Mutations in the 1A Domain of Keratin 9 in Patients with Epidermolytic Palmoplantar Keratoderma

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Epidermolytic palmoplantar keratoderma is an autosomal dominant skin disorder characterized by hyperkeratosis of the palms and soles. Ultrastructurally the disease exhibits abnormal keratin filament networks and tonofilament clumping like that found in the keratin disorders of epidermolysis bullosa simplex and epidermolytic hyperkeratosis. The disease has been mapped to chromosome 17q11-q23 in the region of the type 1 keratin gene locus and more recently mutations have been found in the palmoplantar specific keratin, keratin 9. We have analyzed six unrelated incidences of epidermolytic palmoplantar keratoderma for mutations in their keratin 9 genes. In two of these, we have identified mutations that alter critical residues within the highly conserved helix initiation motif at the beginning of the rod domain of keratin 9. In a three-generation Middle Eastern kindred we found a C to T transition at codon 162 that results in an arginine to tryptophan substitution at position 10 of the 1A alpha-helical domain, thus confirming this codon as a hot spot for mutation in keratin 9. The other mutation found involves a T to C transition at codon 167 that results in the expression of a serine residue in place of the normal leucine at position 15 of the 1A segment and is the first documentation of this mutation in this gene. The identification of these substitutions extends the current catalog of disease causing mutations in keratin 9. *Key words: intermediate filaments/disease/genetics. J Invest Dermatol 104:430–433, 1995*

Epidermolytic palmoplantar keratoderma (EPPK) is an autosomal dominant genodermatosis (MIM number 144200 [1]) first described by Vörner in 1901 [2]. It is characterized by a diffuse thickening of palmar and plantar epidermis that first becomes apparent shortly after birth (reviewed in [3]). Eventually these surfaces are covered by thick hyperkeratotic plates that are yellow-brown in color. The keratoderma is sharply defined with a distinctive erythematous border at the wrists and ankles [4]. In some patients, keratodermic islands cover the knuckles but the remaining aspects of the dorsal surfaces of the hands and feet are never involved. Patients complain of increased sensitivity of their palm and soles to mechanical insult resulting in fissuring, particularly at the joints. Scaling, blistering, and erosions are not common but may be present at the margins [5,6]. Histopathology is characterized by a marked expansion of the granular and stratum corneum layers [5–9]. Keratinocytes of the upper spinous and granular layers of affected areas show perinuclear vacuolization and cytolysis with abnormal keratohyalin granules. Ultrastructural analysis of these cells reveals the presence of abnormal keratin filaments and tonofilament clumping often with a perinuclear distribution [5–9].

The similarity of these ultrastructural features to those of the known keratin disorders of epidermolysis bullosa simplex (EBS) and epidermolytic hyperkeratosis (EHK) [10], together with its dominant mode of inheritance, suggested that EPPK is also caused by a keratin defect. Earlier linkage analyses mapped the candidate gene for EPPK to chromosome 17q11-q23 [11], in the region of the type 1 keratin gene cluster. Of the type 1 keratins, only keratin 9 (K9) has been shown to be expressed exclusively in the epidermis of palms and soles [12]. On this basis, K9 was proposed as the candidate gene for EPPK and recently mutations in the K9 gene have been identified in EPPK patients [13–16].§¶ All of these mutations have been found within the first alpha-helical segment of K9. Here we report two more incidences of EPPK bearing mutations within the helix initiation motif at the beginning of the rod domain of K9.

MATERIALS AND METHODS

PCR and DNA Sequencing Genomic DNA was extracted and purified from whole blood as described previously [17]. At the time that these studies were initiated, the K9 gene sequence was unavailable. Therefore genomic sequence analysis was performed using oligonucleotide primers designed from the cDNA sequence [18]. The primers used to amplify exon 1, which contains the 1A region, including the highly conserved RLASYL motif, were 5'-CTCAACCCGTGAGCTACCAGCTGTG-3' and 5'-GATAGCAGCAGGTCCCTTCTTGTCG-3' corresponding to bp 40–64 and to 630–654 of the published sequence [18]. The 5' oligonucleotide was biotinylated to facilitate the purification of single-stranded DNA for se-

§ Bowden PE, Watts CE, Marks R: Mutation of human keratin 9 (HK9) gene in epidermolytic tylosis (abstr). *J Invest Dermatol* 102:541, 1994.

¶ Shamsher M, Navsaria HA, Ratnavel R, McLean I, Lane EB, Griffiths WAD, Leigh IM: Mutation of keratin 9 gene in epidermolytic palmoplantar keratoderma (EPPK) (abstr). *J Invest Dermatol* 103:427, 1994.

quencing [19]. The sequence primer used was 5'-CTTGTCGTACCAATC-CTG-3' corresponding to bp 619–636 of the published sequence [18]. Polymerase chain reactions (PCR) were preheated at 95°C for 5 min prior to the addition of 1 U of Taq DNA polymerase (AmpliTaq, Perkin-Elmer). Amplification conditions consisted of 30 cycles of 1 min at 94°C, 2 min at 55°C, and 3 min at 72°C and a final elongation step of 15 min at 72°C. PCR products were first-column purified (Magic PCR Preps, Promega) and the biotinylated DNA strands were captured on streptavidin-coated magnetic beads (Dynal) and directly sequenced using Sequenase T7 DNA polymerase (US Biochemicals). The reaction products were separated on 6% acrylamide denaturing gels.

AS-PCR Analysis Allele-specific PCR was performed using a primer specific for the T to C mutation at bp 566. The primer used was 5'-TAGAGCCTGCACCTTATCCG-3', corresponding to bp 566–585 of the published cDNA sequence [18], and was paired with the 5' biotinylated primer used for generating the sequencing template. PCR conditions consisted of 95°C for 5 min followed by 35 cycles of 30 seconds at 94°C, 1 min at 64°C, and 3 min at 68°C, and a final elongation step of 15 min at 72°C. PCR products were visualized on a 1% agarose gel. A fragment of 545 bp was generated in the presence of the mutant nucleotide.

RESULTS

Clinical Description of the EPPK Patients The EPPK-I/D family (**Fig 1**) is of Middle Eastern heritage. All affected family members (I.1, II.2, II.4, II.5, and III.1) show a similar clinical picture with diffuse thickening of palmar and plantar surfaces characterized by a yellow-brown hyperkeratosis with fissuring occurring mainly at the joints. There is some involvement of the dorsal surfaces, predominantly over the joints of the fingers and to a lesser extent of the toes. No other skin alterations were found, and the other structures of the integument, such as nails, hair, and teeth, were all normal. There was no hyperhidrosis and no evidence of deafness or mental retardation. Onset of the disease in all patients was within the first weeks after birth. All patients report exacerbation of the disease with blister formation and subsequent maceration (infection) after mechanical trauma. Histopathology revealed hyperkeratosis with acanthosis and epidermolysis. The EPPK-S family (**Fig 1**) is African-American. The proband is a 4-year–old boy (II.1). The skin of his palms and soles appeared normal up to the age of approximately 3 months, when thickening and roughness of his palms and soles were first noticed. He now exhibits thick hyperkeratotic plaques on his palms and soles with some scaling.

EPPK - I/D

EPPK - S

Figure 1. Pedigrees of the EPPK families. *Solid symbols,* affected individuals; *open symbols,* unaffected; *squares,* male; *circles,* female.

There was no involvement of the nails. A biopsy from the palm showed histologic features of epidermolysis and hyperkeratosis. The mother stated that his lesions were very similar to ones present on the father's side of the family. The father was not available for clinical examination. Family history is remarkable according to the mother for an affected father, an affected paternal grandfather, a paternal great aunt, and possibly a distant cousin with similar physical findings.

Sequence Analysis of K9 We analyzed the K9 gene for mutations in six unrelated incidences of EPPK. Because all previously described keratin mutations have been found in the H1, 1A, L12, and 2B regions of these proteins (see [20,21] for a review), we focused our analysis on these regions of the K9 gene. Primer pairs were chosen to amplify the amino-terminal end of K9 including the 1A segment of the rod domain, the carboxyl-terminal end of the 2B segment and a fragment that included the L12 linker, the 2A segment, and the L2 linker. Sequence analysis of these PCR-generated fragments revealed a mutation in the 1A region of the K9 gene in two of the six EPPK families. Affected individuals of the EPPK-I/D family have a mutation of the highly conserved arginine residue at position 10 of the rod domain. The C to T transition at bp 550 in these patients results in an arginine (R) to tryptophan (W) substitution (**Fig 2a**). The affected individual of the EPPK-S family carries a T to C transition at bp 566, resulting in a leucine (L) to serine (S) substitution at residue 15 of the 1A segment (**Fig 2b**). In both families the mutations were only present in affected individuals. To insure that these mutations were not spurious alterations introduced by the polymerase chain reaction, each reaction was repeated and sequenced independently. Apart from these mutations no other substitutions were observed in the H1, 1A, L12, and 2B regions of K9 in the six patients analyzed. A sequence polymorphism (A–C) was observed in the V1 region at bp 492 in both affected and unaffected individuals; however, this base substitution affects the third position of a glycine codon and does not result in an amino acid change. This inconsequential polymorphism was also detected in the study by Reis *et al* [13] and found to occur frequently.

Mutation Analysis Further analysis of the T to C mutation at bp 566 was undertaken to exclude the possibility that this base change is present in the normal population as a silent polymorphism. Because this mutation did not create or destroy a restriction enzyme site, we used an oligonucleotide primer specific for this substitution to assess its presence in the DNA of 50 unrelated control individuals using allele-specific PCR (AS-PCR). The 545-bp fragment present in the affected individual of the EPPK-S family was not observed in these normal DNAs (**Fig 3** and data not shown).

DISCUSSION

In this study we have identified mutations of two critical residues within the helix initiation motif of K9 in EPPK patients, a highly conserved arginine residue at position 10 of the 1A segment and a leucine residue at position 15 at the end of the helix initiation motif. The helix initiation motif comprises the first 15 residues of the 1A segment and is highly conserved in all intermediate filament types [22]. A number of recent studies have shown that these residues are important for filament assembly and stability and are thought to be involved in lateral associations with residues of the TYRXLLEGE motif at the end of the 2B segment of neighboring coiled-coil molecules [23]. It is also apparent from both *in vitro* analyses [24,25] and patient studies [21] that mutations in these conserved sequences at the ends of the rod domain are more deleterious in terms of filament disruption and phenotypic severity than those occurring at internal residues. Significantly, most of the keratin mutations identified to date affect residues of the helix initiation motif with almost 50% involving the arginine residue at position 10 of the type I keratins [21].

The availability of the nucleotide sequence for K9 has enabled direct sequence analysis of the gene in EPPK patients. Previous to this study, seven different point mutations resulting in substitutions
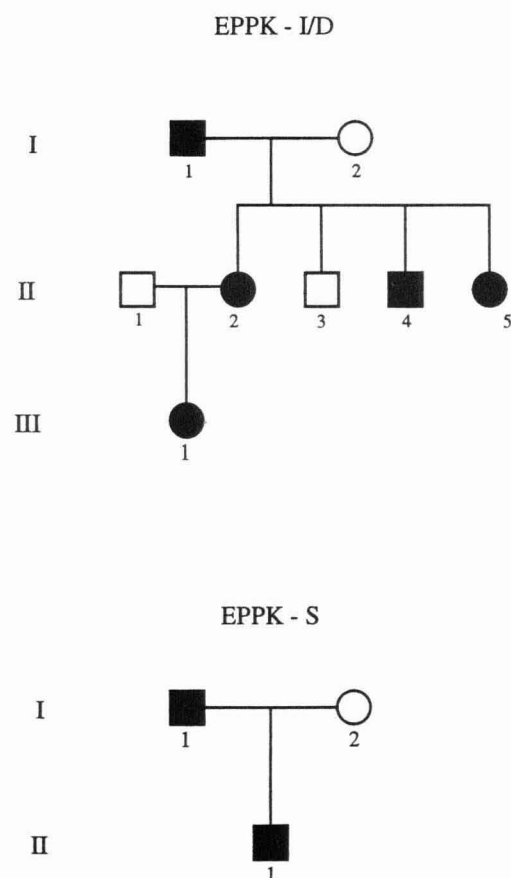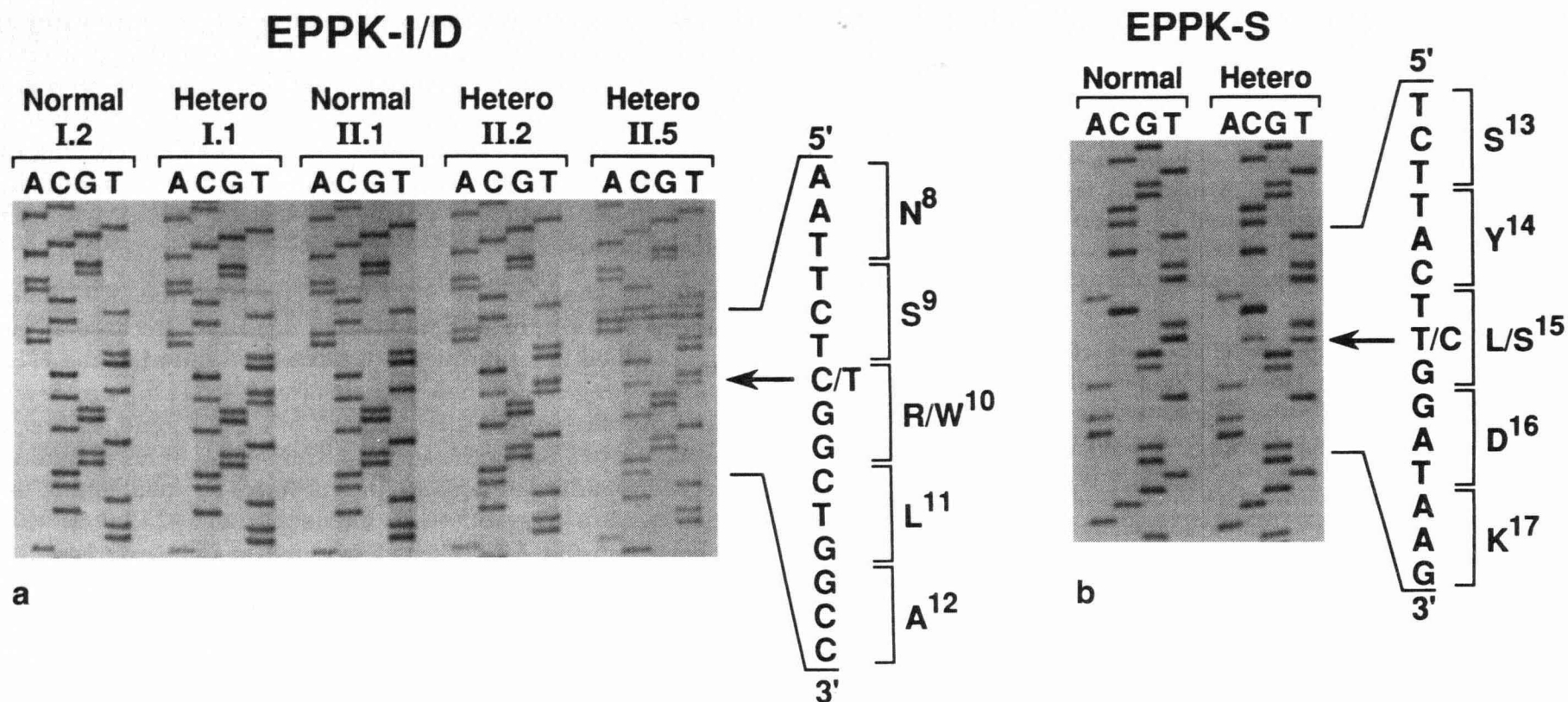
EPPK-I/D

a

EPPK-S

b

Figure 2. Genomic sequence analysis of the 1A region of K9 from individuals affected with EPPK. Normal, sequence obtained from an unaffected family member. Hetero, sequence from an affected individual who carries the mutation and is therefore heterozygous for the K9 allele. Numbering of the amino acids is with respect to the 1A segment of the rod domain. *a*) Sequence from members of the EPPK-I/D family. Affected individuals show a C to T transition, resulting in the expression of a tryptophan residue (W) at position 10. *b*) Sequence from the EPPK-S family. The affected individual shows a T to C transition that results in a leucine (L) to serine (S) substitution at residue 15 of the 1A segment. Residues 10 and 15 correspond to codons 162 and 167 respectively, of the published K9 sequence [17].

within the 1A segment of K9 had been identified [13–16].§¶ The R10W substitution identified in this study was detected in five unrelated German kindreds by Reis *et al* [13]. Haplotype analysis of these families could not rule out the possibility of a common origin, i.e., a founder effect, for some of these patients [13]. Therefore, our finding of the same mutation in a family with a different ethnic origin supports the thesis that this arginine codon is a hot spot for mutation in the K9 gene [13], just as it is for the K10 and K14 genes [26,27]. In fact during the course of these studies, a further three R10W substitutions in K9 from EPPK patients were reported¶ [16] making a total of ten incidences found to date involving mutation of this arginine residue. The L15S substitution found in the EPPK-S family is the first report of this mutation in K9, although the same substitution has been identified in K10 in an EHK family [17].

Figure 3. AS-PCR analysis of the T to C transition at bp 566. Genomic DNAs from 50 unrelated control individuals as well as the normal mother and affected son of the EPPK-S family were analyzed for the presence of a cytosine at bp 566. *Lanes*: M, ØX174/Hae III markers; 1, affected individual (II.1 of EPPK-S); 2, unaffected individual (I.II of EPPK-S); 3–7, representative sample of unrelated and unaffected individuals.

Functional data showing that the K9 mutations identified in this study are causal for the disease remain to be unequivocally established through transgenic studies. However, a number of *in vitro* studies, where mutant intermediate filament proteins have been introduced into cultured cells, have implicated the arginine residue at position 10 of the 1A segment as critical to filament integrity [28,29]. Furthermore, an *in vitro* filament disassembly assay determined that non-conservative substitutions, of the arginine at position 10 and the leucine at position 15 of the 1A region (including the serine substitution), were not tolerated in terms of their ability to interact with wild-type intermediate filaments [30]. The arginine residue at position 10 of the 1A segment is conserved in 47 of 51 published IF protein sequences [22,29], and the leucine at position 15 is completely conserved in all type I keratins and conservatively substituted (isoleucine) in type II keratins and other IF proteins [22]. The high degree of conservation of the R10 and the L15 residues suggest that few, if any, changes of these residues are tolerated and underscores the importance of their respective side-chains to filament biology. In addition, the lack of polymorphic variation in these sites in unaffected individuals strongly suggests that the two K9 mutations identified in this study do in fact cause EPPK.

The residues in the a and d positions of the 7 residue repeat making up the alpha-helical forming sequences of intermediate filament proteins are the most highly conserved, followed by the residues of the e and g positions [22]. Residues of the a, d, e, and g positions are sited on the internal face of the helix and are positioned to interact (via hydrophobic and ionic bridges) with residues on the neighboring helix of the two-chain coiled-coil complex. It is not surprising then that these positions should be the most sensitive to disease causing substitutions [21]. The R10W and L15S substitutions in the helix initiation motif of K9 occur at g and e positions, respectively. In case of the arginine to tryptophan substitution at position 10 both the charge and shape of the side chain are altered, whereas in the leucine to serine substitution the change remains neutral but both the hydrophobicity and size of the side chain at position 15 are altered. Since the residues at e and g positions are thought to contribute to higher-order interactions, both substitutions can be expected to perturb the function of the
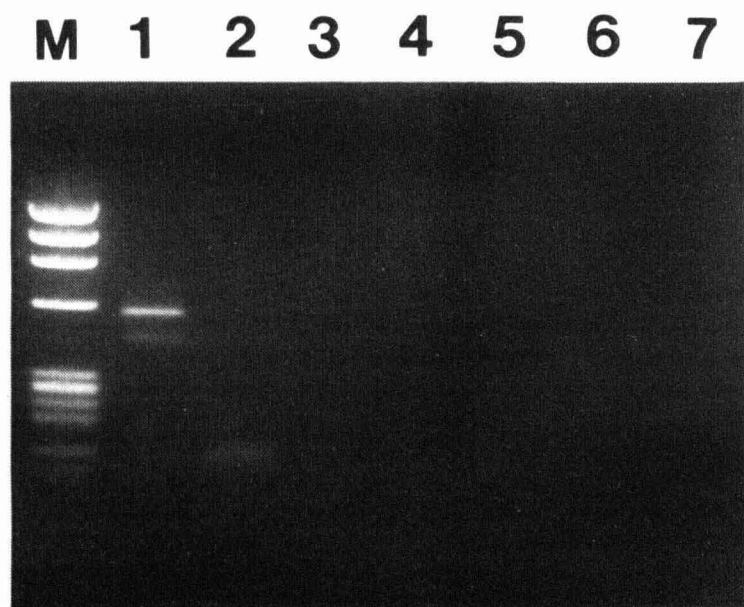
helix initiation motif and thereby disrupt the keratin-filament cytoskeleton.

It is noteworthy that we were unable to identify mutations within the highly conserved regions of the rod domain of K9 in four of the EPPK families studied. It is possible that affected individuals of these families bear mutations elsewhere in the rod domain of K9 at residues not previously associated with disease-forming substitutions. However, it should be noted that in the studies by Reis and colleagues [13,15] nearly half of the EPPK probands analyzed did not carry a mutation in their K9 genes. Similar observations have been reported for the other keratin disorders of EBS and EHK. About 25% of EHK patients do not harbor mutations in their K1 or K10 genes and we have shown recently that some of these individuals actually carry mutations within their K2e genes [31], suggesting the possibility of other keratin genes being involved in EPPK. Both EBS and EHK can be caused by mutations in either of two keratin chains making up the mature filament. The natural type II partner of K9 has yet to be identified but, because over half of EPPK families analyzed to date do not carry K9 mutations, it seems likely that they harbor a mutation in a type II keratin. To date a type II keratin with expression limited to palms and soles has yet to be identified. It is possible, however, that these EPPK patients bear mutations in the more ubiquitous type II keratins such as K1 and K2e, which only manifest themselves in disease at sites of constant and prolonged mechanical stress. Because the hands and feet are the surfaces most prone to these forces, expression of disease would appear limited to these regions. An analogous situation has already been shown with the K5 and K14 mutations found in EBS-Weber-Cockayne patients [32]. Other candidate keratin genes include K6 and K16 whose expression is normally limited to palmar and plantar epidermis and the outer root sheath of the hair follicle [33]. Finally, the proteins that associate or otherwise interact directly with the keratin filament cytoskeleton should not be overlooked as possible sites of disease causing mutations in these patients.

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