A Usual Frameshift and Delayed Termination Codon Mutation in Keratin 5 Causes a Novel Type of Epidermolysis Bullosa Simplex with Migratory Circinate Erythema

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We report here two unrelated families in Japan and Korea having patients with a unique type of epidermolysis bullosa simplex and a novel mutation in the keratin gene *KRT5*, i.e., a frameshift and delayed stop codon inconsistent with any subtype described before. The patients showed migratory circinate erythema and multiple vesicles on the circular belt-like areas affected by erythema. Electron microscopy of skin biopsies showed a reduction in the number of keratin intermediate filaments in the basal cells without tonofilament clumping. We identified a novel heterozygous deletion mutation (1649delG of *KRT5*) in both cases. This deletion is predicted to produce a mutant keratin 5 protein with a frameshift of its terminal 41 amino acids and 35 amino acids longer than the wild-type keratin 5 protein due to a delayed termination codon. As the same abnormal elongated mutant *KRT5* gene was found in the independent families, the predicted abnormal elongated keratin protein is likely to lead to an atypical clinical phenotype that has never been reported, possibly by interfering with the functional interaction between keratin and its associated proteins. ***Key words: Dowling–Meara/pigmentation/tail domain/V2 domain. J Invest Dermatol 121:482–485, 2003***

Epidermolysis bullosa simplex (EBS) is an autosomal-dominant inherited blistering skin disease, commonly known as a keratin disease, caused in most cases by point mutations in *KRT14* or *KRT5* genes, and categorized into three main subtypes, i.e., EBS Dowling–Meara (EBS-DM, OMIM 131760), EBS Kübner (EBS-K, OMIM 131900), EBS Weber–Cockayne (EBS-WC, OMIM 131800) (Fine *et al*, 2000). Among these three primary EBS subtypes, EBS-DM is the most severe, with clustered blisters appearing at any body site following mild trauma; EBS-K is associated with generalized blistering, whereas the mildest subtype, EBS-WC, shows blistering restricted to the hands and feet (Horn and Tidman, 2000).

For all EBS types, the site and type of amino acid substitution within the keratin protein generally correlates with phenotypic severity in this disorder (Letai *et al*, 1993). Every keratin protein has a central α-helix rod domain consisting of approximately 310 amino acids residues, which is believed to be organized into coiled-coil dimers through the hydrophobic associations of a heptad repeat unit. The mechanism by which dimers associate to form mature 10 nm intermediate filaments is unknown (Steinert and Roop, 1988). This rod domain exists in four segments (i.e., 1A, 1B, 2A, and 2B) interrupted by three nonhelix linkers, termed the L1, L12, and L2 regions. The rod domain is flanked on each end with nonhelical head (amino-terminal) domains, i.e., V1 and H1, and tail (carboxyl-terminal) domains, i.e., V2 and H2, although type II keratin does not have H2. The function of these domains remains unclear, although they are likely to reflect in part the tissue specificity of keratins. These nonhelix domains are highly variable in size and sequence even for members within the same intermediate filament subtype. In addition, two very highly conserved regions at the beginning of the 1A region and the end of the 2B region are termed the helix initiation motif (HIM) and the helix termination motif (HTM), respectively (Irvine and McLean, 1999).

The mutations ascribed to the subtype EBS-DM have primarily involved changes in the highly conserved regions (i.e., HIM and HTM). Mutations associated with EBS-WC primarily are restricted to the nonhelix regions of the keratin polypeptide, in particular the H1 domain and L12 linker region. Interestingly, *KRT5* and *KRT14* mutations have been reported less frequently in EBS-K than in the other main EBS subtypes. The mutations of *KRT5* and *KRT14* in EBS-K are mainly located to the nonpolar residues of the HIM and HTM regions of the rod domains. Previous studies also have suggested that the keratin rod domains play important roles in intermediate filament assembly and organization (Steinert *et al*, 1993), whereas the functions of the head and tail domains are less clear. The head region of the keratin 5 (K5) protein, however, appears to be required for both filament elongation and lateral alignments (Wilson *et al*, 1992). In addition, an 18 amino acid residue stretch in the K5 head that is conserved only among type II epidermal keratins has been demonstrated to be critical for the binding to desmoplakin (Kouklis *et al*, 1994). Indeed, mutations in the V1 domain of K5 cause relatively mild clinical phenotypes, i.e., EBS with mottled pigmentation (OMIM 131960) (Uttam *et al*, 1996). Several mutations in the keratin V1 domain other than K5 have also been reported recently (Kimonis *et al*, 1994; Terrinori *et al*, 2000). Conversely, mutations in the V2 domain have been reported only in K1 (Sprecher *et al*,

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2001; Whittock *et al*, 2002). In this study, we noted a heterozygous deletion mutation in the K5 V2 domain that caused a less severe phenotype that resembled EBS-DM.

MATERIALS AND METHODS

Patients' characteristics

Case 1 A Japanese girl, currently aged 2 y, presented to Gifu University Hospital with symptoms of annular migratory multiple erythema circinatum and multiple vesicles on the hands, feet, and legs from birth. The skin blisters continued to appear mainly on the hands, feet, and occasionally the trunk (**Fig 1*a*, *b***). The lesions were exacerbated in summer. The nails, ocular epithelia, and mucosae were not affected, and laryngeal involvement was not seen. The lesions healed without scarring but with brown pigmentation, and their severity lessened with age. No other family members are affected by the same disorder.

Case 2 A Korean girl, currently aged 1 y, presented to Yongdong Severance Hospital with symptoms of erythematous annularly spreading vesicles on the trunk and extremities since birth. The patient was born at term and there was no abnormality at birth. The blisters resolved without scars or milia as the new blisters annularly spread, but brown pigmentation appeared in the central clearing lesions (**Fig 2*b***). Again, the nails, ocular epithelia, and mucosae were not affected, and laryngeal involvement was not present. The mother had a history of similar blisters in her youth, reported that spontaneous improvement coincided with puberty, and now only occasionally develops vesicles. On examination, the mother had generalized hypopigmented and hyperpigmented patches on the trunk and extremities (**Fig 2*c***), but occasionally only small vesicles were observed in these same areas. Pruritus reportedly develops only when new blisters appear, and the lesions tend to worsen in summer time. There was no hyperkeratosis of the palms and soles, and no nail dystrophy. Seven individuals of the family for three generations had a history of blistering (**Fig 2*a***), and all affected adult members in the family had similar pigmentary changes as the mother's skin lesion. At the beginning of this study, informed consent was obtained with a protocol approved by each University Review Board.

Immunohistochemistry The immunofluorescence method (Dako, Carpinteria, CA) was carried out on fresh tissue by using monoclonal antibody C-50 against K5/8 (Laboratory Vision, Fremont, CA); each section was incubated for 1 h at room temperature in a 1:40 dilution with phosphate-buffered saline. The avidin–biotin immunoperoxidase method (Dako) was performed on formalin-fixed, paraffin-embedded tissue by using monoclonal antibody RCK102 against K5/8 (Sanbio, Uden, The Netherlands); each section was incubated for 1 h at room temperature in 1:10 dilution with phosphate-buffered saline. AEC+ substrate chromogen was used as the color reagent for paraffin-embedded specimens. Immunostaining for K14 was also performed on fresh tissue by using monoclonal antibody LL002 (Laboratory Vision). Normal skin was used simultaneously as a control.

PCR amplification and DNA sequence Genomic DNA was isolated from ethylenediamine tetraacetic acid treated blood samples using DNA extractor WB-rapid kit (WAKO, Osaka, Japan) and kept at –20°C prior to use. PCR amplifications of all exons of *KRT5* and *KRT14* were carried out using primer pairs as described previously (Sorensen *et al*, 1999) and based on information obtained from the published sequence of the *KRT5* gene (GeneBank Accession no. M21389) and *KRT14* gene (GeneBank Accession no. J00124). The cycle sequence reaction was performed using Thermo Sequenase cycle sequencing kit (USB, Cleveland, OH). Prior to the cycle

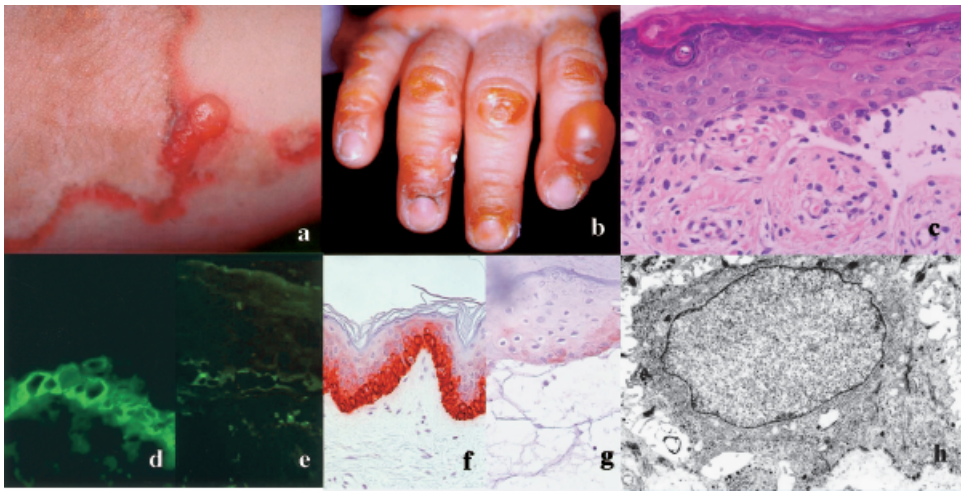

Figure 1. Clinical spectrum of the Japanese case. (*a*) Annular multiple circinate erythema with migration over the thigh of case 1 (age 1 y). (*b*) Multiple vesicles on the dorsal side of the left hand of case 1 (age 1 y). (*c*) A skin biopsy discloses an extensive split through the basal cell layer. (*d*) Normal control demonstrates strong staining with anti-K5 antibody (C50). (*e*) No reactivity to anti-K5 antibody (C50) in the epidermis of case 1 specimen. (*f*) Normal control demonstrates strong staining with anti-K5 antibody (RCK102). (*g*) Faint reactivity to anti-K5 antibody (RCK102) in the basal cells of case 1 specimen. (*h*) Electron microscopy of skin biopsy of case 1 discloses acantholysis of spinous cells and no aggregation of tonofilaments.

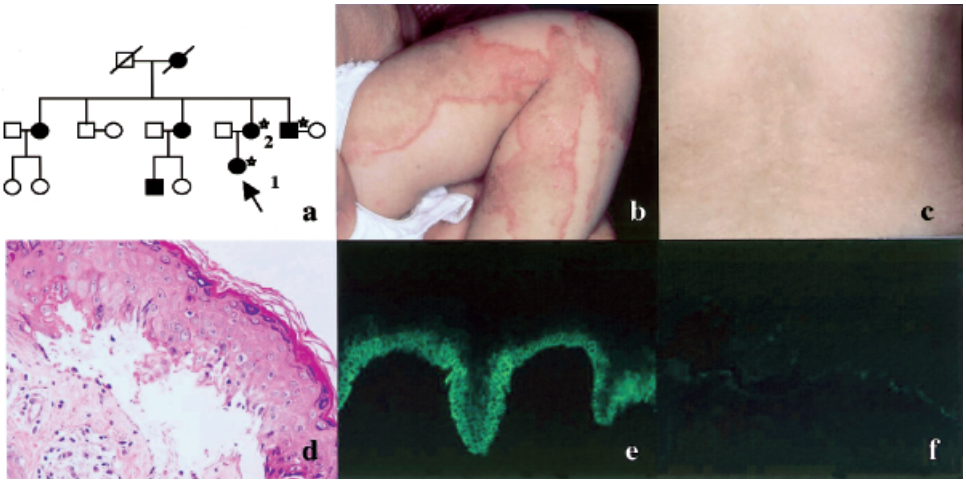

Figure 2. Clinical spectrum of the Korean family. (*a*) *Filled symbols* in the pedigree represent affected individuals. An *arrow* indicates the index patient. All of the affected individuals indicated by *asterisks* were found to contain the mutation. (*b*) Migratory circinate erythema and vesicles on the area affected by erythema over the thigh of individual 1 (age 1 y). (*c*) Generalized hypopigmented and hyperpigmented patches on the trunk and extremities of individual 2; all affected adult members in the family had similar pigmentary changes as her skin lesion. (*d*) A skin biopsy discloses an extensive split through the basal cell layer. (*e*) Normal control skin demonstrates strong staining against anti-K5 antibody (C50). (*f*) No reactivity to anti-K5 antibody (C50) in the epidermis of case 1 specimen.

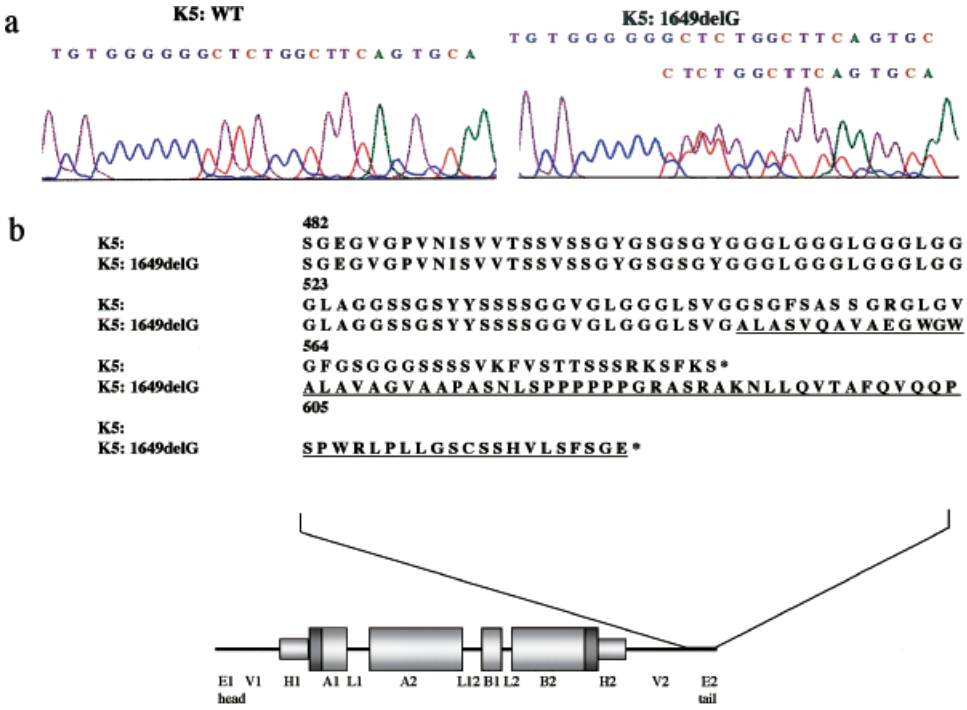

Figure 3. Mutation analysis of the EBS patients. (*a*) In the affected Japanese girl, sequence analysis of PCR fragments discloses a heterozygous deletion mutation (1649delG) in exon 9 of *KRT5*. This mutation leads to a frameshift and delayed termination codon. (*b*) Comparison of amino acid sequence of wild-type and mutant K5 spanning the V2 domain. Numbers denote the amino acid positions. The region affected by the mutation is indicated in the schematic drawing of K5.

sequence reaction, the PCR products were purified for sequencing using MicroSpin Columns (Amersham Pharmacia Biotech, Piscataway, NJ). In order to exclude the possibility of DNA polymerase slippage during PCR amplification, PCR was performed in triplicate for each sample. Sequence analysis was performed on an automated sequencer (ALOKA, Tokyo, Japan) using the primer-labeled method; the phage M13 (–21) forward and reverse primers were used as the universal labeled primer. The gene fragment carrying the 1649delG mutation was amplified with the following primers: forward, 5′-TGTAAAACGACGGCCAGTAAGGGGTCCAGTAGAGTGC-3′, and reverse, 5′-CAGGAAACAGCTATGACCATTTGACGCTGGAGCTGCTA-3′; the underlined tails represent the M13 forward and reverse sequences. Every reaction was repeated three times. A total of 50 control individuals were tested by the same methods.

RESULTS

Histology Hematoxylin and eosin staining of specimens obtained from small vesicles on the leg showed an extensive split through the basal layer of the epidermis (**Figs 1*c*, 2*d***). Electron microscopy revealed a marked reduction in the numbers of both desmosomes and keratin intermediate filaments in the basal cells, and no evidence of tonofilament clumping, resulting in acantholysis of the spinous cells immediately above the basal cell layer (**Fig 1*h***).

Immunohistochemistry showed extremely faint K5 staining in basal cells using the anti-K5 monoclonal antibody (RCK102), and no staining with monoclonal antibody C-50 in case 1 (**Fig 1*e*, *g***). Conversely, normal control skin yielded positive staining with both antibodies (**Fig 1*d*, *f***). In contrast, normal K14 staining in basal cells was found in both the control and patient skin samples (data not shown). These results suggest an underlying defect in the K5 molecule. The same results were observed in the Korean cases (**Fig 2*e*, *f***).

Mutation analysis This analysis revealed a single deletion mutation (1649delG) in exon 9 of the *KRT5* gene for all affected individuals. As other members in the Japanese family neither were affected by the same disorder nor had the same mutation, this deletion mutation appears to have occurred as a *de novo* event. Interestingly, the same mutation (1649delG) in exon 9 was observed in each of the affected Korean family members examined, i.e., the proband, her mother, and her uncle (**Fig 2*a***). No other mutations were detected in the entire *KRT5* and *KRT14* genes. The heterozygous deletion mutation (1649delG) in the K5 V2 domain of these patients is predicted to produce a mutant K5 with a frameshift of its terminal 41 amino acids, resulting in a protein 35 amino acids longer than the wild-type protein and caused by a delayed termination codon (**Fig 3**).

DISCUSSION

We examined two unrelated cases with EBS, i.e., an infant Japanese female and a Korean family. In each case, the patient had a phenotype that was less severe than EBS-DM but rather unusual, showing peculiar migratory circinate erythema and multiple vesicles on the area affected by the erythema (**Figs 1**, **2**). The lesions healed with brown pigmentations but not scars. Mucous membranes and nails were not affected. Although the patients have clinical findings resembling EBS-DM in terms of circular arrangement of vesicles, their clinical features of markedly circinate and prominent erythema with active migration were quite different from this classical EBS subtype. Electron microscopy findings also are distinct from those in EBS-DM, in which spherical clumpings of keratin filaments are seen. The diagnostic criteria of EBS-DM are the presence of herpetiform blisters and the finding of clumped tonofilaments on electron microscopy (Irvine and McLean, 1999). As such, we propose the terminology "EBS with migratory circinate erythema" to describe the present EBS subtype, as this accurately describes these findings. These unrelated patients in Japan and Korea with a unique type of EBS have a novel mutation in the keratin gene *KRT5*, i.e., a frameshift and delayed stop codon inconsistent with any subtype described previously.

Mutations in *KRT5* or *KRT14* that produce a termination codon positional change have been reported in several cases (Muller *et al*, 1999; Gu *et al*, 2002). The mutation reported here is the farthest downstream, however, creating a delayed termination codon not previously reported. It is interesting to note that an elongated mutant of another cornified envelope matrix protein interferes with nuclear functions of differentiating keratinocytes and causes Vohwinkel syndrome (Ishida-Yamamoto *et al*, 2000). Thus, the

elongated mutant K5 reported here might either interfere with the normal function of unaffected K5 or have effects on other matrix protein interactions. This may explain the negative results in immunohistochemistry for K5; i.e., the elongated tail region may mask the normal K5 epitope produced by heterozygous normal gene.

Although V2 mutation in K5 has never been reported, two different frameshift mutations were recently demonstrated in the V2 domain of K1 (Sprecher *et al*, 2001; Whittock *et al*, 2002). One was a very severe form of ichthyosis hystrix Curth–Macklin and the other was mild striate palmoplantar keratoderma. Their phenotypes were quite different due to the subtle difference in the number of glycine loops and only six amino acid residues (Whittock *et al*, 2002). These cases suggest that the faint defect in V2 of K1 might prohibit or interfere with the normal functional interaction between keratin intermediate filaments and other proteins, thus leading to the disease pathogenesis.

In the milder forms of EBS, the mutations occur outside the highly conserved helix boundary motifs, and filaments appear to be essentially normal on electron microscopy (McLean and Lane, 1995). Electron microscopy of skin biopsies in our case showed a reduction in the number of keratin intermediate filaments in the basal cells but no tonofilament clumpings (**Fig 1*h***). In addition, although K14 was detectable using immunohistochemistry, K5 expression was either faint or undetectable. It is possible that the frameshift mutation may interfere with normal protein folding due to the aberrant extended tail region. This also may explain why the present mutation, although occurring in a non-hot-spot and nonhelix region V2 domain, may interfere with the function of the protein. This elongated mutant keratin protein, having an abnormal 41 amino acid sequence and being 35 amino acids longer than wild-type K5 protein, is likely to interfere with functional interaction between keratin and associated proteins, leading to an atypical clinical phenotype, which has never been reported. This interpretation is quite feasible, as the same novel mutation of *KRT5* gene and the same unique clinical phenotype of migratory circinate erythema and vesicles were found in the two unrelated families in Japan and Korea. Additional detailed studies of the aberrant keratin protein in these cases must be completed to understand the critical function for the tail region of the keratin protein.

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