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Identification of a Novel *KRT9* Frameshift Mutation in a Chinese Pedigree with Epidermolytic Palmoplantar Keratoderma

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ABSTRACT Epidermolytic palmoplantar keratoderma (EPPK) is a genodermatosis with autosomal dominant inheritance model. It results from variants of keratin 9 (*KRT9*) or *KRT1* gene. In this study causative gene mapping in a Chinese EPPK family was performed with Two-point linkage analysis and haplotyping. Positive linkage results were obtained on 17q (Z_{max}=2.06, θ_{max}=0.0) at D17S799, which indicated *KRT9* to be the most responsible gene for the family. Subsequently, direct sequencing identified a novel frameshift mutation caused by a 5bp deletion (ΔGGAGG) in *KRT9* in all affected individuals but neither in the unaffected subjects nor in the 50 healthy unrelated controls. The frameshift changed the encoding of the following nine amino acids and resulted in a readthrough translation in exon 7. The data revealed that the novel frameshift mutation in *KRT9* was responsible for the Chinese EPPK pedigree. The researchers' findings broaden the spectrum of KRT9 variants and provide further evidence for the highly genetic heterogeneity of EPPK.

INTRODUCTION

Palmoplantar Keratoderma (PPK) is a conventional genodermatosis disease characterized by hyperkeratosis of the scarfskin over palms and soles (Hennies et al. 1995). According to the clinical symbols and evaluations, PPK includes epidermolytic and nonepidermolytic subtypes, and is also classified into diffuse, focal, and punctate forms (Lucker et al. 1994; Risk et al.1 994). Epidermolytic palmoplantar keratoderma (EPPK, OMIM 144220) is a hyperkeratosis disorder with autosomal dominant inheritance in human, which has an incidence rate of almost 1.0-4.4 in 100, 000 without sex difference (Xiao et al. 2018). It usually begins at birth or develops gradually thickened waxy skin involved the whole surface of palms and soles, and manifests as clearly defined erythematous boundary. Knuckle pads and Blaschko lines are also seen in some or rare cases (Torchard et al. 1994; Terrinoni et al. 2000; Lu et al. 2003; Du et al. 2011). Electron microscopy demonstrates cytolysis of keratinocytes in spinous and granular layers and abnormal concentration of tonofilaments in the epibasal layers of the epidermis in EPPK. Diffuse PPK is restricted in the palm and toes, with no impairments on the lateral of volar skin, especial no follicular or oral impairments (Stevens et al.1996).

So far, the pathogenesis of PPK remains unclear, which involves keratinized envelope (loricrin), cohesion (desmoplakin), communication be-

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ween cells (connexins), transmembrane protein for signal transduction (cathepsin C), and structural proteins (keratins) (Li et al. 2014; Chen et al. 2018; Li et al. 2019). Keratins are the most crucial proteins in PPK. They not only preserve epithelial cells against mechanical stress, but also contribute to cell migration, proliferation, and differentiation (Magin et al. 2007). Causative mutations have been identified in the genes related to epidermolytic hyperkeratosis, namely *KRT1*, *KRT9*, and *KRT10* (Rothnagel et al.1992; Liang et al. 2014; Has and Technau-Hafsi 2016). However, *KRT1* and *KRT9* were considered the only two genes responsible for EPPK owing to its unique expression in the keratinocytes of the vola epidermis (Reis et al. 1992). *KRT9* (NM_000226.3) on chromosome 17q21 was first identified in a large EPPK family from Northwest Germany in 1994 (Reis et al.1994). Subsequently, more and more mutations of *KRT9* were found in multiple EPPK families in different populations. Meanwhile, various mutations in *KRT1* were observed in both epidermolytic and nonepidermolytic palmoplantar keratoderma (Lane and McLean 2004). Currently, 82.1 percent of EPPK mutations in the *KRT9* gene were missense mutations situated in helix boundary motifs (Xiao et al. 2018). In this study, the researchers identified a 5bp deletion (ΔGGAGG) of *KRT9* in a Chinese diffuse EPPK family. This novel mutation caused a frameshift mutation in exon 7 of *KRT9* and was supposed to have the structural and functional impacts in *KRT9* and induced hyperkeratosis of epidermis over palms and soles. The finding provides another evidence that EPPK may result from different variants in the *KRT9*, which further supports genetic heterogeneity in EPPK study.

Objective

EPPK is a common genetic skin disease and its inheritability and pathogenesis remain elusive. The aim of our study is to ascertain the inheritance and pathogenesis of a Chinese pedigree with EPPK by Two-point linkage analysis, haplotyping and direct sequencing.

METHODOLOGY

Clinical Material

A pedigree affected by epidermolytic palmoplantar keratoderma was recruited from Hunan province in China. Medical information of family was completely recorded. Clinical examinations and evaluation of all patients were performed by the experienced dermatologists in Ruijin Hospital of Shanghai Jiao Tong University. Thirteen blood samples (8 affected and 5 unaffected individuals) were extracted for further genetic analysis. Fifty unrelated subjects without diffuse palmoplantar keratoderma were also collected as normal controls for the study.

Ethics Statement

All participants in the study signed informed consent complying with the Helsinki Declaration. This study was confirmed by the ethics committee of Bio-X institutes, Shanghai Jiao Tong University in China. Blood sample collection procedures conformed to the ethical principle of human experimentation.

Genes Scan

Genomic DNA was separated from peripheral leukocytes of all participants applying the QIAamp Blood Mini DNA kit (Qiagen, Santa Clara, CA). Eleven microsatellite markers (D17S799, D17S921, D17S1857, D17S798, D17S1293, D17S1836, D17S800, D17S1868, D17S787, D17S949 and D17S785) involved the type I keratin gene cluster on 17q21 on the basis of the NCBI (http://www. ncbi.nlm.nih.gov) were singled out for gene scan. Genotyping was carried out by capillary electrophoresis of the touchdown PCR fragments.

Touchdown PCR program was performed through the Gene Amplification PCR System 9700 (PE Applied Biosystems, USA) as follows: initial denaturation of 96^0C for 3 min; 14 cycles at 94^0C for 35 s, 64^0C for 30 s (temperature reducing from 64^0C to 57^0C by 0.5^0C per cycle), extension at 72^0C for 30 s; followed by 30 cycles of 94^0C for 35 s, 57^0C for 30 s, 72^0C for 30 s, and final extension of 72^0C for 5 min.

Standard PCR reaction contained 0.1μM of each primer, 20ng template DNA, 300μM dNTP, 1μL of 10×PCR buffer and 7mM $MgCl_2$, 0.3U Hotstart Taq, add up to 10 μL volume. The size of PCR products were electrophoresed using a 96-capillary automated genetic analyser (MegaBACE 1000, Amersham, Freiburg, Germany) after denaturation at 95^0C for 1 min. Genotyping data analysis were done using Genetic Profiler version 1.5 software (Amersham).

Linkage Analysis and Haplotyping

First two-point linkage analysis was done utilizing the MLINK subprogram of the LINKAGE package (version 5.1) based on genotyping data. Recombination distances between the microsatellite marker loci and allele frequencies were on the basis of the UCSC Database (http://genome.ucsc.edu) and the Marshfield Database (http://www.marshfieldclinic.org). The Chinese EPPK pedigree was explored as an autosomal dominant inheritance with full penetrance and a causative gene allele frequency for 0.0001. Subsequently, haplotyping was constructed utilizing Cyrillic software (version 2.1) and confirmed by inspection. The standard for establishing linkage have been described previously (Lander and Kruglyak 1995).

Mutation Screening

KRT9, a candidate gene which has been reported to be responsible for EPPK was selected to sequence first. The primers for PCR amplification and PCR conditions were used as previously reported (Reis et al.1994). Sequencing reactions were carried out employing the BigDye Terminator Cycle Kit v3.1 (Applied Biosystems, Foster City, CA) by an ABI PRISMTM 3730 automated sequencer. Sequencing data analysis were done utilizing Sequence Scanner v1.0 software referring to the NCBI GeneBank (GeneID: 3857). Subsequently, cosegregation analysis was performed using available DNA samples from the Chinese family individuals. The single nucleotide polymorphism was confirmed in 50 unrelated controls.

RESULTS

Clinical Features

A three-generation Chinese epidermolytic palmoplantar keratoderma family consisted of fifteen members, including eight affected (I:2, II:1, II:4, II6, II:7, III:2, III:3, III:5) and seven normal individuals (I:1, II:2, II:3, II:5, II:8, III:1, III:4). The inheritance pattern of all affected members distributed in three successive generations in this family conformed to typical autosome dominance (Fig. 1). The proband (II:4) was a female over eighty years old having dispersive hyperkeratosis with

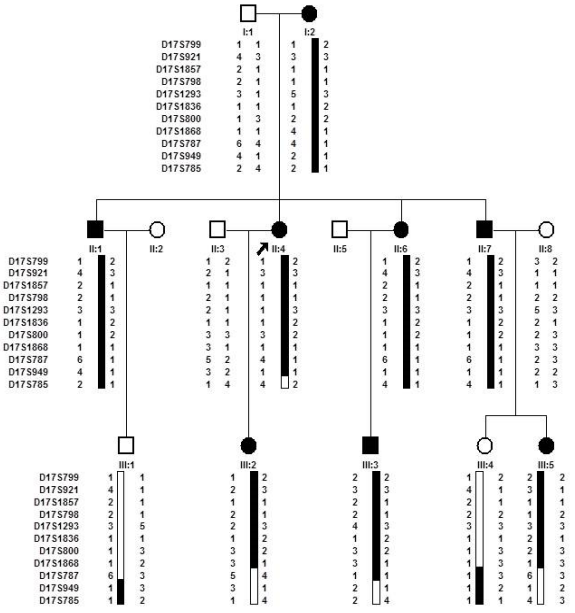

Fig. 1. Pedigree and haplotype diagram of the EPPK family. Black arrow indicates the proband (II:4) of the family, and the filled symbols represent affected members. Blackened filled bars show the chromosomal regions that are originated from the ancestral disease-associated haploty
Source: Authors

clearly demarcated borders on her palms and soles (Fig. 2). Her lesions appeared at 6 months of age, and then progressively worsened until 3 years of age. After 3 years old, the lesions stabilized. Scales was observed sometimes. There were painful fissures on her palms in winter. The nails were thickened with her ages. The knuckle pads were also observed on her finger joints at the age of five. Medical history showed that all affected individuals were suffering from similar clinical courses but to varying degrees in their lives. The general health examination showed that the oral mucosa tissue, teeth, hair, and else organs remained unchanged in all patients. No clinical symptom was found on the mucous membranes, extremities, and trunk .

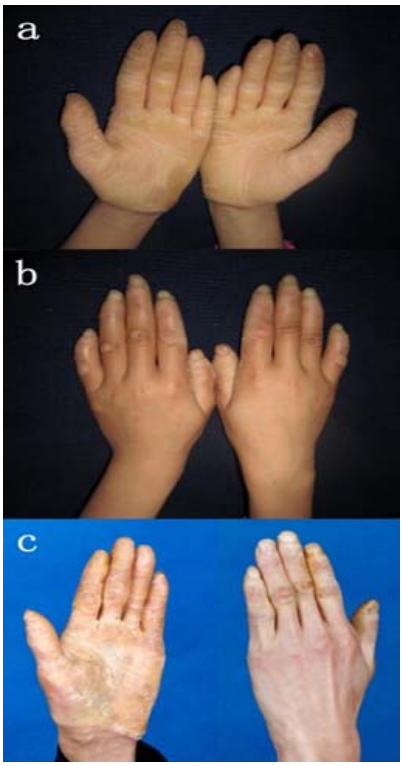

Fig. 2. Clinical photographs showing (a) palmar keratoderma, (b) knuckle pads, and (c) hyperkeratosis yellow thickening plaques on the palms
Source: Authors

Linkage and Haplotype Analysis

Positive findings were obtained on chromosome 17 in the filtrating of PPK associated loci, though the maximum LOD score (Z_{max}) only reached 2.06 at D17S799 (θ_{max}=0.00) (Table 1). This locus was located in the same site as type I keratin gene group. After a series of STR markers were added, six informative recombination events were seen in the family by Haplotyping. Among them, crossovers occurred in Ⅱ:2, Ⅲ:4, and Ⅲ:5 narrowed the proximal border between D17S799 and D17S787. According to the haplotying, the researchers finally defined the chromosome region resulting in the family EPPK to 17q12-21, where type Ⅰ keratin gene cluster located (Fig.1).

Mutation Analysis

A 5bp deletion (ΔGGAGG) in exon 7 was found by direct DNA sequencing result of the exons, UTRs, and exon/intron boundaries of *KRT9*. The deletion produced a frameshift mutation and

Table 1: Two point LOD scores for palmoplantar keratoderma on chromosome 17p

		LOD Score at θ =						
Marke	*CM*	*0.0*	*0.1*	*0.2*	*0.3*	*0.4*	*Zmax*	*θ max*
D17S799	31.96	2.06	1.70	1.30	0.86	0.40	2.06	0.0
D17S921	36.14	1.16	0.98	0.78	0.56	0.30	1.16	0.0
D17S1857	43.01	0.58	0.49	0.39	0.28	0.15	0.58	0.0
D17S798	53.41	0.58	0.49	0.39	0.28	0.15	0.58	0.0
D17S1293	56.48	1.20	0.97	0.72	0.44	0.17	1.20	0.0
D17S1836	60.40	1.31	1.00	0.67	0.35	0.1	1.31	0.0
D17S800	62.01	1.16	0.98	0.78	0.56	0.30	1.16	0.0
D17S1868	64.16	0.90	0.72	0.51	0.30	0.09	0.90	0.0
D17S787	74.99	-7.05	-0.75	-0.23	-0.06	-0.04	-0.04	0.4
D17S949	93.27	-0.12	0.16	0.17	0.10	0.01	0.17	0.2
D17S785	103.53	-3.51	-2.64	-1.58	-0.87	-0.38	-0.38	0.4

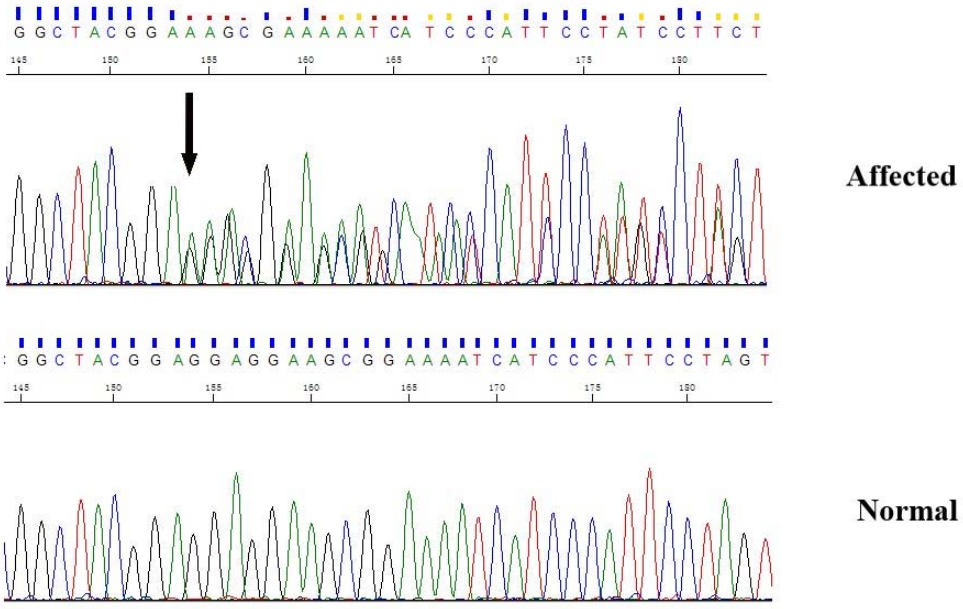

Fig. 3. Sanger sequencing chromatograms. The heterozygous 5bp deletion mutation in KRT9 gene was found in the patient, not in the control. The arrow indicated the mutated site
Source: Authors

changed the encoding sequence including the following nine condons and the stop codon of exon 7. Thus the original nine amino acids Gly-Gly-Ser-Gly-Lys-Ser-Ser-His-Ser (GGSGKSSHS) changed to Lys-Arg-Lys-Ile-Ile-Pro-Phe-Leu-Val-Phe (KRKIIPFLVF), and a readthrough translation that skipped the stop codon of exon 7. This mutation resulted in a frameshift existed in all patients in this EPPK pedigree but neither in the unaffected individuals nor in the 50 unrelated controls, which indicated the 5bp deletion was co-segregated with this family EPPK and not a natural polymorphism (Fig. 3). Besides, the 5bp deletion was novel because it had not been reported previously nor was it described in Exome Variant Server, HGMD, or dbSNP .

DISCUSSION

As an important member of various families of intermediate filaments (IF) proteins, Keratins represent the typical function in epithelial cells and skin tissue (Moll et al. 2008; Knöbel et al. 2015). After the first research of keratin began in sheep in 1952 (Crick 1952), serials significant findings of Keratin were made in vitro, including human keratin family identifying, keratin protein disclosing, and keratin gene sequences revealing, etc. (Rogers et al. 2006; Balmer et al. 2017). The important function of keratins is to protect the mechanical integrity and stability for the epithelial cells and tissues, for example, stability between epithelial cells, attachment of the basement membrane, and compartment of reticular tissue of a specific epithelium (Moll et al. 2008). Some keratins are also critical to intracellular signaling pathways regulation, cell proliferation, differentiation, migration and transformation in different types of epithelia (Magin et al. 2007; Karantza 2011; Fujiwara et al. 2020). Moreover, the characteristic expression of some keratins (K5, K7, K19, and K20 etc.) can even be the important sign in immunohistochemical diagnosis and clinical classification in tumor (Dmello et al. 2019).

Total 54 functional genes were identified in human keratin family, which encode proteins with molecular weight ranges from ~44 to ~66 kDa. In order to classify them clearly, a more reasonable and systematic nomenclature was improved and

adopted in 2006 based on the previous catalog in 1982 (Moll et al. 1982; Schweizer et al. 2006). Keratin family comprises type I/II keratins categories. Twenty-eight type I keratins genes encode 11 hair keratins and 17 epithelial keratins including K9 to K10, K12 to K28, and K31 to K40. Other 26 type II keratin genes encode 6 hair keratins and 20 epithelial keratins including K1–K8 and K71–K86. Keratin family shares common tripartite domain structure of α-helical central rod domain with 310 amino acids of highly conserved region. The central rod domain is flanked by the non-helical N-terminal head and the C-terminal tail domains. It is the essential structure unit of the polymeric filament, composed of two conserved sequence motifs: α-helical segments (coils 1A, 1B, 2A, and 2B) and linkers (L1, L2, and L12). The head domain is composed of subdomains V1 and H1, while the variable length tail domain comprises subdomains V2 and H2. Conserved structures including repeating heptad sequence motif are critical to biological function maintaining of keratins (Wu et al. 2000; Lane and McLean; Norlén et al. 2007; Parry et al. 2007; Kirmse et al. 2010; Chernyatina et al. 2012). Single keratin deviating from equimolar amounts of type I/II is fastly decomposed (Lu and Lane 1990; Rogel et al. 2010). Three levels of assembly between the basic units of the keratin filaments are necessary in obligate formation of heterodimers: the α- helix domains is essential for protein accumulation ; N- and C-terminal domains of the interaction keratins is indispensable for stable filament formation , and type I-type II pairing plays a crucial role in construction of the cytoplasmic network .

The human keratin gene clusters are located on different chromosomal sites respectively: I keratins genes are on 17q21.2, while II keratin genes are on 12q13.13 (Lessin et al. 1988; Reis et al. 1992). More and more reorganizations of structural functions of Keratins have been supported by studies of human keratin disorders and transgene mouse models. *KRT9* (OMIM 607606) is known as an important type I keratin expression pairing with K1 to play a significant role in keratinocyte differentiation related to special mechanical enhancement (Magin et al. 2007). High expression of K9 in the epidermis suprabasal cells of palm and sole is considered essential to maintaining mechanical integrity of palmoplantar epidermis, which is reasonable to explain why variants of *KRT9* gene are associated with EPPK. *KRT9* consists of 8 exons and encodes K9 with 623 amino acids to construct typical keratin domains: an α helical central rod domain with non helical tail and head domains at both ends. Nearly 30 kinds of *KRT9* mutations were reported deeply involved in EPPK in different population, including more than 20 kinds in highly conserved central á helical 1A domain and no more than 10 kinds in 2B regions. Most of these mutations were reported to occur within two short highly conservative border sites between the helix termination motif (HTM) and the helix initiation motif (HIM), the structurally and functionally significant regions for keratin heterodimerization (*www.interfil.org*) (Liu et al. 2019). Variants within 1A domain are related to EPPK in numerous pedigree studies, including M157V, M157T, M157R, M157K, L160V, L160F, N161Y, N161H, N161S, N161I, N161K, P162S, R163W, R163Q, R163P, R163L, L164P, Y167insW, L168S, K170X, V171M, and Q172P, exactly in amino acid 154-188 of 1A domain (Chen et al. 2017; Fukunaga et al. 2018; Liu et al. 2019). Codons 163, 161, 160 and 157 of KRT9 within 1A domain are regarded as mutation hotspots (Liu et al. 2012). Among these mutations, R163W and R163Q in HIM are the mostly reported in various studies. A CpG methylation site in amino acid 163 may play a significant role in structural function of 1A domain for heterodimerization. Meanwhile, the neighboring codon 164 located in the HIM, was also reported significant for keratin filament assembly (Liu et al. 2019). It was supposed that mutations in position 163 and 164 may decrease the hydrophobic effect and result in the delay initiation of coiled coil conformation (Liu et al. 2019). In addition, an unusual mutation L11V in head of 1A was showed to be causative by activation of a hidden donor splice site, leading to 162 amino acids deletion in an Ashkenazi Jewish patient (Fuchs-Telem et al. 2013). The rod domain 2B located in amino acid 345-465 of KRT9 comprises 121 amino acids, much more than amino acids in 1A domain. However, mutations associated to EPPK in 2B domain are less frequently than those in 1A. All known mutations, including C406R, Q428X, Y454H, 454_455insH, L457F and L458P, are located in the half part of 2B domain close to terminal sites of the rod domain (Coleman et al. 1999; Shimomura et al. 2010; Wang et al.

2010; Du et al. 2011; Umegaki et al. 2011; Xiao et al. 2018). An early step of IF assembly of keratin is considered through end-to-end interactions between the last 10–11 residues in the 2B rod domain of a keratin overlapping with the inital 10–11 residues in the 1A rod domain of its paring keratin (Steinert 1993; Terron-Kwiatkowski et al. 2004). However, all known mutations in 2B domain show at least 60 residues at the end of the 2B rod domain segment is indispensable for IF assembly and molecular stability.

In this study, the researchers report a novel 5bp deletion (ΔGGAGG) of *KRT9* in a Chinese PPK pedigree. The researchers further define the disorder as EPPK via the identification of *KRT9* mutation in all affected family members. The mutant site within exon 7 in this family is at amino acid 615, the end of open reading frame of *KRT9*. A 5bp deletion (ΔGGAGG) produces a frameshift mutation and changes the encoding sequence including the following nine codons and the stop codon of exon 7. The original nine amino acids between the mutant site and the stop condon are Gly-Gly-Ser-Gly-Lys-Ser-Ser-His-Ser (GGSGKSSHS), exactly in HTM of 2B domain, the essential region to form keratin heterodimerization. In mutants, a 5bp deletion results in a substitution of Lys-Arg-Lys-Ile-Ile-Pro-Phe-Leu-Val-Phe (KRKIIPFLVF) and a readthrough translation which skips the stop codon. It should be verified whether the frameshift mutation results in 53 amino acids in addition to the mutant Polypeptide chain until it is stopped by the next stop codon. As a exclusive character of keratins, the pairing of heterodimers via rod domains aligned in parallel between type I and type II keratin monomers is very important to Keratins biological function (Herrmann et al. 2002; Haines and Birgitte 2012). Both ends of the rod domain, HIM at N-terminal and HTM at C-terminal, are the highly conservative helix motifs respectively in the interacting keratins. The specific interactions between 2B domains play an important role during 10nm IF assembly (Lee et al. 2020). The mutant proteins are assumed to impair the motile and dynamic keratin network by abnormal aggregation in the periphery or the entire cytoplasm (Xiao et al. 2018). Therefore, the mutations in 2B domain may severely impact the structure and function of Keratin. Though the mutation found in this study is nearly 150-200 amino acids away from all known mutations in 2B domain, they may share the similar or same structural and functional change of K9. The researchers suggest the mutant protein may alter the highly conserved HTM of 2B domain severely and has a significant effect on keratin heterodimerization, consequently it causes EPPK in this family.

EPPK is known as the discord of significant phenotypic and genetic variability. Besides common clinical presentation of waxy hyperkeratosis confined to the vola encircled by an erythematous boundary, knuckle pads and camptodactyly are also observed in some cases. Phenotypic variation is usually thought to be associated with gene interaction, epigenetic or environmental factors. On the other hand, genetic heterogeneity has been commonly observed in EPPK studies (Liu et al. 2019). Clinical feather of EPPK are usually various with additional symptoms in cases carrying the same mutation in different pedigrees or even in cases within the same pedigree (Liu et al. 2019). For instance, the most common mutation R163W is not only present EPPK, in some cases also along with knuckle pad, friction related impairments and palmar constriction (Xu et al. 2009). A new 5bp deletion variant in 2B domain of *KRT9* responsible for EPPK in this study provides a further evidence for the highly genetic heterogeneity of EPPK. In order to address the relation of disease-causing mutations and clinical appearance, genetic pathogenesis investigations might be an important insight into EPPK. Molecular studies not only provide an approach in clinical diagnosis, classification, and genetic counseling, but also may afford a hopeful and powerful tool in potential gene- or mutation-specific therapy in the disorders like EPPK in future. Moreover, the researchers require further investigation of the potential effect of the frameshift mutation caused by a 5-bp deletion (ΔGGAGG) in exon 7 of KRT9 on keratin IF assembly.

CONCLUSION

In short, through a combination of linkage analysis and direct sequencing, the researchers identified a new frameshift mutation induced by a 5-bp deletion (ΔGGAGG) in exon 7 of *KRT9* in a Chinese EPPK pedigree, which was predicted to seriously interfere the assembly of the keratin IF. The current study broadens the mutant spectrum

of *KRT9* gene, provides a significant evidence for the highly genetic heterogeneity of EPPK, and should be helpful in genetic counseling and prenatal diagnosis for the Chinese family.

RECOMMENDATIONS

In order to explore the relation of *KRT9* mutation and clinical appearance, discovery of more cases carrying novel mutations within *KRT9* gene is required to completely describe the entire phenotypic spectrum related to EPPK. The potential effect of the frameshift mutation caused by a 5-bp deletion (ΔGGAGG) in exon 7 of *KRT9* on keratin IF assembly warrants further study.

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AUTHORS' CONTRIBUTIONS

Haiou Jiang and Xiaoqing Zhao contributed equally to this work.(1) Study design and administrative support: Anli Shu and Hai Luo; (3) Experiments design and data analysis and interpretation: Haiou Jiang, Xiaoqing Zhao and Linghan Gao; (2) Data collection: Xiaoqing Zhao, Dan Yin, Juan He, Jie Li and Song Wang; (4) Manuscript writing (drafting and revision): Linghan Gao and Haiou Jiang.

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