A Mutation in the V1 Domain of K16 is Responsible for Unilateral Palmoplantar Verrucous Nevus

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Palmoplantar keratodermas are a group of heterogeneous diseases characterized by thickening, and marked hyperkeratosis, of the epidermis of the palms and soles. Palmoplantar keratodermas can be divided into four major classes: diffuse, focal, punctate, and palmoplantar ectodermal dysplasias. All forms are genetic diseases inherited as autosomal dominant disorders. We studied a patient exhibiting a localized thickening of the skin in parts of the right palm and the right sole, following Blaschko's lines, that does not fit into any classes already described. We sequenced the keratin 16 cDNA derived from skin biopsy material from affected and non affected palms. The keratin 16 cDNA sequence from lesional epidermis showed a 12 base pair deletion (309–320del), which deletes codons 104–107. The mutation is predicted to delete four amino acids, GGFA, from the V1 domain of the keratin 16 polypeptide, close to the 1A domain. Full-length keratin 16 cDNA sequence derived from the unaffected palm was completely normal, consistent with a postzygotic mutation as is suggested by the mosaicism observed. We defined this new clinical entity, "unilateral palmoplantar verrucous nevus", rather than localized or focal epidermolytic palmoplantar keratodermas, as the lesions are present only on one side of the body and follow Blaschko's lines. This study is a report of a mosaic mutation in keratin 16 and also the association of a mutation in the V1 domain of a type I keratin associated with a human disease. *Key words: intermediate filaments/keratin 16/keratin/mutation/palmoplantar keratoderma. J Invest Dermatol 114:1136–1140, 2000*

Palmoplantar keratodermas (PPK) are a group of clinically and genetically heterogeneous diseases, all characterized by marked hyperkeratosis and thickening of the epidermis of the palms and soles (Itin and Lautenchlager, 1995). These diseases were originally classified only by inheritance patterns and clinical criteria such as morphology of the skin lesions and differential involvement of different body sites (Stevens *et al*, 1996). More recently, the classification has been modified to include histopathologic findings and molecular defects, when known. Based on the most recent classification, PPK are now subdivided into four major classes (Christiano, 1997): diffuse PPK, focal PPK, punctate PPK, and palmoplantar ectodermal dysplasias. Diffuse PPK can be further subdivided into epidermolytic PPK (Voerner type, EPPK; OMIM 144200) and nonepidermolytic PPK (Unna–Thost, NEPPK; OMIM 148400). Both forms are inherited as autosomal dominant disorders with variable penetrance. EPPK is clinically characterized by a diffuse thickening of the skin of the palms and soles with an erythematous border (Vorner, 1901; Klaus

et al, 1970). Peeling and blistering can also be present. Histologically, the affected skin shows hyperkeratosis and vacuolar cytolysis of the granular layer (Baden *et al*, 1984; Moriwaki *et al*, 1988), in a pattern very similar to that found in epidermolytic hyperkeratosis. Electron microscopy shows vacuolization of keratinocytes of the granular layer and clumping of keratin filaments. Although NEPPK has a similar clinical presentation to EPPK, in this condition the lesions lack an erythematous border (Thost, 1880; Unna, 1883). Histologically, the lesions show orthokeratosis and acanthosis but no vacuolar degeneration.

Recently, mutations in differentiation-specific keratin genes have been shown to be the cause of some types of PPK. Interestingly, different mutations of the same keratin have been associated with completely different phenotypes (**Table I**). Keratins and their attachment complexes represent the major structural cytoskeleton in keratinocytes and are essential for maintaining the integrity of the epidermis. Keratins can be divided into two classes: type I (acidic) and type II (basic), whose genes are clustered on chromosomes 17 and 12, respectively. Keratins are expressed in a differentiation-specific and tissue-specific manner. They are always coexpressed in type I and type II pairs, which form heterodimers. These dimers undergo further polymerization to form a dense meshwork of keratin intermediate filaments (Coulombe and Fuchs, 1990; Hatzfeld and Weber, 1990). Dominant–negative mutations in keratin genes, largely affecting the central α-helical domain, result in disorders characterized by epithelial fragility and/or hypertrophy. The distribution of the lesions in skin diseases reflect the expression pattern of the affected keratin (Corden and McLean, 1996).

Table I. PPK with known genetic mechanisms

Disorder	Protein	Reference
EPPK, Vorner type	K9	Reis et al, 1992; Reis et al, 1994
NEPPK, Unna–Thost type	K1	Kimonis et al, 1994
Erythrokeratoderma Variabilis	Cx31	Richard et al, 1998
Focal NEPPK	K16	Shamsher et al, 1995
Pachyonychia congenita type 1	K6a/K16	Bowden et al, 1995; McLean et al, 1995
Pachyonychia congenita type 2	K6b/K17	McLean et al, 1995; Smith et al, 1997
Vohwinkel syndrome	Cx26	Maestrini et al, 1999
Camisa syndrome	Loricrin	Maestrini et al, 1996; Korge et al, 1997

Mutations in K9 are responsible for diffuse EPPK (Reis et al, 1992, 1994). K16 mutations have been found in focal NEPPK (Shamsher et al, 1995) and pachyonychia congenita type 1 (PC-1; McLean et al, 1995). The latter disorder has also been shown to be caused by K6a mutations (Bowden et al, 1995). The cause of diffuse NEPPK still remains unclear, although in one case a mutation in the V1 domain of K1 has been described (Kimonis et al, 1994). Another form of diffuse NEPPK has been mapped to a locus near to, but distinct from, the type II keratin gene cluster on chromosome 12 (Kelsell et al, 1999).

Here we report a case of unilateral palmoplantar nevus, with histologic features similar to EPPK, which appears to be caused by a postzygotic mutation in the V1 domain of the K16 gene.

MATERIALS AND METHODS

Light and electron microscopy Ethical approval and informed consent was obtained before skin biopsy. Biopsy samples from the proband were processed for light and electron microscopy. Electron microscopy samples were prefixed with 2% (wt/vol) glutaraldehyde for 2 h at 4°C; complete fixation was achieved by incubation with 1% (wt/vol) osmium tetroxide in 0.1 M cacodylate buffer, 4.5% (wt/vol) sucrose, for 1 h at 4°C. Samples were dehydrated in ethanol and embedded in epoxy resin. Semithin sections (1 μm) of embedded samples were stained with uranyl acetate 1% (wt/vol). Light microscopy samples were paraffin embedded and stained by standard methods.

Molecular genetic analysis Genomic DNA were extracted from patient blood, from parents and 20 unrelated controls according standard protocols. Total RNA was extracted from two 3 mm skin biopsies, from the left affected palm and the right unaffected palm, using the Qiagen (Crawley, UK) RNeasy minikit. Reverse transcription (reverse transcription–PCR) reactions were performed using the reverse transcription–PCR One Step System (Life Technologies; Paisley, UK), using 100 ng of total RNA, according to the manufacturer's instructions. The entire coding region of K9 gene was amplified by reverse transcription–PCR using primers K9F1 (5′-AGCCGGTAGCACTCCTATCACTGCTT-3′, + strand) and K9R5 5′-GACCACTGGTTCTACTCTGTTTTCC-3′, − strand). Similarly, the coding region of K1 was amplified using primers K1F1 (5′-GGCTCGTAAAAATCTTGTATGGCTGC-3′, + strand) and K1B6 (5′-GATGCTGGGGGAGAACTAG-3′, − strand) and directly sequenced. The V1 and the entire rod domain of K16 were amplified using specific primers reported by Shamsher et al (1995). The following PCR program was used in all cases: 51°C for 30 min; 94°C for 2 min; followed by 40 cycles of: 94°C for 35 s; 59°C for 2 min and 30 s; 70°C for 30 s. PCR fragments were gel purified with the Qiaex II extraction kit (Qiagen; Crawley, UK) prior to sequencing. In all cases, PCR products were directly sequenced using the amplification primers and additional internal primers. Approximately 100 ng of purified template DNA was sequenced with the BigDye Termination Reaction Kit (Perkin Elmer; Foster City, CA) on an ABI-PRISM 377 DNA sequencer (Perkin Elmer) according to the manufacturer's recommended protocols.

For mutation confirmation, a 204 bp PCR fragment of the K16 gene was amplified using primers K16F1 (5′-GTGCTGGCTTTGGTGGTGGT-3, + strand), K16R1 (5′-GGGACTGTAGTCTTTGATCTCACTGG-3′, − strand) using the conditions described above. The resultant PCR products were end-labeled using [^{33}P]γdATP; were resolved on non-denaturing 7.5% polyacrylamide/tris borate EDTA (TBE) gels; and visualized by autoradiography.

For exclusion of the mutation from normal individuals, PCR was performed using genomic DNA using primers K16F1 (above) and K16R1

(above) and the following conditions: standard PCR buffer with 1.5 mM MgCl$_2$ (Promega; Madison, WI) and 2.5 U of Taq polymerase (Promega). The following PCR program was used: a primary step of 4 min at 95°C; 40 cycles consisting of 94°C for 40 s, 59°C for 40 s, 72°C for 40 s; followed by 1 cycle of 72°C for 10 min.

Protein expression analysis The effects of the K16 deletion mutation on keratin assembly was investigated by transfection of normal and mutant K16 cDNA into epithelial cell line PtK2 (Franke et al, 1978). A normal K16 cDNA expression construct based on plasmid pCR3 was used as reported previously (Smith et al, 2000). The deletion mutation was introduced into this plasmid using the Transformer site-directed mutagenesis system (Clontech; Palo Alto, CA). Normal or mutant cDNA were transfected into 50% confluent cultures of PtK2 cells using the Fugene liposome system (Boehringer; Mannheim, Germany). Transfection was carried out for 4 h, after which the cultures where washed to remove the plasmid DNA/Fugene mixture. At 24 and 48 h post-transfection, duplicate cultures were stained by indirect immunofluorescence for K16 expression using monoclonal antibody LL0025 (a gift from E.B. Lane, Department of Anatomy and Physiology, University of Dundee). The stained cultures were examined for signs of filament abnormality in a "blinded" study where the mutation status of the cultures was unknown to the observer. More than 50 transfected cells were examined in each culture at each time point.

RESULTS

Case report We studied a 15 y old female patient exhibiting skin thickening localized to discrete parts of the right palm (**Fig 1***a*) and right sole (**Fig 1***b*), following Blaschko's lines (Bolognia et al, 1994). The lesions were clearly demarcated from adjacent normal skin. No lesions were present on the left palm or sole or other body sites. A skin biopsy of the affected area, including a portion of unaffected tissue, was taken from the right palm. Light microscopy showed hyperkeratosis; vacuolar degeneration of keratinocytes in the upper spinosum and granular layer; pyknotic nuclei; and a thickened granular layer containing an increased number of keratohyaline granules. Furthermore, the boundary with normal skin was well defined (**Fig 1***c*) and the unaffected portions of the skin were histologically normal. Ultrastructural analysis of affected palmar skin (**Fig 1***d*), showed marked tonofilament clumping and cytoplasmic vacuolization in suprabasal cells. Tonofilament aggregates in the granular layer, were seen to connect with keratohyaline granules. The histologic findings were consistent with the diagnosis of a disease similar to localized palmoplantar epidermolytic hyperkeratosis, or focal EPPK, and the ultrastructural analysis suggested a keratin defect. The peculiar nevoid distribution of the lesion and the absence of affected relatives suggested mosaicism due to a somatic mutation in early development.

Mutation analysis We adopted the strategy of analyzing mRNA derived from the patient's hyperkeratotic lesion by reverse transcription–PCR followed by direct sequencing. In all cases, comparisons were made with mRNA derived from uninvolved skin from the patient. A number of candidate keratin genes were fully sequenced by this reverse transcription–PCR approach. As mutations of the K9 gene have been reported to be responsible for EPPK (Bonifas et al, 1994; Reis et al, 1994; Rothnagel et al, 1995), we first examined this gene. Complete sequencing of K9 cDNA, however, revealed no mutations. Therefore, we sequenced other keratin genes known to be responsible for diseases with
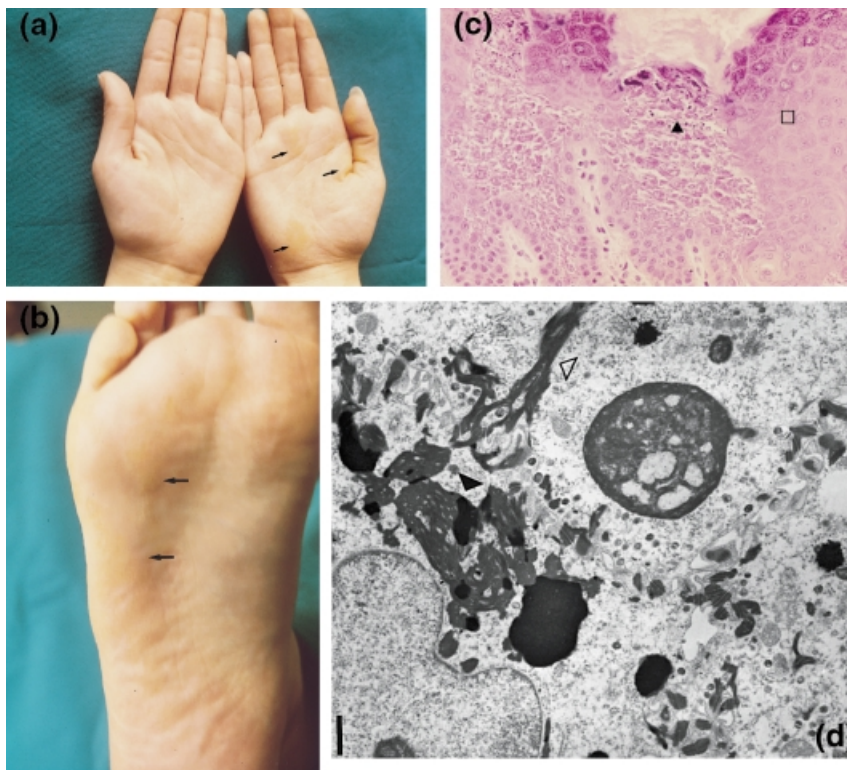
Figure 1. Clinical presentation and histopathology. (*a*) Hyperkeratotic lesions on the right palm and (*b*) right sole. The lesions (*arrows*) are well demarcated from the adjacent normal skin and follow the lines of Blaschko. (*c*) Histopathology of a skin biopsy from the right palm, including both affected (▲) and nonaffected (□) tissue. Hyperkeratosis and vacuolar degeneration of the granular layer are present in the affected region. The adjacent unaffected skin is normal. (*d*) Electron microscopy shows marked tonofilament clumping (△). The tonofilament aggregates are connected (▲) with keratohyaline granules (*scale bar*: 0.8 μm).

palmoplantar involvement. Specifically, we analyzed the K1 cDNA, mutations in which can cause a form of NEPPK (Kimonis *et al*, 1994), and the K16 cDNA, mutations in which can cause focal NEPPK (Shamsher *et al*, 1995) or PC-1 (McLean *et al*, 1995; Smith *et al*, 1999). No sequence changes were detected in the complete K1 coding sequence. A 12 bp in-frame deletion, however, was detected in the K16 cDNA (**Fig 2a–d**). The mutation was designated 309–320del, according to the recent guidelines for human mutation nomenclature (Antonarakis, 1998). This mutation deletes four amino acids (GGFA) from the V1 domain, close to the 1A domain (**Fig 2c**). The mutation was only present in cDNA derived from the affected skin of the palm, the K16 sequence from the unaffected area being completely normal (**Fig 2a**, **b**). This is consistent with this change being a postzygotic mutation. Direct sequencing of the entire K16 coding sequence revealed no other mutations. Additionally, the sequence of the K1 cDNA was found to be normal in our patient; also we sequenced the mutation hotspot regions of the K6a gene (Bowden *et al*, 1995; Corden and McLean, 1996) and no mutations were detected (data not shown). To confirm the sequencing results, a 204 bp fragment of K16 including the deleted area was amplified by reverse transcription–PCR from the patient's lesional and nonlesional skin. **Figure 2**(*e*) shows a single band of the expected size of 204 bp in the sample from the unaffected palm. In contrast, PCR products obtained from the lesion showed bands of 204 and 192 bp, consistent with the presence of a somatic mutation in the affected skin.

In order to demonstrate that this is a somatic mutation, we performed the same analysis using peripheral blood lymphocyte DNA from the patient, her parents, and 20 normal, unrelated control individuals. All the DNA amplified showed only the normal 204 bp band (**Fig 2f**), demonstrating that the mutation is (i) not detectable the proband's blood, and (ii) unlikely to be a common polymorphism.

Protein expression analysis Eukaryotic expression constructs were made corresponding to the normal and mutant K16 cDNA. These were expressed in epithelial cell line PtK2, which express simple epithelial keratins K8, K18, and K19 (Franke *et al*, 1978),
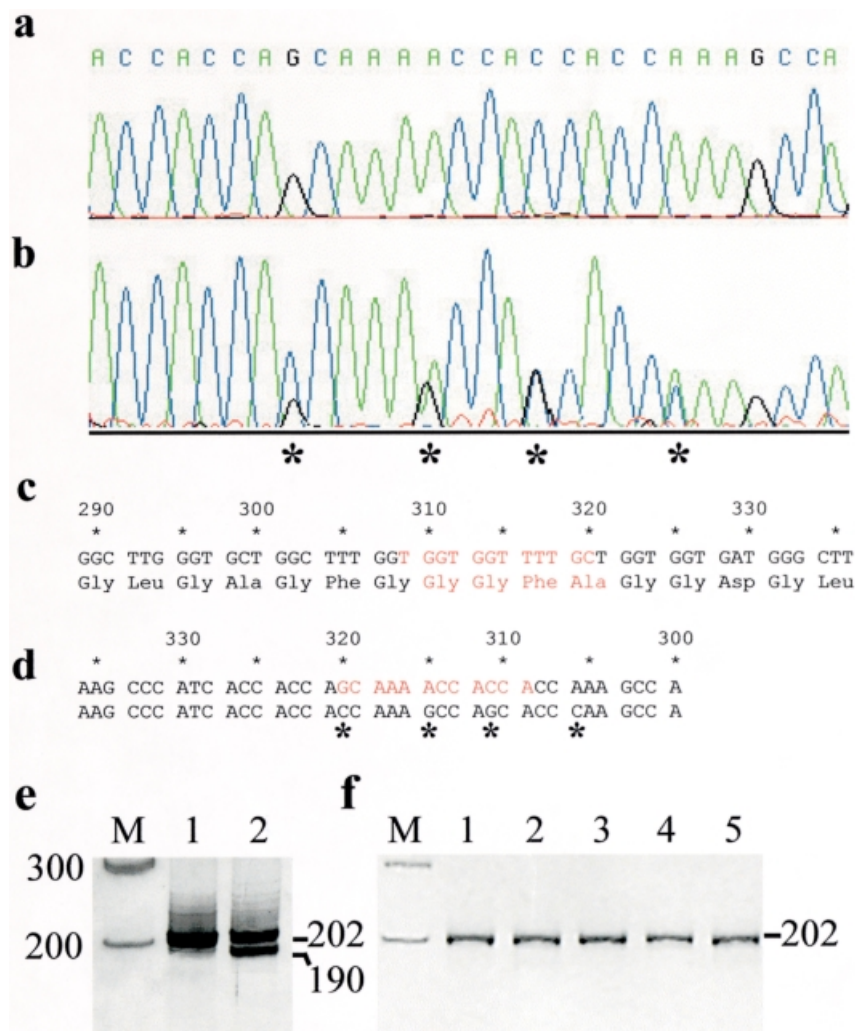
according to a modification of the previously reported protocol (Smith *et al*, 2000). Immunofluorescence examination of the transfected cultures was performed as a ''blinded'' study where more than 50 cells were examined in duplicate transfections at two time points: 24 and 48 h post-transfection. By this means, no overt changes, such as the filament aggregation previously described for rod domain mutations in K16 (Smith *et al*, 2000), could be detected between the normal and mutant transfected cultures (data not shown).

DISCUSSION

Here, we report a mutation in the V1 domain of K16 in a case of unilateral palmoplantar nevus with the histologic features of epidermolytic hyperkeratosis. K16 gene mutations have already been associated with focal NEPPK (Shamsher *et al*, 1995) and PC-1 (McLean *et al*, 1995; Smith *et al*, 1999), showing that different mutations of the same keratin can be responsible for different phenotypes. Our results demonstrate again that there is not a simple correlation between the keratin affected and a particular phenotype. Mutations in the K16 gene can generate both a nonepidermolytic phenotype (NEPPK), as previously reported (McLean *et al*, 1995; Shamsher *et al*, 1995), as well as the epidermolytic pathology seen in some PC-1 cases (McLean *et al*, 1995) and here in our patient.

This study is a report of a mutation affecting the V1 domain of a type I keratin. Keratin mutations are usually clustered in the 1A and 2B domains (Letai *et al*, 1992) and less frequently on the H1, L12, and central 2B domains (Irvine and McLean, 1999). These domains are known to be important for keratin filament assembly and therefore mutations that affect these regions are likely to be pathogenic and only a few mutations have been described in other domains (Corden and McLean, 1996; Irvine and McLean, 1999). The V1 region consists mostly of short glycine repeats in which are interspersed aromatic or large apolar residues. These residues aggregate to form a ''glycine loop'' structure that may modulate flexibility or other unknown physical attributes of keratin filaments (Steinert *et al*, 1991, Steinert, 1995). In addition, it has been suggested that the glycine loops of keratins interact with similar structures in loricrin, integrating these proteins into the cornified envelope (Steinert *et al*, 1991; Steinert, 1993; Candi *et al*, 1998).

Figure 2. Mutation analysis. Sequence analysis of the K16 gene using the anti-sense primer K16R1: (*a*) from cDNA obtained form normal tissue (*left hand*); (*b*) from affected tissue. Stars indicate peaks derived from the overlap of the wild-type allele with the mutated allele. (*c*) Portion of the K16 gene sequence showing (*in red*) the nucleotides and predicted amino acids deleted. (*d*) Schematic representation of the sequence pattern derived from the overlap of the wild-type and mutated alleles. Stars indicate the predicted heterozygous nucleotides corresponding to those visible on the sequence trace above. Electrophoretic analysis (*e, f*) of a PCR-amplified 204 bp fragment containing the 12 bp deletion, using: (*e*) mRNA of normal (*lane 1*) and affected tissue (*lane 2*); (*f*) genomic DNA of the proband (*lane 1*), parents (*lanes 2* and *3*), and normal controls (*lanes 4* and *5*). Two bands of 204 bp and 192 bp are visible only in the mRNA from the affected palm. The deletion is absent in the mRNA from the unaffected palm, the genomic DNA of the patient and in normal genomic DNA.

These theories are supported by experiments of keratin pairing and filament assembly, such as *in vitro* reconstitution of keratins filaments and *in vivo* transfections of type I and II keratins bearing mutations of the V1 domain (Lu and Lane, 1990; Wilson *et al*, 1992). These experiments have shown that the V1 domain plays a subtle but important part in the assembly of the filaments from the tetramer to structures of higher order, up to the 10 nm filament (Hatzfeld and Burba, 1994). Here, our transfection experiments revealed that this V1 deletion mutation in K16 does not produce overt filament aggregates in cultured epithelial cells, which is the case for mutations involving the rod domain of K16 (Smith *et al*, 2000) and other keratins (Lu and Lane, 1990; Letai *et al*, 1992; Wilson *et al*, 1992). Therefore, this mutation is unlikely to act at an early stage of the polymerization process and presumably acts in a more subtle manner than previously described mutations.

It is also interesting that size polymorphisms have been found in the V2 domain of K1 and K10 (Korge *et al*, 1992a, b; Mischke, 1993), but have not been reported in the V1 domain or type I keratins, perhaps because such deletions are pathogenic. Two polymorphisms in the V1 domain of type II keratins have been described. Both are insertions with no associated phenotype: a 42 bp insertion in K4 (Wanner *et al*, 1993) and an 18 bp insertion in K2e (Smith *et al*, 1998). In the case reported here, we found a similar 12 bp in-frame postzygotic deletion in the V1 domain of a type I keratin, which may compromise the formation of filament bundles or higher-order structures and/or cross-linking with other proteins. Dominant–negative mutations in this domain of type II keratins have been associated with other skin diseases. Specifically, a mutation on the V1 region of K1 have been associated with

NEPPK (Kimonis *et al*, 1994); and a mutation in the V1 domain of K5 with EBS-MP (Uttam *et al*, 1996; Irvine *et al*, 1997). The precise molecular interactions that allow V1 mutations, including the one reported here, to act as dominant–negative disrupters of keratin filament assembly and/or integrity remains to be elucidated.

In conclusion, we describe a new form of epidermolytic hyperkeratosis and present data which indicate an important role for the V1 domain of type I keratins in intermediate filament assembly and/or integrity. The new phenotypic entity we term as ''unilateral palmoplantar verrucous nevus'', rather than focal EPPK, because the lesions are present only on the left anteroposterior half of the sagittal division and because they follow the Blaschko lines (Paller *et al*, 1994). This phenotype appears to be caused by a novel postzygotic mutation (309–320del) in the K16 gene. If this mutation had occurred in the germ line or a very early developmental stage, the resulting phenotype might have had more widespread clinical features, as previously reported (McLean *et al*, 1995; Shamsher *et al*, 1995; Smith *et al*, 1999). Additional mutations need to be identified to establish whether different phenotypes are due to specific mutations or if the genetic background of the affected individual plays a major part in the phenotypical variability of the disease.

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