (HGMD) [22].

In this study, we identified a novel heterozygous c.1369C>T transition (p.Leu457Phe) in the *KRT9* gene in a four-generation Han Chinese pedigree with EPPK, and the variant co-segregated with the disease condition in the family and was absent in 200 normal controls, supporting that it may be a disease-causing mutation for EPPK in this family. *In vitro* studies further indicated the involvement of the *KRT9* gene mutation in the pathogenic mechanism responsible for EPPK in this pedigree.

Materials and Methods

Pedigree and participants

A four-generation Han Chinese pedigree consisting of 15 individuals was referred from the Third Xiangya Hospital, Central South University, China (Fig. 1A). Peripheral blood samples and clinical data were collected from 11 pedigree members, consisting of four individuals affected with EPPK (II:1, II:5, III:2, and III:3) and seven unaffected members (I:2, III:1, III:4, III:5, III:6, IV:1, and IV:2). Blood specimens were also obtained from 200 unrelated ethnically-matched normal controls (male/female: 100/100; mean age, 34.2±4.5 years). Written informed consent was obtained from all the participating individuals or their parents. This study was carried out according to the Declaration of Helsinki, and had received approval from the Institutional Review Board of the Third Xiangya Hospital, Central South University, China.

Exome capture

Peripheral blood genomic DNA (gDNA) was extracted from leukocytes as previously described [23]. Paired-end DNA library was generated according to the manufacturer's instructions (Agilent Technologies Inc., USA). Genomic DNA was fragmented into 180-280 bp with the use of sonication. Capture enrichment was performed by using an Agilent SureSelect Human All Exon V5 Kit. The enriched DNA library was sequenced on a HiSeq 2000 platform (Illumina Inc., USA) [24].

Variant analysis

Following sequencing on the HiSeq 2000 platform, the primary data was converted to a FASTQ format and processed to retrieve high quality paired-end reads, which were aligned to human reference genome sequence (UCSC database, GRCh37/hg19) using Burrows-Wheeler Alignment tool [25]. High quality alignment was essential to guarantee variant calling accuracy (greater than 0) so as to identify single nucleotide polymorphisms (SNPs) and insertions-deletions (indels). The analysis-ready Binary Alignment/Map (BAM) alignment results were produced after being processed through SAMtools, Genome Analysis Toolkit, and Picard. SNPs were called using parameters as previously described [26, 27, 28]. SNPs and indels were annotated using Annotate Variation (ANNOVAR), and filtered against databases including the SNPs build 142 (dbSNP142), the 1000 Genomes Project (2014 September release), the NHLBI exome sequencing project (ESP) 6500, and an in-house exome database from Novogene Bioinformatics Institute using 700 ethnically-matched controls. A prioritization process was used as previously described [28]. After this step was performed, any retained variants were taken as “novel” variants. A functional prediction was performed by submitting non-synonymous SNPs to Polymorphism Phenotyping version 2 (PolyPhen-2) and Sorting Intolerant from Tolerant (SIFT, <http://sift.jcvi.org/>) as previously described [28, 29].

Variants were selected for validation when the bioinformatics tools predicted that a variant was disease-causing. Primer3 program was used to design locus-specific primers, and Primer-BLAST (Basic Local Alignment Search Tool) to determine Primer specificity. The forward primer sequence was 5'-CCCCAGGATGAAGAAGTCCT-3', and the reverse primer sequence was 5'-CAAGAGATCGAGTGCCAGAA-3'. Sanger sequencing was performed in the proband, available pedigree members and the 200 normal controls to verify the potential pathogenic variant [30, 31].

Multiple protein sequence alignment among a variety of species was performed using Clustal Omega (<http://www.ebi.ac.uk/Tools/msa/clustalo/>). SIFT, PolyPhen-2, MutationAssessor, and MutationTaster were used to predict the probable influence of the amino acid residue substitution on protein function in order to further analyse the variant's assumed pathogenicity [29, 32, 33].

Plasmid construction

To generate expression constructs for wild-type and mutant KRT9 fusion proteins tagged with EGFP, a human KRT9 cDNA was amplified with primers 5'-TACCGGACTCAGATCTCGAGCGCCACCATGAGCTGCAGACAGTTCTCCTC-3' and 5'-GATCCCGGGCCCGGTACCGTGGGAATGGGATGATTTCCGCTTC-3', and cloned into the *XhoI/KpnI* restricted pEGFP-N1 vector. The identified missense mutation (c.1369C>T, p.Leu457Phe) was introduced into the wild-type KRT9 expression clone by site-directed mutagenesis using primers 5'-CTACCACAACCTTCCTTGAGGGAGGCCAGGAAG-3' and 5'-TCCCTCAAGGAAGTTGTGGTAGGTCTCGATTTC-3'. The cDNA clones were sequenced in both directions to confirm that no additional non-synonymous mutations had been introduced during mutagenesis.

Cell culture, transfection and immunofluorescence staining

HaCaT cells were maintained in Dulbecco's modified Eagle's medium (Gibco, Carlsbad, CA, USA) supplemented with 10% fetal calf serum (FCS) at 37°C and 5% CO₂. HaCaT cells were grown on glass coverslips in a 6-well plate (Costar, Cambridge, MA, USA). Transient transfection was performed at 70–80% confluency using Lipofectamine™ 3000 (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocols. At 72 h post-transfection, cells were fixed in 4% paraformaldehyde and permeabilized in 0.2% Triton X-100 for 30 min. Transfected cells were processed to recognize the tubulin network by staining with mouse monoclonal anti- α -tubulin antibody T6199 at a dilution of 1:200 (Sigma, St Louis, MO, USA). A Cy3-conjugated sheep anti-mouse IgG C2181 (Sigma, St Louis, MO, USA) was used as the secondary antibody at a dilution of 1:100. Nuclei were stained with DAPI. Observations were performed with a Carl Zeiss LSM 800 confocal laser scanning microscope.

Image processing and statistical analysis

Cells containing particles were visualized using an Olympus IX71 microscope (Olympus, Tokyo, Japan), and images were captured using an imaging software (Olympus cellSens Standard ver. 1.2.1). To quantify and compare particle sizes in wild-type and mutant KRT9 groups, we assumed that fluorescence area is directly proportional to the particle size. Fluorescence areas for 3287 particles (60 cells) in mutant group

and 2147 particles (50 cells) in wild-type group were measured using ImageJ programme (https://imagej.net/Particle_Analysis). Statistical analyses were performed using the PASW Statistics 18 (SPSS Inc., Chicago, IL, USA), and comparison of particle sizes in these two groups was performed using the independent-samples *t*-test to calculate the *P*-value. Significance level was set at *P* < 0.05.

Results

Clinical data

The proband (III:2) was a 43 year-old man having diffuse hyperkeratosis with well-demarcated erythematous margins on his palms and soles since infancy (Fig. 1B). Skin scaling was also observed on his soles. All other family members affected with EPPK had clinical appearances similar to the proband but to varying degrees. No evidence of knuckle pads, digital mutilation, camptodactyly, hyperhidrosis, or other symptoms were observed in the pedigree. The clinical characteristics of EPPK-affected family members are summarized in Table 1.

Mutation analysis

By exome sequencing of proband gDNA, a total of 37.85 million reads were generated, with 37.80 million reads (99.88%) mapped to the human reference genome, and 3,605.20 Mb of effective sequences mapped to the target sequence region. The average sequencing depth on the target region was 71.55 [28]. Aligned bases covered 99.7% (50.24 million bases) of the target region, 97.0% of which was covered at 10× or greater. In total, 96,123 SNPs and 9,659 indels were detected. The previously-described prioritization scheme was used to identify the probable variant in the patient [28, 34]. It was eliminated that commonly-known variants documented in public databases, consisting of the 1000 Genomes Project with a frequency > 0.5%, the dbSNP142 (minor allele frequency, MAF > 1%), and the NHLBI ESP6500, and synonymous variants. PolyPhen-2 and SIFT were used to predict the possible effects of non-synonymous variants on protein function. No known disease-causing variants in palmoplantar keratoderma-associated genes were found, except for a missense variant c.1369C>T (p.Leu457Phe) in the *KRT9* gene was discovered for EPPK.

Sanger sequencing validated the heterozygous c.1369C>T variant in exon 6 of the *KRT9* gene identified in the proband. This variant was also identified in other affected family members (II:1, II:5, and III:3), but was absent in the unaffected family members (I:2, III:1, III:4, III:5, III:6, IV:1, and IV:2) and the 200 normal controls (Fig.

Fig. 1. Genetic analysis of the EPPK family. (A) Pedigree analysis of the EPPK family. Squares and circles indicate male and female family members, respectively. Individuals with symptoms are shown with black squares or circles. Arrow indicates the proband (III:2). Deceased family members are indicated with "/". N, normal; M, the *KRT9* c.1369C>T transition mutation. (B) Clinical presentation of the proband. The diffuse yellowish hyperkeratosis on the surface of palms and soles was sharply bordered with erythematous margins. (C) Heterozygous c.1369C>T (p.Leu457Phe) mutation in the *KRT9* gene. (D) Unaffected family member (III:1) of the family.

1C,D). Based on multiple sequence alignment of the KRT9 protein sequences, the leucine at position 457 (p.Leu457) was shown to be highly conserved among the ten vertebrates (Fig. 2). SIFT returned a score of 0.00, implying that the transition was predicted to be damaging. PolyPhen-2 produced a score of 0.999 based on the HumVar database (sensitivity, 0.09; specificity, 0.99). MutationAssesor produced a functional impact score of 4.105, predicting that the variant would be functional (high). MutationTaster predicted that the transition was disease-causing, with a high probability of 0.908.

Immunofluorescence analysis of transfected HaCaT cells

At 72 hours after transfection, the cytoplasmic particle formation was present in about 10% of the Leu457Phe-transfected HaCaT cells, while approximately 3% of the cells transfected with wild-type KRT9 showed this phenotype (Fig. 3). The particles in mutant group were larger than that in wild-type group (P -value < 0.05). However, tubulin indicated by red signal was not apparently different in these two groups.

Table 1. Clinical characteristics of four family members affected with EPPK. EPPK, epidermolytic palmoplantar keratoderma

	II:1	II:5	III:2	III:3
Age (years)	66	57	44	43
Age of onset	Infancy	Infancy	Infancy	Infancy
Gender	Female	Male	Male	Female
Diffuse hyperkeratosis confined to palms or soles, with well-demarcated erythematous margins	Palms and soles	Palms and soles	Palms and soles	Soles and slight hyperkeratosis on palms
Knuckle pads on the finger joints	No	No	No	No
Painful fissuring	Yes	No	No	No
Scaling	Yes	Yes	Yes	No
Digital mutilation	No	No	No	No
Campodactyly	No	No	No	No
Hyperhidrosis	No	No	No	No
Increased sensitivity of palms and soles to mechanical insult	Yes	No	No	No
Decreased sensitivity to heat	Yes	No	No	No
Other affected areas (wrists and nails)	No	No	No	No
Associated diseases (tumour and heart disease)	No	No	No	No

Fig. 2. Conservation analysis of the KRT9 p.Leu457 amino acid residue.

Fig. 3. Immunofluorescence assay of transfected HaCaT cells. Particles in Leu457Phe-transfected cells (top row) were distributed in the cell periphery, with a fiber structure partially remained in the central cytoplasm. Expression of wild-type KRT9 proteins in wt-transfected cells showed discrete particles distributed in the entire cytoplasm (second row) or a fiber structure (third row). The tubulin was not apparently different in these two cell groups. Note: KRT9, tubulin and nucleus were indicated by green, red and blue signals, respectively. wt, wild-type.

Fig. 4. Schematic representation of the reported *KRT9* gene mutations in epidermolytic palmoplantar keratoderma. The *KRT9* (NM_000226.3, NP_000217.2) protein structure is a central α -helix rod domain flanked by nonhelical head and tail domains at both ends. The rod domain is composed of four coils (1A, 1B, 2A, and 2B) isolated by nonhelical linkers (L1, L12, and L2). Most of the *KRT9* gene mutations are located in the coils 1A and 2B.

Discussion

EPPK is the most common hereditary keratoderma [4]. It is characterized by uniform epidermal thickening, with well-demarcated erythematous margins, confined to palms and soles [8, 15, 35]. Mutations in the *KRT9* gene and less frequently in the keratin 1 gene (*KRT1*, OMIM 139350) were reportedly responsible for EPPK [8, 36, 37]. Presently, 82.1% (23/28) of the *KRT9* gene mutations are missense mutations occurred in helix boundary motifs (shown in Fig. 4, according to the HIFD and the HGMD).

The *KRT9* gene belongs to the type I keratin gene cluster, includes eight exons, and encodes 623 amino acids (NCBI GenBank database). Keratins are part of the intermediate filament superfamily, constituting the major portion of structural proteins in epithelial cells [38]. They not only provide support to resist traumatic damage to the cell [5], but also are involved in cell migration, differentiation, and proliferation [39]. The common keratin structure is a central, primarily α -helix rod domain flanked by nonhelical head and tail domains at both ends [40]. The rod domain is composed of four coils (1A, 1B, 2A, and 2B) isolated by nonhelical linkers (L1, L12, and L2) (Fig. 4) [41]. Type I and type II keratin proteins form heterodimers, via a coiled-coil interaction of two α -helices. The interaction is promoted by heptad repeats located in the central rod region [2]. Two short highly-conserved boundary regions, the helix initiation motif (HIM) and the helix termination motif (HTM), in which most causative mutations occur, are located at the beginning and the end of the rod domain [40-42]. EPPK is generally believed to be caused by the dominant negative effect of the *KRT9* mutants located in the HIM and HTM regions [41-43].

This study identified a novel p.Leu457Phe variant of the *KRT9* gene in a Han Chinese family with EPPK. The variant co-segregated with the EPPK phenotype in this family, and was absent from public databases and the 200 normal controls, implying that this variant may be disease-causing. Though both leucine and phenylalanine are hydrophobic amino acids, p.Leu457 is phylogenetically conserved among various vertebrates (Fig. 2). Bioinformatics tools predicted that the variant was "disease-causing". The final 10-11 residues of the 2B rod domain of one keratin molecule overlap with the first 10-11 residues of the 1A rod domain of its neighbor [20]. This is considered important to keratin intermediate filament assembly through end-to-end interactions [36]. The p.Leu457 residue of the *KRT9* gene is the second of the last ten amino acids in the 2B rod segment [44]. A mutation of it may produce dominant negative effects by interrupting the formation of keratin intermediate filaments [41]. An adjacent mutation (p.Leu458Phe, previously mislabeled "p.Leu457Phe") in the same segment was reported to be responsible for EPPK in a Japanese kindred [45], confirming the importance of the region (HIFD). Data recorded in the HIFD and the HGMD showed that 92.0% (80/87) of the *KRT9* gene mutations identified in independent EPPK families were located in exon 1 ranging from p.Met157 to p.Gln172, and 6.9% (6/87) in exon 6. This study tends to show that the 2B rod domain of the *KRT9* protein is a conserved

domain, and that exon 6 of the *KRT9* gene is a second potential target for EPPK therapies except for exon 1 [5].

Keratin filament sources consist of newly produced keratin particles and soluble subunits [46]. Keratin filament networks are highly motile and dynamic [46, 47], following the keratin cycling model with keratin filament assembled primarily in the periphery, centripetal keratin flux, and disassembly in the cell center [46, 48]. In our study, at 72 hours after transfection of HaCaT cells, 10% of the transfected cells in the mutant group and 3% of that in the wild-type group both showed perturbed endogenous keratin filament networks. Instead of forming a fiber structure, particle formation was observed in the cytoplasm. Particularly, the particles were more pronounced in the periphery of some cells, while the central part of the keratin filament network was partially remained, similar to previous reports [47, 49]. The newly formed mutant and wild-type *KRT9* proteins may result in abnormal aggregation by integrating into preexisting keratin network in the periphery or the entire cytoplasm [46, 47, 49]. Although the cytoplasmic particle formation was also observed in the wild-type group, the affected cells were about one-third of that in the mutant group, and the particles were smaller than that in the mutant group (P -value < 0.05). Therefore, the keratin network was probably more severely impaired in the mutant group by previously claimed dominant negative effects [37, 43]. The amount of over-expressed wild-type *KRT9* proteins may exceed the cell abilities to eliminate surplus material, leading to a significant shift in the dynamic equilibrium of keratin cycling model [46, 47], which is consistent with microinjected type I keratin and transient transfection of wild-type *KRT1* or *KRT5* plasmids showing discrete particles and filament aggregation [37, 47]. However, in our two transfected cell groups, the overall pattern of tubulin showed no apparent perturbations, similar to previous studies [50, 51].

Other *in vitro* and *in vivo* studies showed that the formation of intermediate filament network was perturbed by transfecting cells and injecting into mouse skin with the *KRT9* p.Arg163Gln mutant [38, 43]. *Krt9*^{-/-} mice developed hyperpigmented calluses confined to their footpads, whereas *Krt9*^{+/-} mice had normal palmoplantar epidermis, suggesting that the *KRT9* gene is critical for terminal differentiation and structural integrity of palmoplantar epidermis [52]. *Krt9*^{+/-mut} and *Krt9*^{mut/mut} knockin mice both developed EPPK-like phenotypes by inducing an imbalance in a subset of palmoplantar keratins, demonstrating a dominant negative effect of the *KRT9* mutations [53].

Among the seven *KRT9* gene mutations identified in the 2B rod domain (including our mutation) [9, 10, 42, 45, 54, 55], five of them are closely distributed, ranging from p.Tyr454 to p.Leu458 [9, 42, 45, 54] (Fig. 4). All the patients suffered from diffuse palmar and plantar hyperkeratosis [9, 10, 42, 45, 54, 55]. Camptodactyly [9], hyperhidrosis [9, 55], and knuckle pads [9, 10, 55], were observed in one, two, and three families, respectively. Similar to our pedigree, patients with the same *KRT9* mutation showed variability of disease severity in their pedigrees [9, 55]. This suggests that the clinical appearance may be influenced not only by the *KRT9* mutations, but other genetic, epigenetic and environmental factors [36, 56].

Prenatal genetic diagnosis would be able to take advantage of identifying the *KRT9* gene mutation in this family, and help minimize the EPPK risk [57, 58]. The exclusive expression of the *KRT9* gene, and the dominant-negative interference in EPPK may favor gene-targeted therapies delivered *in situ* as pachyonychia congenita [59-61]. The feasibility of this potentially personalized medicine has been shown in mouse models [53, 59], however, any potential delivery system awaits further investigation [60].

Conclusion

In summary, a novel *KRT9* c.1369C>T (p.Leu457Phe) transition mutation was identified in a Han Chinese family containing EPPK sufferers. Further *in vitro* studies showed that intracellular stress caused by expression of the mutated *KRT9* gene may function as an important factor in determining the course and outcomes of EPPK in this family. This study

may promote a better understanding of the EPPK's molecular basis and contribute to prenatal diagnosis and potential gene-targeted therapies for EPPK.

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Disclosure Statement

The authors declare no conflict of interest.

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