Mutations in the 1A Rod Domain Segment of the Keratin 9 Gene in Epidermolytic Palmoplantar Keratoderma

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Palmoplantar keratodermas (PPK) constitute a heterogeneous group of diseases marked by the thickening of palms and soles of affected individuals. They are divided into autosomal dominant and autosomal recessive groups by the mode of transmission. The autosomal dominantly transmitted group is further divided into epidermolytic (EPPK, Voerner) and non-epidermolytic (NEPPK, Unna-Thost) types according to the histopathologic findings. Recent development of molecular approaches has confirmed that EPPK and NEPPK are caused by the mutations in keratin 9 and 1 genes, respectively. We have studied three families of EPPK to find the mutation in the keratin 9 gene. DNA sequence analyses revealed single base changes in sequences encoding the highly conserved 1A rod domain segment of the keratin 9 gene in two of the three families. These mutations caused Arg (CGG) to Glu (CAG; R162Q) and Arg (CGG) to Try (TGG; R162W) substitutions. The same arginine position has been mutated in the keratin 10 gene in epidermolytic hyperkeratosis, the keratin 14 gene in epidermolysis bullosa simplex, and the keratin 9 gene in hereditary EPPK in Western patients. In this study we show that unrelated Korean patients have similar mutations. *Key words: keratin 9; mutation; epidermolytic palmoplantar keratoderma.*

Hereditary palmoplantar keratoderma (PPK) defines a group of heterogeneous disorders characterized by severe thickening of the palms and soles. PPK is divided into autosomal dominant and autosomal recessive groups by the mode of transmission. The autosomal dominant group is subdivided on the basis of histopathology into epidermolytic type (EPPK, Voerner type-McKusick #144200) and non-epidermolytic type (NEPPK, Unna-Thost type-McKusick #148400) (1) by the presence or absence of epidermolysis in the epidermis. The epidermolytic variety of PPK was first described by Voerner in 1901 (2). Since then, families with this disorder, as well as sporadic cases, have been reported in the literature (3–5). The histological presence of epidermolysis in the spinous and granular layers, and ultrastructurally abnormal keratin intermediate filament network and tonofilament clumping, are the characteristics in this disease.

Keratin intermediate filaments constitute the major differentiation product of epidermal cells. They are assembled from acidic (type I) and neutral/basic (type II) keratin chains. Specific type I/type II pairs are expressed which are characteristic of the type and stage of differentiation of the keratinocyte. Keratins 5/14 (K5/K14) are expressed in the basal layer, and K1/10 in the spinous layer (6, 7). Recent experiments have demonstrated that the mutations in the keratin chains expressed in the epidermis and appendages are etiologically responsible for several distinct types of autosomal dominant genetic skin diseases, including: epidermolysis bullosa simplex (K5/14) (8–10), epidermolytic hyperkeratosis (K1/10) (11–14), ichthyosis bullosa of Siemens (K2e) (15), pachyonychia congenita (K16, 17) (16–18), EPPK (K9) (19–24) and NEPPK (K1, K16) (25, 26). Keratin 9 is expressed only suprabasally, principally in the epidermis of the palms and soles. Using genetic linkage analyses, Reis et al. (27) showed that EPPK maps to a gene in the type I keratin cluster on chromosome 17q12-q21. Subsequent sequencing revealed point mutations in the K9 gene as the cause of the disease in families of northern European heritage.

We have investigated three pedigrees of EPPK in Korean patients and have identified mutations in two families. These mutations cause amino acid substitutions in the 1A domain segment of K9, and are highly consistent with other keratin 9 mutations reported so far.

MATERALS AND METHODS

Patients

Three families were approached as part of a continuing effort to identify the etiology of a variety of epidermal genetic skin diseases in Korea. Blood and skin samples were obtained with informed consent. Diagnosis was made by examination by a dermatologist, and skin biopsy samples were obtained from patients and examined with a light microscope.

Source of DNA

DNA was isolated as earlier described (28) from freshly drawn blood obtained from both affected and unaffected family members. Genomic DNA was also extracted from 50 unrelated individuals from the normal population for use as controls.

Direct PCR amplification of K9 gene and sequencing procedures

Oligonucleotide primers (19) and the conditions of PCR to amplify the K9 exons have been described previously (29). For every 100-μl reaction, 500 ng of genomic DNA was used with 1.5 mm $MgCl_2$, 200 mm dNTPs, 0.1 μM concentration of each of the primers, and 2.5 units of Taq polymerase (AmpliTaq, Perkin-Elmer Cetus). Amplifications were done on a DNA Thermal Cycler (Perkin-Elmer) for 35 cycles (1: denaturation for 60 s at 94°C; 2: a 90-s transition to 55°C; 3: annealing for 30 s at 55°C; 4: transition for 30 s to 72°C; and 5: extension for 90 s at 72°C). Amplifications were preceded by a hot start: 5–10 min incubation of the DNA and primer mix at 95–100°C, and equilibriation to 80–85°C, at which time the premixed dNTPs, PCR buffer, $MgCl_2$, and Taq polymerase were added and cycling started. To increase the

yield of PCR product for direct sequencing, reamplification was performed on a 1:100–1:1000 dilution of the first amplification reaction.

Direct sequencing of the PCR-amplified DNA was done in reactions using single-stranded binding protein (US Biochemicals) to prevent strand reassociations (30). The primers used for amplifying exon 1 were: primer 1a(+), 5′-ACT CCT ATC ACT GGT GCA ACC C-3′, BK11B(+), 5′-TTG GCT ACA GCT ACG GCG GAG GAT-3′ and BK21(−), 5′-TGA GAT CAT CAA TAG TGT TAT AAT-3′. Primers 1a(+)/BK21(−) were used for the first PCR amplification reaction, and BK11B(+)/BK21(−) for the reamplification reaction. The latter primer was also used for the sequencing. The numbering of bp and amino acids is according to Langbein et al. (31).

Screening assay for K9 mutations

We developed assays that did not require DNA sequencing to facilitate the detection of each identified mutation in the patients, and to screen for the occurrence of that mutation in the normal population. The R162Q substitution of the family EPPK-J results in the creation of a Pvu II restriction enzyme site in DNA amplified from exon 1. Completed PCR reaction products were purified from the gel and digested with 2 units of Pvu II for 2–4 h, and analysed in 6% polyacrylamide gel. Since the R162W substitution in family EPPK-KS did not destroy or create any restriction enzyme sites, a PCR amplification of specific alleles assay was developed (32). In this assay, PCR amplification was obtained specifically from the wild type or the mutant allele using primers that differ only at the 3′-most nucleotide. The primers KSwt(−), specific for the wild type base C, and KSmt(−), specific for the mutation T, were each used with 1a(+) as the second primer. Amplification was performed with previously described materials for 30 cycles of 1 min denaturation at 94°C, 1 min annealing at 55°C, and 1 min extension at 72°C. The primers used for PASA assay were: KSwt(−), 5′-TCC AAG TAA GAG GCC AGC C**G**-3′, and KSmt(−), 5′-TCC AAG TAA GAG GCC ACG C**A**-3′.

Electron microscopy

Peptides corresponding in the sequence to the first 18 residues of the 1A rod domain of the K9 chain were synthesized, and included both the wild type and peptides containing either a glutamine or tryptophan substituted for arginine in position 10. These were used for keratin intermediate filament disassembly assays, as earlier described (25, 26).

RESULTS

Clinical description of patients

The diagnosis of EPPK was confirmed by histopathology from skin biopsy specimens obtained from at least one affected person in each family. All affected patients in EPPK families had confluent thickening of the palms (Fig. 1) and soles shortly after birth. The borders of the normal skin were surrounded by an erythematous halo. Usually, skin lesions thickened with age, but parents denied any kind of previous blistering. The skin at the other sites, oral mucosa, teeth, and nails were normal. No consanguinity was observed between the parents of affected persons. Histopathologic findings showed characteristic epidermolytic hyperkeratosis marked by tonofilament changes and vacuolar degeneration of keratinocyte in the granular and spinous layers of the epidermis (Fig. 2).

EPPK-J family

This family consisted of a single 4-year-old Korean girl (II-2), her sister (II-1) and two normal parents (I-1 and I-2), indicating this may be a sporadic case. By sequencing analyses, we found a G to A substitution at bp 551 resulting in the substitution of glutamine for arginine in codon 162 in exon I of the

Fig. 1. Clinical appearences of palms of EPPK-J patient. A diffuse, symmetric, sharply defined hyperkeratosis of the palms and soles, and an erythematous halo at the border of the lesion are noted.

K9 gene (R162Q, **C**GG to **CA**G) (Fig. 3a). This substitution creates a restriction site for Pvu II (CAG/CTG),which cuts the PCR-amplified product of 429 bp (wild type allele) into 284 bp and 145 bands (mutant allele) (Fig. 3b).

EPPK-KS family

This is a four-generation family with nine affected individuals (Fig. 4a). First, we saw the patient (III-3) and his son (IV-1). In both patients, we found a C to T substitution at bp 550 resulting in the substitution of tryptophan to arginine in codon 162 of exon I of the K9 gene (R162W, **C**GG to **T**GG) (Fig. 4b). As this substitution does not create or destroy any restriction enzyme site, a PCR amplification of specific allele assay was used to selectively amplify the wild type allele or the allele carrying the mutation (see MATERIALS AND METHODS). Only the affected individual had a PCR product with both the mutant and wild type-specific primers, whereas all of the unaffected individuals in this family and normal controls were negative for the primer bearing the mutation (Fig. 4c).

Using an established keratin intermediate filament disassembly assay with a synthetic peptide corresponding to the first 18
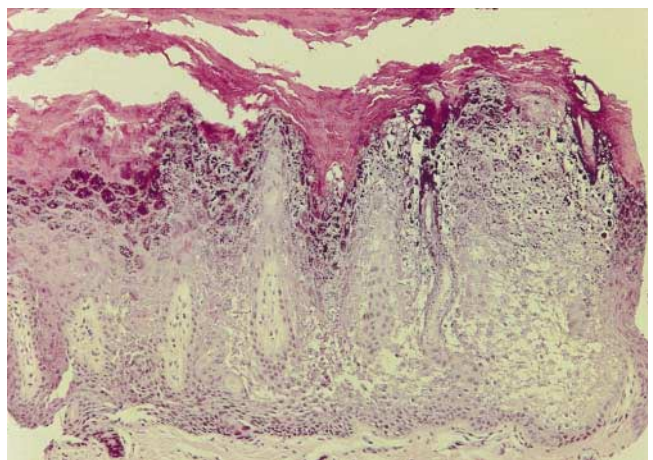

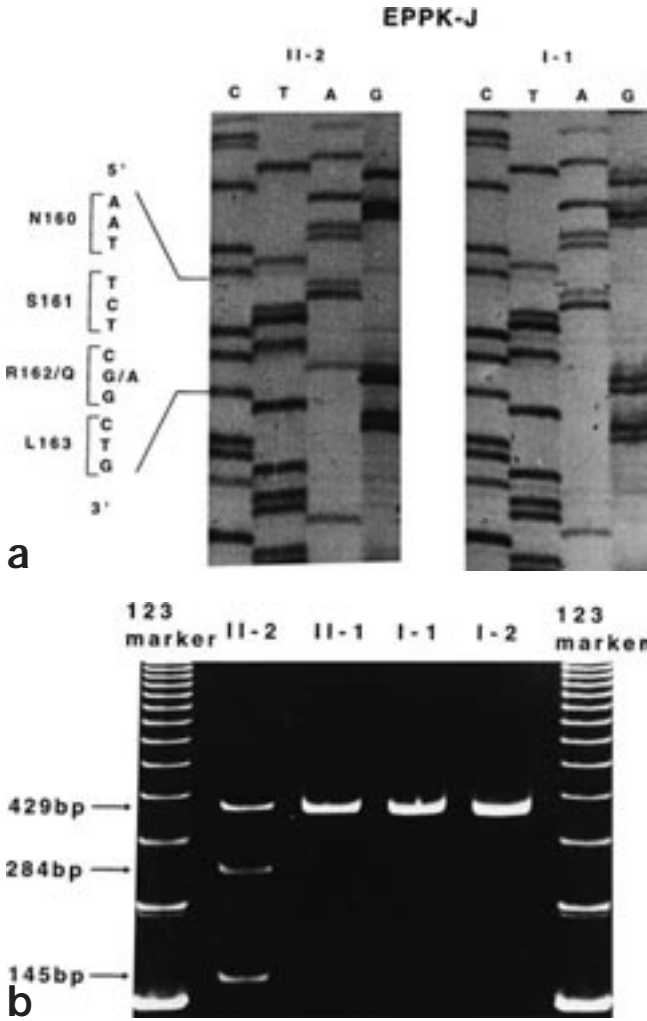*Fig. 2.* Histopathology. Epidermolytic changes in the spinous and granular layers are noticeable.

Fig. 3. (*a*) DNA sequences of EPPK-J. This shows a G to A change, i.e. R162Q mutation. (*b*) Pvu II digestion of PCR-amplified DNA of the whole family members. The 429 bp fragment of only the affected individual is digested.

residues of the 1A rod domain segment of the K9 chain, K1/K10 KIF (Fig. 5a) were rapidly disassembled into protofilamentous structures within 30 min (Fig. 5b). However, a peptide bearing a glutamine (data not shown) or tryptophan (Fig. 5c) substitution in position 10 instead of arginine did not significantly alter the keratin intermediate filament structure within 6 h. These data suggest that the structures of keratin chains containing either of these substitutions varies significantly from the wild type chain, and no longer favours the formation of normal keratin intermediate filaments *in vitro* or *in vivo*.

DISCUSSION

EPPK is a genodermatosis with an autosomal dominant mode of transmission, first described by Voerner in 1901 (2), although there has been one report of an autosomal recessive case, perhaps due to consanguinity (35). To date, all cases of this disease map to the type I keratin gene cluster on chromosome 17q12-q21 (27) and have been found to be caused by mutations in sequences encoding the 1A rod domain segment of the keratin 9 gene (19). EPPK is distinguishable from
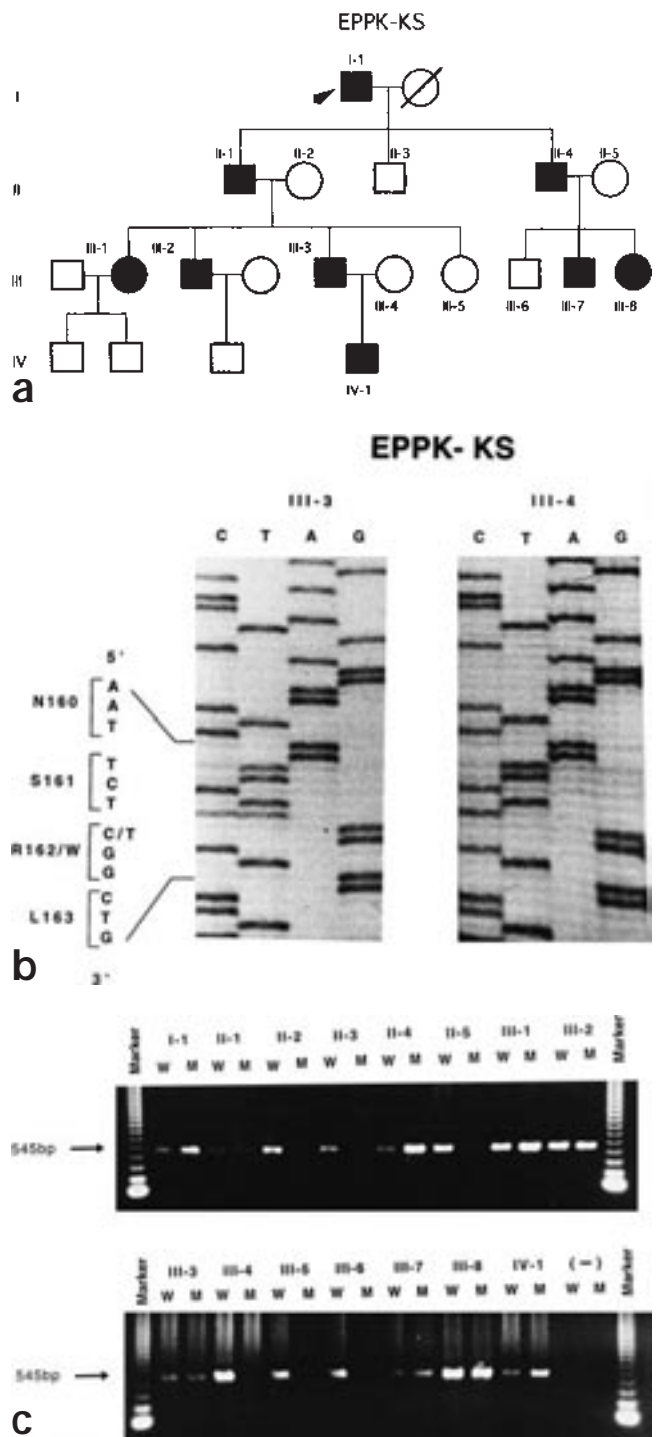

Fig. 4. (*a*) Pedigree of the EPPK-KS family. Arrow indicates proband; numbering above symbol indicates individuals whose DNA was analysed. (*b*) DNA sequences of EPPK-KS. This shows a C to T change which causes R162W substitution. (*c*) A PASA assay using primers specific for either the normal K9 gene sequence (a 3′ C), or the mutant sequence(a 3′ T). Only DNA from the affected family members yields an amplified band of 545 bp with both the mutant (M) and wild type (W) primers.

NEPPK by the following criteria (1, 2, 36): (i) the presence of epidermolytic changes in the epidermis; (ii) the disease being apparent at birth, in comparison to two years of age for
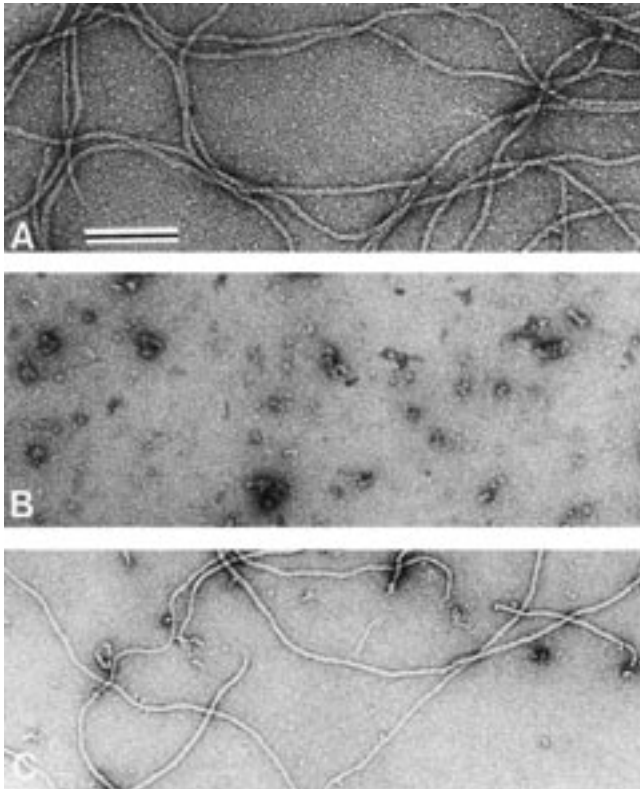
Fig. 5. Electron microscopy of keratin intermediate filament. (*a*) Normal keratin intermediate filament composed of K1/K10 chains. (*b*) As (*a*), but reacted with an 18-residue peptide of wild type human K9 sequences for 30 min. (*c*) As (*b*), but reacted with a peptide containing R→W substitution for 6 h. Similar data were obtained for a peptide containing the R→Q substitution (25). Negatively stained with uranyl acetate. Bar: 100 μm.

NEPPK; and (iii) the mutations being reported so far in the keratin 9 gene only, in comparison to either the keratin 1 or 16 genes for NEPPK (25, 26).

Since Reis et al. (19) reported that mutation of the K9 gene causes EPPK, nine different mutations have been reported, and all located in sequences encoding the 1A domain (19–24). In this study, we have identified two different point mutations in Korean patients in K9 by direct DNA sequencing. These mutations, R162W and R162Q, are the most frequently reported mutations in K9. This highly conserved arginine residue occupies position 10 of the 1A rod domain segment of the type I keratins, and mutations of this arginine have only been found in the type I keratins never in a type II keratin, e.g. in K14 causing epidermolysis bullosa simplex (9, 10), in K10 causing epidermolytic hyperkeratosis (11–14), in K16 causing focal NEPPK (26), and in K17 causing pachyonychia congenita (18). This arginine residue when altered in other keratins is converted into histidine (9, 11–13, 18), cysteine (9, 11–13, 26), serine (10, 14), leucine (13), and proline (14), but R162W and R162Q have only been observed in K9. The discovery of point mutations at this position in several distinct diseases draws attention to the basis of these mutations and to their functional consequences. The arginine codon in this position is CGG, which is known to be a candidate for mutations within the CpG dinucleotide. The frequency of C-T transitions in

CpG dinucleotides within coding regions is 42 times higher than expected from random mutation (37).

Earlier work has demonstrated that the beginning of the 1A rod domain segment of keratin (and other intermediate filament) chains is especially important for keratin intermediate filament assembly and maintenance of keratin intermediate filament structural integrity (34). These sequences are thought to be involved in overlaps with neighbouring molecules within the keratin intermediate filament hierarchy. Inappropriate amino acid substitutions in the first few residue positions provide the molecular basis of most keratin diseases (38, 39), apparently because they alter critical three-dimensional structure. The tryptophan and glutamine substitutions in the two cases of EPPK disease described here are likely to significantly alter structure, as determined by our indirect keratin intermediate filament disassembly assay (Fig. 5). The resultant weakened keratin intermediate filaments are presumably no longer able to fulfil their structural requirements in the epidermis, leading ultimately to epidermolysis and notable skin diseases.

We investigated three families, but could not find any mutation in the K9 gene in our third family, despite completely sequencing all the rod domain exons. We could not perform the linkage analysis for the type II keratins because our third family also might be a sporadic case. Hennies et al. (21) reported that not all the families with PPK showed linkage to the type I keratin gene cluster at chromosome 17q21, and that only half of the cases of EPPK had the mutation in the K9 gene. However, it is not yet known which of the type II keratins forms the heterodimer with K9 in native keratin intermediate filaments. Furthermore, no type II keratin has yet been described whose expression pattern is restricted to the palms and soles. Nevertheless, it seems reasonable to speculate that mutations in one of the type II keratin genes co-expressed in the palm-sole epidermis might be the basis of these uncharacterized cases of EPPK.

In summary, we report mutations in the K9 gene, leading to the substitutions R162W and R162Q, as the probable cause of EPPK in one unrelated individual patient, and in one large pedigree from Korea. These findings provide further evidence for mutational heterogeneity in EPPK and for the functional importance of the 1A rod domain segment in the structural integrity of keratin intermediate filaments.

ACKNOWLEDGEMENTS

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