A Premature Stop Codon Mutation in the 2B Helix Termination Peptide of Keratin 5 in a German Epidermolysis Bullosa Simplex Dowling–Meara Case

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Epidermolysis bullosa simplex (EBS) is a group of hereditary skin disorders with mostly autosomal dominant inheritance, characterized by intraepidermal blister formation due to cytolysis of basal keratinocytes after minor mechanical stress. Blistering is more frequent in warm and humid weather and generally improves with advancing age. Epidermolysis bullosa herpetiformis Dowling–Meara (EBS-DM) is the most severe subtype of EBS, characterized by extensive blistering since birth with herpetiform marginal spreading and central healing, leaving light-brown postbullous pigmentations. Oral mucosal involvement, nail dystrophy, onychogryposis, formation of milia, and palmar-plantar hyperkeratosis are common features (Anton-Lamprecht and Schnyder, 1982; Fine et al, 1991; Gedde-Dahl and Anton-Lamprecht, 1996). In contrast to the milder forms of EBS, skin biopsies of EBS-DM reveal distinctive ultrastructural abnormalities: aggregation and clumping of basal keratins resulting in a total collapse of the keratin cytoskeleton of basal keratinocytes (Anton-Lamprecht and Schnyder, 1982; Anton-Lamprecht, 1983, 1992).

In 1991, Vassar et al (1991) showed that expression of a truncated K14 gene in transgenic mice led to clinical and morphologic findings resembling EBS-DM. Subsequent sequence analysis of keratin genes in EBS patients revealed that point mutations in the basal keratins K5 and K14 are clustered in distinct regions of the central rod domain (for review see Corden and McLean, 1996; Fuchs, 1996; Korge and Krieg, 1996; Paller, 1996). Almost all EBS-DM mutations are located in the highly conserved hydrophilic peptides of either the amino or the carboxy end of the central α-helical rod domain with the majority of mutations residing either in the 1A helix initiation peptide of K14 or in the 2B helix termination peptide of K5. Here we report a novel K5 mutation (E477stop, residue 93 of the 2B helix, the last residue of the K/LLEGE motif) in a patient showing the characteristic clinical and ultrastructural features of EBS-DM. The location of this K5 mutation is consistent with previous reports but also provides additional in vivo evidence that partial K/LLEGE truncation of K5 indeed disturbs normal K5/K14 keratin filament assembly, which is in agreement with the in vitro assembly studies by Wilson et al (1992).

**MATERIALS AND METHODS**

**Case report (Fig 1)** A 38 y old woman was affected since birth by generalized blister formation with herpetiform spreading and central healing, leaving faint brown postbullous pigmentations. Hemorrhagic blisters occurred on trunk and extremities including palms and soles, on the face, and on the oral mucosa after minor trauma. Some improvement was noted after the fourth year of life. Palmar and plantar hyperkeratoses were reported to have existed already before walking and often caused painful problems in periods of plantar blistering. Subungual blisters and irregular nail plate thickening were present, and the patient experienced recurrent generalized blistering even in adulthood. The diagnosis of the Dowling–Meara type was stated by electron microscopy of a skin biopsy sample at the age of 19 and confirmed clinically some time later on clinical inspection in Heidelberg. As no other family member is affected, she represents a spontaneous new DM mutation. Her son is unaffected.

**Electron microscopy** A 1 d old blister induced experimentally by friction by the patient, had been excised elsewhere for electron microscopy at the age of 19 y. The sample was subdivided prior to fixation, then fixed in phosphate-buffered 2.5% glutaraldehyde solution, dehydrated in graded ethanols, and embedded in Durcupan. Cured tissue blocks were later sent to Heidelberg for electron microscopic analysis and diagnosis. Ultrathin sections were investigated using a Philips EM 400 electron microscope.

**Mutation detection** Genomic DNA isolated from ethylenediamine tetraacetic acid blood samples was used to amplify exon 7 of KRT5 by polymerase chain reaction (Taq DNA polymerase, Boehringer, Mannheim, Germany). The primer set was 5’ CCC ACT CTC CTC CTT TCT...
ATC 3' and 5' CGC TTT ATC AAC TGA AGG CC 3'. A 50 μl reaction solution containing 250 ng DNA, 200 nM of each primer, 200 μM of each dNTP, 10 mM Tris–HCl, 1.25 U Taq DNA polymerase with optimized pH, MgCl2 and KCl concentrations were initially denatured at 94°C for 2 min, followed by 35 cycles of 1 min at 94°C, 1 min at 56°C and 1.5 min at 72°C, followed by a 7 min final extension step at 72°C. Direct sequencing of polymerase chain reaction products was preceded by shrimp alkaline phosphatase and exonuclease 1 treatment (Sequenase PCR product sequencing kit, USB/Amersham Life Science, Cleveland, OH) as described (Müller et al., 1998). An additional sequencing primer was 5' CAA GCA GGA CAT GGC CCG 3'.

RESULTS

Skin ultrastructure Advanced blister formation with partial or complete destruction of basal cells, small intrabasal blisters, and exocytosis of lymphocytes were seen in the blister area. Most affected basal cells were necrotic. In the upper dermis, increased numbers of small capillaries, a lymphohistiocytic infiltrate, and pigment-loaded melanophages reflected the intense inflammatory reaction to blister formation. In less destroyed areas and adjacent to the blister basal cells revealed round or irregular clumps and aggregation of their keratin filaments as a result of a collapse of the entire basal keratin network. Most of the clumps were distributed irregularly in the cytoplasm that otherwise was entirely normal, and only few of them were attached to desmosomes (Fig 2). Upper epidermal keratinocytes were normal apart from some unspecific high-level paranuclear vacuoles due to friction. Clumping of basal keratins is unique among EBS subtypes and pathognomonic of the DM type, thus permitting the diagnosis of EBS-DM for the patient.

Mutation analysis The entire H1, 1A, and 2B domains of keratin K5 and K14 were screened for potential mutations (Fig 3). The sequence analysis detected a G to T transition at base 2334 (EMBL accession code: M28496) in residue 93 of the 2B helix of KRT5 leading to an exchange of a glutamic acid to a stop codon at position 477 of K5 (2B rod domain position 93, the last residue of the K/LLEGE motif). This premature stop codon results in a truncated K5 protein lacking the last four amino acid residues of the helix termination peptide (residues 477–480) and the entire tail domain. The parents, the two sisters, and the son of the patient, who were all clinically unaffected, did not show the mutation and neither did 23 normal nonrelated controls.

DISCUSSION

EBS-DM mutations are generally located in the highly conserved hydrophilic residues of either end of the central α-helix, mainly in the helix termination peptide of K5 or in the helix initiation peptide of K14. Previously, two stop codon mutations were also reported in the 1A/1B region of K14 in two patients with autosomal recessive EBS (Chan et al., 1994; Rugg et al., 1994). In these mutations the truncated K14 proteins as well as the corresponding mRNA are obviously unstable, which results in a functional K14 knockout in homozygous individuals. In heterozygous individuals the defects can be compensated by the remaining unaffected K14 gene. In contrast, the stop codon mutation in K5 found in our autosomal dominant case most probably leads to a stable truncated protein which participated in intermediate filament formation. This notion is supported by our electron microscopic data, revealing in our patient the basal clumping of keratins which is characteristic for EBS-DM, while basal keratinos were missing in both cases of K14. How the defective keratins impair normal keratin intermediate filament (KIF) stability is still speculative. Several lines of experimental evidence suggest that the mutation described here will alter
various stages of KIF formation and help to understand the rather severe clinical phenotype of our patient.

The basic feature of intermediate filaments including keratins is the periodicity of heptad repeats in the central α-helix, which allows to form a coiled-coil dimer (Cohen and Parry, 1986). The residues in the a and d position are mostly occupied by apolar amino acids which form a hydrophobic core between interacting coiled-coil molecules. Oppositely charged residues in the c and g position are able to stabilize the coiled-coil by forming salt bridges (Conway and Parry, 1990). Site-directed mutagenesis of the d position at the end of the 2B rod domain of K8/K18 caused the heptad structure to resolve and perturbed heterodimer formation in vitro (Hatzfeld and Weber, 1990). Based on these observations the occurrence of a premature stop codon mutation in the d position of the K5 helix termination peptide as reported here is therefore likely to destabilize the formation of coiled-coil heterodimers.

Further experimental support for this notion was given by Hatzfeld and Weber (1991) in using a competing synthetic peptide representing the K/LEgE consensus sequence; this caused a loss of assembly of preformed KIF in vitro suggesting that this motif may be crucial for alignment of tetramers during elongation and protofibril formation. Additional in vitro assembly studies by Wilson et al. (1992), who paired wild-type K14 chains with mutant K5 chains lacking the entire tail domain in addition to the K/LEgE motif resulted in substantially fewer, shorter, and thicker filaments compared with wild-type filaments. It remains to be discussed, to what extent the additional absence of the H2 domain in our patient contributes to the severity of the disease phenotype.

This aspect was partly addressed in in vitro assembly studies with mutant truncated K5 chains lacking the entire tail domain plus the last residue of the 2B rod domain: electron micrographs revealed significantly shorter than normal filaments which had the tendency to associate laterally into cables of two or three intertwined filaments (Wilson et al., 1992). The fact that the amino acid sequence of the H2 peptide has also been highly conserved might implicate an additional obligatory role of the H2 peptide in KIF assembly and stabilization.

The present model of KIF structure postulates linear arrays of keratin heterodimers (Steinert et al., 1993). The beginning of the 1A rod domain of one dimer is connected by a 10–11 residue head-to-tail overlap to the 2B end of the next dimer in line. These overlap regions correspond exactly to those locations where keratin mutations occur in EBS-DM patients. Therefore, the model might explain the high evolutionary conservation of these sequences at both ends of the rod domain in all intermediate filaments and the deleterious effect of these mutations for higher order filament formation, elongation, and stability.

Transgenic mice expressing a truncated K14 with lack of the last third of the C-terminal rod domain produce a severe EBS-DM phenotype leading to early neonatal death (Vassar et al., 1991). The K5 truncation at the very end of the C-terminal rod in our case is nonlethal in humans but may explain the rather severe clinical phenotype with lack of improvement with advancing age.

The mutation reported here affects the same codon as a previously described recurrent point mutation (G/A2334 substitution, E477K) in sporadic EBS-DM cases (Stephens et al., 1997) who presented with the typical clinical EBS-DM phenotype and keratin clumping in basal keratinocytes. The frequency of sporadic mutations at this position is likely due to a common mechanism for human mutagenesis involving spontaneous deamination of a methylated cytidine residue in a Cpg dinucleotide (Cooper and Vousoufian, 1988). The Cpg site in nucleotide position 2333/2334 of KRT5 can therefore be considered as a mutational hot spot for EBS-DM. This common mechanism gets further support from several mutations in the corresponding residue of keratin 2e in ichthyosis bullosa of Siemens (Kremer et al., 1994; McLean et al., 1994; Rothnagel et al., 1994; Jones et al., 1997) and of the basic hair keratins 1 and 6 in monilethrix (Winter et al., 1997a; 1997b; Korge et al., 1998).

It will be of interest for the future whether corresponding mutations can be found in other disorders caused by keratin defects.

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