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der Albert-Ludwigs-Universität Freiburg i.Br.

# Genetic and biological characterization of keratin 5 and 14 mutations in EBS

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# Contents

# Abbreviations

| | |
|---|---|
| BMZ | basement membrane zone |
| DEBS | dominant epidermolysis bullosa simplex |
| EBS | epidermolysis bullosa simplex |
| EBS-DM | epidermolysis bullosa simplex Dowling-Meara |
| EBS-K | epidermolysis bullosa simplex Koebner |
| EBS-MP | epidermolysis bullosa simplex with mottled pigmentation |
| EBS-WC | epidermolysis bullosa simplex Weber-Cockayne |
| HIP | helix initiation peptide |
| HTP | helix termination peptide |
| IIF | indirect immunofluorescence |
| IFs | intermediate filaments |
| K5 | keratin 5 protein |
| K14 | keratin 14 protein |
| KIF | keratin intermediate filament |
| *KRT* | Keratin gene |
| *KRT5* and *KRT14* | genes encoding for K5 and K14, respectively |
| PCR | polymerase chain reaction |
| PTC | premature termination codon |
| REBS | recessive epidermolysis bullosa simplex |

# 1. Abstract – Zusammenfassung

## Abstract

Epidermolysis bullosa simplex (EBS) represents a heterogeneous group of inherited blistering diseases with epidermal cytolysis due to fragility of the basal layer of epidermal keratinocytes. EBS has a predominantly autosomal dominant mode of inheritance. The clinical spectrum of EBS ranges from relatively mild blistering of the hands and feet, to generalized blistering, which can be fatal. Keratins are obligate heterodimer proteins that form the intermediate filament cytoskeleton of all epithelial cells. Genetic defects in the epidermal expression of keratins are known to form the basis of EBS. It results from mutations in either the keratin 5 (*KRT5*) or keratin 14 (*KRT14*) gene, which consist of 9 and 8 exons, respectively. We performed mutation analysis and indirect immunofluorescence (IIF) staining of the skin in 20 patients with the clinical diagnosis of EBS to determine the molecular basis of the disease. Genomic DNA was extracted from peripheral blood leukocytes, then amplified by PCR followed by restriction enzyme analysis, direct automated DNA sequencing and analysis with sequence analysis software. IIF of skin cryosections was performed with antibodies to keratin 5 and keratin 14. In this study 18 different mutations were identified, 10 of them novel. We found two novel homozygous *KRT14* nonsense mutations in 2 patients with recessive EBS. We observed certain correlations between the genotype and the phenotype in our patients, but the results also show that it is not possible to predict the EBS phenotype only based on the location of the mutation. Finally, we discuss possible implications of the novel mutations on protein structure and summarize the spectrum of mutations reported so far in EBS. These findings should be helpful for genetic counselling and may contribute to the spectrum of the EBS mutation database and pave the way for gene therapy.

## Zusammenfassung

Epidermolysis bullosa simplex (EBS) ist eine heterogene Gruppe vererbter, blasenbildender Erkrankungen, mit epidermaler Zytolyse aufgrund von Fragilität der basalen Schicht der Keratinozyten. EBS wird vorwiegend autosomal dominant vererbt. Das klinische Spektrum der EBS reicht von relativ milden Blasen an Händen und Füßen zu generalisierter Blasenbildung. Keratine sind obligate heterodimere Proteine, die das intermediäre Filament-Zytoskelet der epithelialen Zellen bilden. Genetische Defekte der epidermalen Expression von Keratinen sind die Ursache von EBS. Sie resultiert aus Mutationen im Keratin 5 (*KRT5*) oder Keratin 14 (*KRT14*) Gen, welche aus 9 bzw. 8 Exonen bestehen. Wir führten Mutationsanalyse und Immunfluoreszenz – Färbung (IIF) in 20 Patienten mit klinisch diagnostizierter EBS durch, um die molekulare Grundlage der Erkrankung zu ermitteln. Genomische DNA wurde aus peripheren Blut-Leukozyten isoliert und dann mittels PCR amplifiziert, mit anschließender Restriktionsenzym Verdauung, direkter automatisierter DNA-Sequenzierung und Analyse. IIF von Haut Kryoschnitten wurde mit Antikörpern gegen Keratin 5 und Keratin 14 durchgeführt. In dieser Studie identifizierten wir 18 verschiedene Mutationen, 10 davon waren bisher unpubliziert. Wir fanden zwei unpublizierte, homozygote Nonsense-Mutationen in *KRT14* in zwei Patienten mit rezessiver EBS. Wir erkannten Genotyp-Phänotyp-Korrelationen bei unseren Patienten, stellten aber auch fest, dass es nicht möglich ist, den EBS-Phänotyp nur anhand des Ortes der Mutation vorherzusagen. Zuletzt diskutieren wir mögliche Auswirkungen der unpublizierten Mutationen auf Proteinebene und fassen das Spektrum bisher bekannter EBS-Mutationen zusammen. Diese Erkenntnisse sind für die genetische Beratung hilfreich und erweitern die EBS - Mutationsdatenbank. Weiterhin bahnen sie den Weg für eine mögliche Gentherapie.

# 2. Introduction

## 2.1. Keratins and keratin filaments

The cytoskeleton of all multicellular organism cells consists of three abundant filament systems which play important roles in the organization, mechanical integrity and strength of cells: actin microfilaments (MFs), intermediate filaments (IFs), and interconnected microtubules (MTs). Each filament system is built from a family of proteins with tissue- or cell-specific regulation of expression, and each protein family is encoded by the corresponding gene family.
The term “intermediate filaments” comes from their diameter of 10-12 nm, between that of MTs (25 nm) and MFs (7-10 nm). IFs are by far the most complex of the cytoskeletal proteins with at least 60 different IF proteins. IFs are subcategorized into six broad types based on tissue-specific expression, sequence similarity and protein structure (Table 1).

Table 1. IF proteins and their distribution

| Class | Protein | Distribution |
|---|---|---|
| I | Acidic keratins | Epithelia K9-K23<br>Trichocyte keratins Ha1-Ha9<br>Inner root sheath of hair follicles K6irs1- K6irs4 |
| II | Basic / Neutral keratins | Epithelia K1-K8<br>Trichocyte keratins Hb1-Hb6<br>IRSa1-IRSa3 |
| III | Desmin<br>Vimentin<br>Glial fibrillary acidic protein<br>Peripherin | Muscle<br>Mesenchyme<br>Astrocytes and glia<br>Peripheral neurons |
| IV | Neurofilaments | Neurons |
| V | Nuclear lamins | Nuclear envelope |
| VI | Nestin | Neuroepithelial stem cells |

source: http://www.ebi.ac.uk/2can/disease/keratins/keratins2.html

Several human disorders are caused by defects in genes which encode IF proteins. Lesion pathogenesis in these diseases is based on fragility of the cells or tissues, reflecting the major function of structural frame fulfilled by IFs which are located in the cytoplasm and nucleus.

Keratins make up a large multigene family, representing three-quarters of known IFs in humans [14, 9]. They form an intricate cytoskeletal network structure that withstands mechanical, chemical, and compression stress.

Keratins range in size between 40 and 67 kDa, and can be divided into acidic (pH 4.5~5.5) and neutral - basic (pH 6.5~7.5) subunits, namely, type I and type II keratins (Table 2). Type I keratins are low molecular weight keratins, and the corresponding genes are located on 2 loci of chromosome 17 (17p12 and 17q12-q21). Type II keratins have high molecular weight, and the genes are located on chromosome 12 (12q11-q13). Type II keratins are generally longer than type I, with the helix being extended by two additional homology subdomains H1 and H2, and the variable regions in the head and tail domains are also longer.

Table 2. Classification of keratins [45]

| **Category** | **Number range** |
|---|---|
| Human type I epithelial keratins | 9-28 |
| Human type I hair keratins | 31-40 |
| Nonhuman type I epithelial and hair keratins | 41-70 |
| Human type II epithelial keratins | 1-8 and 71-80 |
| Human type II hair keratins | 81-86 |
| Nonhuman type II epithelial and hair keratins | 87-120 |
| Type II keratin pseudogene | 121-220 |
| Type I keratin pseudogene | 221→ |

Keratin 5 (K5) belongs to type II keratins, whereas keratin 14 (K14) belongs to type I keratins. In the epidermis, all undifferentiated keratinocytes in the basal layer express K5 and K14 [23]. The keratin 5 gene, *KRT5,* comprises 9 exons, and the genomic length is approximately 5.88 kb. The gene coding for keratin 14 (*KRT14*) consists of 8 exons, and the genomic DNA spans approximately 4.61 kb.

Similar to all IFs, keratins share a head-rod-tail structure. This basic homologous structure consists of a central rod domain with about 310 amino acids, predicted to be α -helical, with an amino terminal “head” and a carboxy terminal “tail”. The rod domain can be further divided into four helical segments (1A, 1B, 2A and 2B), interrupted by three short non-helical flexible linkers (L1, L12 and L2). The terminal regions are usually split into variable (V), end (E) and homologous (H) domains (Figure 1). Type II keratins, such as K5, have an “ISIS motif” within V1, which has been implicated in mediating attachment of keratins to desmosomes.

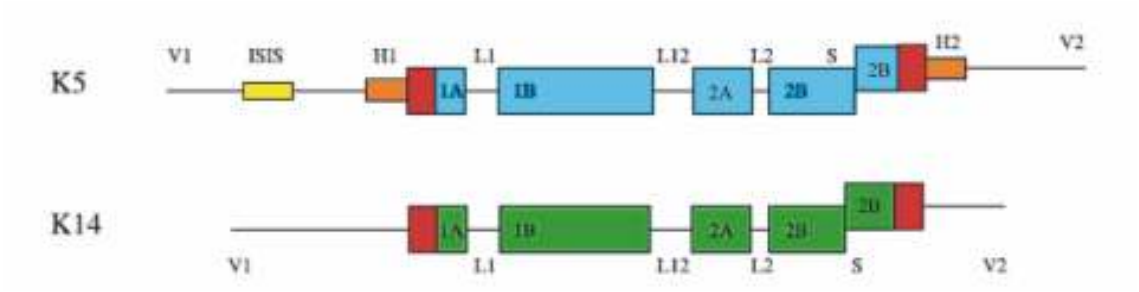


**Figure 1. Schematic representation of the K5 and K14 domains [36].** Blue, respectively green boxes represent the helical fragments (1A, 1B, 2A, 2B), L1, L12, L2 represent the linker regions. Red boxes represent the helix initiation peptide or motif and the helix termination peptide. V1 and V2 are the variable terminal regions. In addition, type II keratins have conserved homology domains H1 and H2; K5 have an 'ISIS motif' within V1, which has been implicated in mediating attachment of keratins to desmosomes.

The α-helical domain reveals seven residue heptad repeat linear arrays ($\underline{a}$-b-c-$\underline{d}$-e-f-g)$_n$. The first and fourth amino acid positions (*a* and *d*) are preferentially occupied by small nonpolar residues like Leu, Ile, Met or Val [53]. In an aqueous environment, *a* and *d* positions provide a hydrophobic seal on the helical surface [12]. Two or more such helices would aggregate to hide the nonpolar residues from water by coiling around each other to form a coiled-coil fold. The keratin chains are further stabilized by hydrophobic residues at the *a* and *d* positions of the heptad [10].

The most conserved helix boundary fractions are two short sequences at the start and end of the rod domain [15;27] the helix initiation peptide or motif (HIP) and the helix termination peptide (HTP) that are critical for polymerization (Figure 1). In addition, a discontinuity in the heptad repeat called the "stutter", the helix inversion feature, may locally alter the character of helix. The "stutter" region near the middle of the 2B rod domain might play specific roles in the elongation of keratins [60].

One type I and one type II keratin form a heterodimer, through coiled-coil interaction of their rod domains [32] (Figure 2). Although *in vitro* some of the type I polypeptides can be induced to associate with any of the type II ones, the composition of the keratin pairs found *in vivo* is highly specific. Within the basal keratinocytes, filaments are composed of K14 and K5, whereas in the suprabasal layers, cells arrest synthesis of K14 and K5, and activate K10, K1 and eventually K2e expression as they leave the basal layer and move to the skin surface [13].

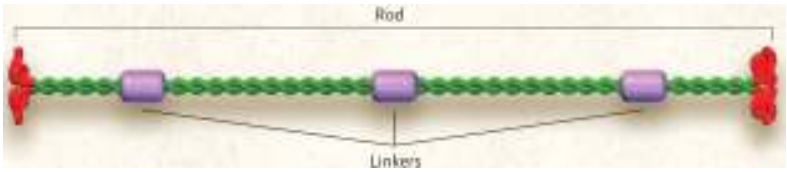


**Figure 2. Schematic representation of a keratin heterodimer [39].** Each intermediate filament molecule consists of a central, α-helical coiled-coil rod domain (green), 310 to 352 amino acids in size that is interrupted by linker regions (purple). The rod domain begins and ends with highly conserved sequence motifs consisting of 8 to 12 amino acids (red) that, when mutated, result in the most severe phenotypes.

Heterodimers associate side-by-side in an antiparallel and staggered mode to form a stable tetramer [13]. These subunits are linked in head to tail mode to produce a protofilament. A group of two protofilaments intertwine to form protofibril. Finally, four protofibrils coil around each other to generate an intact keratin intermediate filament (KIF) (Figure 3).

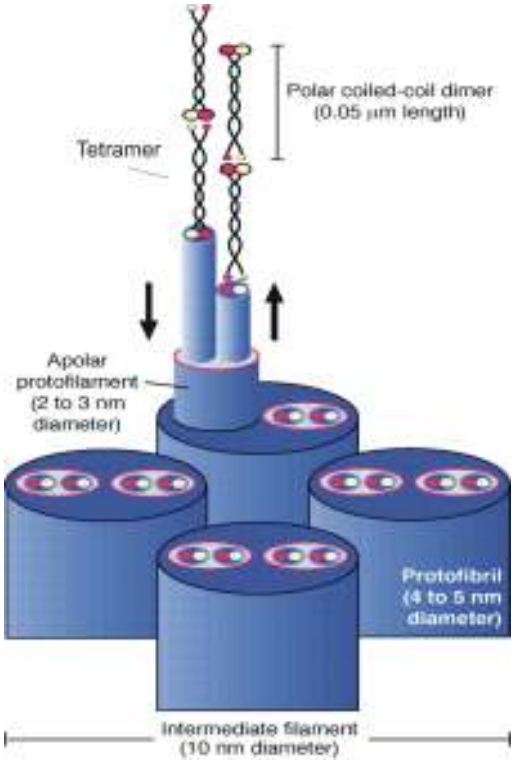


**Figure 3. Structural model of a KIF [13].** Heterodimers associate to form a stable tetramer. This heterodimers associate side-by-side and form a stable tetramer. These subunits built protofilaments and then protofibrils. Finally, four protofibrils build a keratin intermediate filament. KIFs are attached to specialized proteins involved in anchoring the cell to either the basal membrane, the hemidesmosomes, or to an adjacent epidermal cell, the desmosomes.

## 2.2. Keratin mutations and epidermolysis bullosa simplex

The KIFs form an extensive cytoskeleton within the epidermal keratinocytes, to protect the human body from environmental changes. When the synthesis of a keratin protein is defective, the structural integrity of the affected keratinocytes is injured. The keratinocytes become fragile and prone to rupture under stress. Mutations in keratins often impair KIF stability; consequently, this results in tissue breakdown. In the early 1990s [27], mutations in the basal keratinocyte keratins K5 or K14 were shown to be causative in the human blistering disorder, epidermolysis bullosa simplex (EBS), and since then mutations in keratin genes have been identified as the cause of many different disorders (http://www.interfil.org/diseasesTypeInII.php).

Epidermolysis bullosa (EB) refers to a heterogeneous group of skin disorders, characterized by increased fragility of skin and mucous membranes, resulting in blisters and erosion after minimal or no trauma. Depending on the level of the tissue separation within the cutaneous basement membrane zone (BMZ), as determined by transmission electron microscopy and/or immunofluorescence antigen mapping [1;54], EB can be further categorized into three subtypes: epidermolysis bullosa simplex, junctional epidermolysis bullosa (JEB), dystrophic epidermolysis bullosa (DEB) [1;54]. In EBS, blistering at the level of basal keratinocytes is caused by mutations in either the keratin 5 or keratin 14 gene. In JEB, the cleavage level is within the lamina lucida of the BMZ, and is caused by defects in laminin-332, collagen XVII, or α6β4 integrin [1;46]. DEB, characterized by cleavage underneath the BMZ, is caused by mutations in the gene coding for collagen VII [1], the major component of the anchoring fibrils that attach the epidermis to dermis.

EBS is a genetic disease with predominantly autosomal dominant inheritance. It is the most common subtype of EB, with an estimated prevalence of approximately 1 in 30,000-50,000 of the population [59]. The prevalence of EBS in 2001 was determined as 33.2 cases per million of the population, and the incidence during 1960-1999 was 34.4 per million live births in a Scottish study (http://www.medscape.com/viewarticle/499497). The National Epidermolysis Bullosa Registry (NEBR) reported 4.6 cases per million of the population and 10.75 per million live births in the USA [43].

The basal layer of the epidermis and the mucosal epithelia are fragile, leading to intra-epidermal blisters. EBS is considered a non-scarring form of EB, but secondary infection on trauma may cause scarring. Patients present with varying severity. Depending on the clinical criteria, EBS

has been subdivided into four major phenotypic presentations: EBS Dowling-Meara (EBS-DM; OMIM 131760), EBS Koebner (EBS-K; OMIM 131900), Weber-Cockayne (EBS-WC; OMIM 131800) and EBS with mottled pigmentation (EBS-MP; OMIM 131960) (Table 3). The three most common subtypes of EBS were encountered in the group of Scottish patients as follows: EBS-WC represented 42%, EBS-K 53% and EBS-DM 5% [21].

Table 3. Clinical features of the four major EBS subtypes

| **EBS Subtype** | | | **Weber-Cokayne** | **Koebner** | **Dowling-Meara** | **Mottled Pigmentation** |
|---|---|---|---|---|---|---|
| **Age of Onset** | | | Usually ~12-18 months | Birth/infancy | Birth | Birth/infancy |
| **Clinical Feature** | **Blisters** | **Distribution** | Usually limited to hands, feet; can occur at sites of repeated trauma (e.g., belt line) | Generalized | Generalized | Generalized |
| | | **Grouped (herpetiform)** | No | No | Yes | Sometimes |
| | | **Hemorrhagic** | Rare | Occasionally | Yes | No information |
| | | **Mucosal** | No | Occasionally | Often | Occasionally |
| | **Progressive Hyperkeratosis of Palms and Soles** | | Occasionally | Occasionally | Yes | Yes |
| | **Nail Involvement** | | Occasionally | Occasionally | Common | Variable |
| | **Milia** | | Rare | Occasionally | Common | No information |
| | **Hyper/ Hypopigmentation** | | No | Can occur | Common | Always |

source: http://www.geneclinics.org/profiles/ebs

EBS-WC is the mildest, most common subtype, in which blisters are usually restricted to the hands and feet. Onset is delayed until the child begins to walk. The mutations are located in the K14 L12 linker region (e.g. p.D273G), the K5 L12 linker (e.g. p.M327K and p.D328H), and the H1 domain of K5 (e.g. p.P156L) (Figure 4, Tables 4 and 5). The above mutations in mildly affected patients underline the relevance of the keratin linker regions for the EBS Weber-Cockayne phenotype and keratin filament integrity [38]. For example, affected members of two unrelated families with Weber-Cockayne EBS had a T>G point mutation in the second base position of codon 161 of one of two K5 alleles, leading to an Ile>Ser mutation. The mutation generates a potential substrate site for protein kinase C, which could influence intermediate filament architecture, perhaps leading to the intrafilament association seen ultrastructurally in patients with the mutation [4].

The EBS-K subtype is intermediate in severity, characterized by a generalized distribution of blisters, which may present at birth or shortly after birth. Mutations are distributed in a more diffuse manner within the rod domain and in the L12 linker of both keratin genes (Figure 4, Tables 4 and 5).

EBS-DM is regarded as the most severe subtype of EBS. Onset is usually at birth. Blistering is generalized, annular or herpetiform, and often involves the mucous membranes. Some individuals manifest palmoplantar hyperkeratosis, dystrophy of nails, and oral erosions. As with other forms of EBS, blistering tends to improve with age, but the palmoplantar hyperkeratosis tends to worsen. The *KRT5* or *KRT14* mutations in this disease usually cause amino acid substitutions within HIP or HTP (Figure 4, Tables 4 and 5). In a patient with an EBS Dowling-Meara phenotype with severe palmoplantar hyperkeratosis a heterozygous *KRT14* mutation that changes the predicted amino acid at the start of the helix initiation motif, K14 p.M119T was disclosed. This demonstrates that the three major types of EBS can arise from missense mutations in the same codon [47]. The major ultrastructural feature distinguishing EBS-DM from other subtypes is clumping of tonofilaments within basal keratinocytes [59].

EBS-MP may present at birth or shortly thereafter. Progressive brown pigmentation interspersed with depigmented spots, different from post-inflammatory hyper- and hypo-pigmentation, distinct pigmentary changes of the trunk and extremities [48]; (http://www.geneclinics.org/profiles/ebs/). Affected members of two seemingly unrelated families (Swedish and German) with EBS-MP had a C to T point mutation in the second base position of codon 25 of one of two K5 alleles, converting Pro to Leu. This part of the K5 head domain is likely to protrude on the filament surface, perhaps leading to additional aberrations in intermediate filament architecture and/or in melanosome distribution that are seen ultrastructurally in patients with the mutation [56]. A Japanese family with two members affected with EBS-MP, also showed the P25L mutation of *KRT5*, and the authors suggested that this mutation has an effect on filament structure and integrity [16]. Hamada *et al.* detected that P25L mutation in one family initially diagnosed as having EBS-K; the proband started to develop hyperpigmented spots 9 months later and gradually the clinical picture became typical for EBS-MP [17]. Electron microscopy of the pigmented spots demonstrated vacuolization of basal cells and incontinence of pigmentation. Other mutations causing an EBS-MP phenotype are: a heterozygous deletion of a guanine nucleotide in *KRT5* exon 9 at position 1649 [20] and a heterozygous T>C transition at position 356 of *KRT14*. The latter affects a highly conserved residue playing an important role during KIF formation. However, not all patients, heterozygous for M119T have mottled pigmentation, which indicates that this genetic defect is not sufficient in itself to cause epidermal pigmentary changes [18].

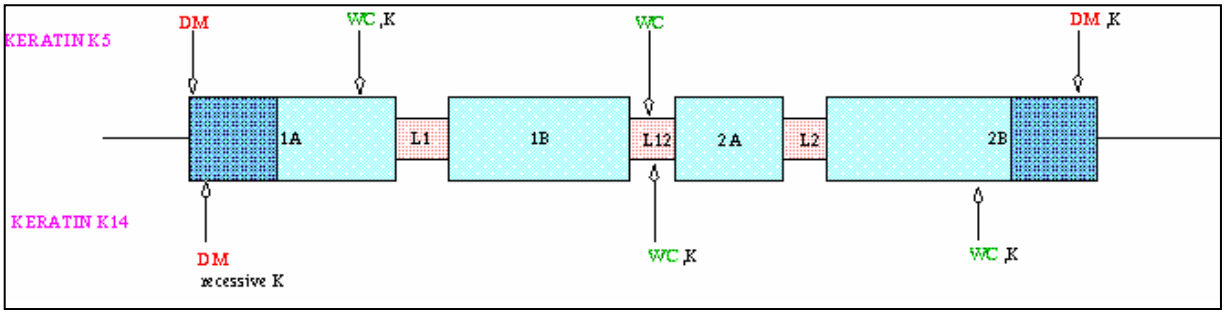


**Figure 4. Genotype – phenotype correlations in EBS**. Schematic representation of a keratin molecule and of the most frequent mutations causing the Weber-Cockayne (WC), Koebner (K) and Dowling-Meara (DM) EBS subtypes. (http://www.biology.iupui.edu/biocourses/Biol540H/EBroberts.html)

Table 4. Published *KRT5* mutations

| No. | Codon position | Nucleotide | Amino acid conversion | Phenotype | Reference |
|---|---|---|---|---|---|
| 1. | 25 | CCG-CTG | Pro → Leu | EBS-MP | Uttam (1996) |
| 2. | 152 | CCC-CTC | Pro → Leu | EBS-WC | Müller (1998) |
| 3. | 161 | ATC-AAC | Ile → Asn | EBS-WC | Müller (1998) |
| 4. | 161 | ATC-AGC | Ile → Ser | EBS-WC | Chan (1993) |
| 5. | 167 | GAG-AAG | Glu → Lys | EBS-WC | Ciubotaru (2003) |
| 6. | 168 | GAG-AAG | Glu → Lys | EBS-DM | Müller (2006) |
| 7. | 169 | CGC-CCC | Arg → Pro | EBS-DM | Müller (2006) |
| 8. | 170 | GAG-AAG | Glu → Lys | EBS-K | Yasukawa (2002) |
| 9. | 170 | GAG-GGG | Glu → Gly | EBS | Rugg (2007) |
| 10. | 173 | AAG-AAC | Lys → Asn | EBS-K | Stephens (1995) |
| 11. | 175 | CTC-TTC | Leu → Phe | EBS-DM | Nomura (1996) |
| 12. | 176 | AAC-AGC | Asn → Ser | EBS-DM | Stephens (1997) |
| 13. | 177 | AAT-AGT | Asn → Ser | EBS-WC | Liovic (2004) |
| 14. | 179 | TTT-TCT | Phe → Ser | EBS-DM | Stephens (1997) |
| 15. | 181 | TCC-CCC | Ser → Pro | EBS-DM | Shemanko (2000) |
| 16. | 183 | ATC-TTC | Ile → Phe | EBS-DM | Pfendner (2003) |
| 17. | 186 | GTG-TTG | Val → Leu | EBS-K | Liovic (2001) |
| 18. | 190 | GAG-AAG | Glu → Lys | EBS-WC | Müller (2006) |
| 19. | 193 | AAC-AAG | Asn → Lys | EBS-WC | Humphries (1996) |
| 20. | 199 | AAG-ACG | Lys → Thr | EBS-WC | Xu (2004) |
| 21. | 199 | AAG-AGG | Lys → Arg | EBS-WC | Abu Sa'd (2006) |
| 22. | 311 | CTG-CCG | Leu → Pro | EBS-WC | Ciubotaru (2003) |
| 23. | 323 | GTG-GCG | Val → Ala | EBS-K | Galligan (1998) |
| 24. | 324 | GTC-GAC | Val → Asp | EBS-WC | Ciubotaru (2003) |
| 25. | 325 | CTC-CCC | Leu → Pro | EBS-K | Sorensen (1999) |
| 26. | 327 | ATG-AAG | Met → Lys | EBS-WC | Muller (1998) |
| 27. | 327 | ATG-ACG | Met → Thr | EBS-WC | Chan (1994) |
| 28. | 328 | GAC-GGC | Asp → Gly | EBS-WC | Li (2004) |
| 29. | 328 | GAC-GTC | Asp → Val | EBS-WC | Matsuki (1995) |
| 30. | 328 | GAC-GAA | Asp → Glu | EBS-WC | Liovic (2000) |
| 31. | 328 | GAC-CAC | Asp → His | EBS-WC | Muller (1998) |
| 32. | 329 | AAC-AAA | Asn → Lys | EBS-WC | Chan (1994) |
| 33. | 329 | AAC-AGC | Asn → Ser | EBS | Rugg (2007) |
| 34. | 331 | CGC-TGC | Arg → Cys | EBS-WC | Rugg (1993) |
| 35. | 331 | CGC-CAC | Arg → His | EBS-WC | Müller (2006) |
| 36. | 404 | AAG-GAG | Lys → Glu | EBS-WC | Schuilenga-Hut (2003) |
| 37. | 418 | GAG-AAG | Glu → Lys | EBS-K | Yasukawa (2002) |
| 38. | 428 | GCC-GTC | Ala → Val | EBS | Rugg (2007) |
| 39. | 438 | GCC-GAC | Ala → Asp | EBS-WC | Schuilenga-Hut (2003) |
| 40. | 463 | CTG-CCG | Leu → Pro | EBS-K | Dong (1993) |
| 41. | 467 | ATC-ACC | Ile → Thr | EBS-DM | Irvine (1997) |
| 42. | 467 | ATC-CTC | Ile → Leu | EBS-WC | Rugg (2007) |
| 43. | 469 | ACT-CCT | Thr → Pro | EBS-DM | Müller (2006) |
| 44. | 472 | AAG-TAG | Lys → Term | EBS-DM | Livingston (2001) |
| 45. | 475 | GAG-GGG | Glu → Gly | EBS-DM | Lane (1992) |
| 46. | 475 | GAG-AAG | Glu → Lys | EBS-DM | Schuilenga-Hut (2003) |

| | | | | | |
|---|---|---|---|---|---|
| 47. | 476 | GGC-GAC | Gly → Asp | EBS-WC | Abu Sa'd (2006) |
| 48. | 477 | GAG-AAG | Glu → Lys | EBS-DM | Stephens (1997) |
| 49. | 477 | GAG-TAG | Glu → Term | EBS-DM | Müller (1999) |

| No. | Position | Mutation | Type | Phenotype | Reference |
|---|---|---|---|---|---|
| 50. | IVS1+1 | G>A | splice site | EBS-DM | Rugg (1999) |
| 51. | IVS1-1 | G>C | splice site | EBS-K | Schuilenga-Hut (2003) |
| 52. | 428 | del15 | in-frame deletion | EBS-DM | Kemp (2005) |
| 53. | 544 | delG | out of frame deletion | EBS-WC | Sprecher (2003) |
| 54. | 548 | delG | out of frame deletion | EBS | Gu (2003) |

*****Source: www.hgmd.cf.ac.uk, with modifications

Table 5. Published *KRT14* mutations

| No. | Codon position | Nucleotide | Amino acid conversion | Phenotype | Reference |
|---|---|---|---|---|---|
| 1. | 116 | AAG-AAC | Lys → Asn | EBS-WC | Sorensen (1999) |
| 2. | 116 | AAG-GAG | Lys → Glu | EBS | Rugg (2007) |
| 3. | 119 | ATG-ACG | Met → Thr | EBS-DM | Shemanko (1998) |
| 4. | 119 | ATG-ACG | Met → Thr | EBS-MP | Harel (2006) |
| 5. | 119 | ATG-ATA | Met → Ile | EBS-WC | Chen (1995) |
| 6. | 119 | ATG-GTG | Met → Val | EBS-K | Cummins (2001) |
| 7. | 120 | CAG-CGG | Gln → Arg | EBS-DM | Chen (1995) |
| 8. | 122 | CTC-TTC | Leu → Phe | EBS-K | Yamanishi (1994) |
| 9. | 123 | AAT-AGT | Asn → Ser | EBS-DM | Sorensen (1999) |
| 10. | 123 | AAT-AAA | Asn → Lys | EBS-DM | Müller (2006) |
| 11. | 125 | CGC-AGC | Arg → Ser | EBS-DM | Chen (1995) |
| 12. | 125 | CGC-TGC | Arg → Cys | EBS-DM | Coulombe (1991) |
| 13. | 125 | CGC-CAC | Arg → His | EBS-DM | Coulombe (1991) |
| 14. | 125 | CGC-CCC | Arg → Pro | EBS-DM | Morley (2003) |
| 15. | 129 | TAC-GAC | Tyr → Asp | EBS-DM | Chan (1996) |
| 16. | 129 | TAC-TGC | Tyr → Cys | EBS-DM; -K | Rugg (2007) |
| 17. | 130 | CTG-CCG | Leu → Pro | EBS-DM | Schuilenga-Hut (2003) |
| 18. | 133 | GTG-ATG | Val → Met | EBS-WC | Hamada (2005) |
| 19. | 133 | GTG-GCG | Val → Ala | EBS | Rugg (2007) |
| 20. | 133 | GTG-TTG | Val →Leu | EBS-K | Müller (2006) |
| 21. | 134 | CGT-CCT | Arg → Pro | EBS-K | Rugg (2000) |
| 22. | 134 | CGT-TGT | Arg → Cys | EBS-WC | Indelman (2005) |
| 23. | 143 | CTG-CCG | Leu → Pro | EBS-K | Sorensen (1999) |
| 24. | 144 | GAA-GCA | Glu → Ala | EBS-WC | Hovnanian (1993) |
| 25. | 148 | CGT-TGT | Arg → Cys | EBS-WC | Wood (2003) |
| 26. | 204 | TAT-TAA | Tyr → Term | EBS-WC | Chan (1994) |
| 27. | 211 | CGC-CCC | Arg → Pro | EBS-WC | Müller (2006) |
| 28. | 247 | GCC-GAC | Ala → Asp | EBS-K | Premaratne (2002) |
| 29. | 270 | GTG-ATG | Val → Met | EBS-WC | Rugg (1993) |
| 30. | 272 | ATG-AGG | Met → Arg | EBS-K | Humphries (1993) |
| 31. | 272 | ATG-ACG | Met → Thr | EBS-K | Schneider(2005) |
| 32. | 273 | GAC-GGC | Asp → Gly | EBS-WC | Muller (1998) |
| 33. | 274 | GCT-GAT | Ala → Asp | EBS-WC; -DM | Chen (1995) |
| 34. | 305 | TGG-TGA | Trp → Term | EBS-K | Corden (1998) |

| | | | | | |
|---|---|---|---|---|---|
| 35. | 377 | ATT-AAT | Ile → Asn | EBS-WC | Chen (1995) |
| 36. | 377 | ATT-ACT | Ile → Thr | EBS-WC | Rugg (2007) |
| 37. | 384 | CTG-CCG | Leu → Pro | EBS-K | Bonfas (1991) |
| 38. | 388 | CGC-TGC | Arg → Cys | EBS-WC | Chen (1995) |
| 39. | 388 | CGC-CAC | Arg → His | EBS-WC | Ciubotaru (2003) |
| 40. | 396 | CAG-TAG | Gln → Term | EBS-K; -WC | Ciubotaru (2003) |
| 41. | 408 | CTG-ATG | Leu → Met | EBS-WC | Schuilenga-Hut (2003) |
| 42. | 411 | GAG-TAG | Glu → Term | EBS-K | Gu (2002) |
| 43. | 413 | GCC-ACC | Ala → Thr | EBS-K | Chao (2002) |
| 44. | 415 | TAC-CAC | Tyr → His | EBS-DM | Rugg (2000) |
| 45. | 415 | TAC-TGC | Tyr → Cys | EBS-WC | Ciubotaru (2003) |
| 46. | 416 | CGC-CCC | Arg → Pro | EBS-DM | Wood (2003) |
| 47. | 418 | CTG-GTG | Leu → Val | EBS | Rugg (2007) |
| 48. | 419 | CTG-CAG | Leu → Gln | EBS-DM | Hut (2000) |
| 49. | 422 | GAG-AAG | Glu → Lys | EBS-WC | Hut (2000) |

| No. | Position | Mutation | Type | Phenotype | Reference |
|---|---|---|---|---|---|
| 47 | IVS1-2 | A>C | splice site | EBS-K | Jonkman (1996) |
| 48 | IVS4+1 | G>A | splice site | EBS-K | Schuilenga-Hut (2003) |
| 49 | 30 | delT | out of frame deletion | EBS | Batta (2000) |
| 55. | 104 | delGC | out of frame deletion | EBS-WC | Rugg (1994) |
| 56. | 127 | delCCT | in-frame deletion | EBS-DM | Wood (2003) |
| 57. | 375 | delGAG | in-frame deletion | EBS-WC | Chen (1993) |
| 58. | 247 | delCinsAG | indels | EBS-K | Lanschuetzer (2003) |
| 59. | 406 | delG | out of frame deletion | EBS-WC | Müller (2006) |
| 60. | 411 | delGAG | in-frame deletion | EBS-WC | Müller (2006) |

*****Source: www.hgmd.cf.ac.uk, with modifications

## Aims of the study

Epidermolysis bullosa simplex is the most common subtype, accounting for one half of all EB cases. EBS can be diagnosed by genotyping, in addition to skin biopsy. Defects in *KRT5* and *KRT14* genes, which encode K5 and K14, cause the different EBS subtypes.

The aim of this study was to optimize the molecular analysis of the causative genes by direct DNA sequencing and to delineate genotype-phenotype correlations by indirect immunofluorescence staining of the skin and by characterization of the clinical manifestations of EBS. Indirect immunofluorescence (IIF) was also the most important diagnostic tool to distinguish EBS from other EB forms. It also allowed a fast first prognosis for the patient.

This study will improve our understanding of the structural roles of keratin intermediate filaments in the epidermis and give new insights into the pathogenesis of EBS.

# 3. Materials and methods

## 3.1. Materials

All items not listed here were standard laboratory products bought from BD Labware, Heidelberg; Eppendorf, Hamburg; Merck, Darmstadt; Neolab, Heidelberg; Nunc, Wiesbaden; Roche, Mannheim; Roth, Karlsruhe; Sigma-Aldrich, Munich.

**Table 6. Technical equipment**

| | |
|---|---|
| Agarose gel chambers mini, midi, large | Roth, Karlsruhe |
| Automated sequencer ABI Prism 3100 | ABI, Darmstadt |
| Centrifuge Labofuge 400 | Heraeus, Hanau |
| Confocal Laser scanning microscope LSM 510 | Carl Zeiss, Oberkochen |
| Cryomicrotome CMI 900 | Leica, Wetzlar |
| Gel documentation system | Intas GDS, Goettingen |
| Immunofluorescence microscope Axiophot & digital Axio Cam Mrc | Carl Zeiss, Oberkochen |
| PH-meter | Sartorius, Goettingen |
| Power supply Power pac 300 | Biorad, Muenchen |
| Power supply P25, P30 | Biometra, Goettingen |
| Spectrophotometer Bio Photometer | Eppendorf, Hamburg |
| Thermal cycler PTC-100, minicycler<br>Thermal cycler Mastercycler gradient | Biozym, Hess. Oldendorf<br>Eppendorf, Hamburg |
| Thermal mixer Thermomixer comfort | Eppendorf, Hamburg |

**Table 7. Reagents**

| | |
|---|---|
| AE-buffer (TE) | Qiagen, Hilden |
| Agarose | Sigma-Aldrich, Munich |
| Big dye terminator DNA sequencing kit 1.1 | ABI, Darmstadt |
| Biogel P100 | Biorad, Munich |
| DNA molecular weight marker (0.07-12.2kbp) | Roche, Mannheim |
| Eukitt | Kindler, Freiburg |
| Millipore multiscreen 96 well plate | Millipore, Schwalbach |
| Mowiol | Calbiochem, Bad Soden |
| Nucleotides dNTP | Eppendorf, Hamburg |
| Primer Synthesis | Biomersnet, Ulm |
| Ready load 100bp DNA ladder | Invitrogen, Karlsruhe |
| Sephadex G50 fine | Amersham, Freiburg |

**Table 8. Buffers and solutions**

| | |
|---|---|
| DNA sample buffer for agarose gel electrophoresis | 50% Glycerol<br>TBE 1x<br>1% Bromphenolblau |
| Mowiol | 20g Mowiol 4-88<br>80ml PBS<br>40ml Glycerin |
| TBS | 0.15M NaCl<br>0.05M Tris/HCl, pH 7.4 |

**Table 9. Enzymes**

| | |
|---|---|
| Hot Master *Hot Start Taq* polymerase (5U/µl) | Eppendorf, Hamburg |
| *Expand Long Template Enzyme* | Roche,Mannheim |
| Restriction enzymes<br>Bae I; Sac I; Pvu II | New England Biolabs; GibcoBRL |

**Table 10. Primary antibodies**

| Primary antibodies | Type | Origin | Dilution | Source |
|---|---|---|---|---|
| Anti-Human cytokeratin 5/6 | monoclonal, Clone D5/16 B4 | mouse | 1:50 | Dako, Hamburg |
| Anti-Human cytokeratin 5 | | | 1:1000 | Prof. Magin, Bonn |
| Anti-Human cytokeratin | monoclonal, Clone LP34 | mouse | 1:500 | Dako, Hamburg |
| Anti-Human cytokeratin 14 | monoclonal, Clone LL002 | mouse | 1:50 | Biogenex, San Ramon, USA |

**Table 11. Secondary antibodies**

| Secondary antibodies(working dilution) | Source |
|---|---|
| Anti rabbit-IgG FITC labeled (1:30) | Dako, Hamburg |
| Anti mouse-IgG FITC labeled (1:30) | Dako, Hamburg |

**Table 12. Ready to use kits**

| | |
|---|---|
| QIA quick Gel extraction kit | Qiagen, Hilden |
| Qiaquick PCR Purification kit | Qiagen, Hilden |
| QiAmp DNA mini kit | Qiagen, Hilden |

**Table 13. PCR primers for *KRT 5* and *KRT 14* amplification from gDNA**

*KRT5*

| Exons | gDNA-Primers | Product size (bp) |
|---|---|---|
| 1 | 5'-GAGCTCTGTTCTCTCCAGCA-3'<br>5'-CCTTCTTTCTCTCTCTTTGGC-3' | 695 |
| 2 | 5'-GATCATAGAACTTGAAATCC-3'<br>5'-CCATCTGGTACCAAGAAGAC-3' | 328 |
| 3 | 5'-TGGCCAGAGGTTCATGCTAC-3'<br>5'-TCAACCTTGGCCTCCAGCTCC-3' | 378 |
| 4 | 5'-GAGAACCAGCAGCCTGCAG-3'<br>5'-TGAGGTGTCAGAGACATGC-3' | 371 |
| 5 | 5'-ATGAGATTAACTTCATGAAGATG-3'<br>5'-CCATTCTTAGTGTCGTCATG-3' | 5F1-5R1 423 |
| 6 | 5'-CTCACTGCCTGTGAACTTTG-3'<br>5'-TTTAGAACTCAGGCCCCTTC-3' | 6F-5R2 250 |
| 7 | 5'-GAGAGCCGAGATTGACAATG-3'<br>5'-TAGAGCAGCCTCGCTTTATC-3' | 562 |
| 8 | 5'-TCGAATCATGAGGATGGGAG-3'<br>5'-GAGGAAACACTGCTTGTGA-3' | 705 |
| 9 | 5'-TAAATGGGCCATGCAGGATC-3'<br>5'-AGAAGAGGCAATCTCCATGG-3' | 700 |

*KRT14*

| Exons | gDNA-Primers | Product size (bp) |
|---|---|---|
| K14spf2<br>K14spr2 | 5'-ATTTCAGGGATGGGACAGAC-3'<br>5'-GAGGGGATCTTCCAGTGGGATCT-3' | 5093 |

**Table 14. Primers for sequencing of *KRT14* PCR products**

*KRT14*

| Exons | Primers | Product size (bp) |
|---|---|---|
| 1 | 5'-CAGCTCCATGAAGGGCTCC-3'<br>5'-GAGCTAGCTGGAATGGTGCC-3' | 534 |
| 2 | 5'-GACAAATTACCTGTGCCTTT-3'<br>5'-GCCCAAGAGTCTTATTCTTT-3' | 261 |
| 3 | 5'-GCACTGTGTTCAACCACGCC-3'<br>5'-TCCTGTCTCAGCCTCCCAAG-3' | 406 |
| 4 | 5'-CAGGCCTAAGGAACACCAAT-3'<br>5'-GAGAATGCCATTCACACCAG-3' | 304 |
| 5 | 5'-GTGTCATTTGAGGTGGAAGG-3'<br>5'-ATTAGTGAGTGTGGCCGTTC-3' | 273 |
| 6 | 5'-GAACGGCCACACTCACTAAT-3'<br>5'-CATTAGATACATGGTGGGGC-3' | 337 |
| 7 | 5'-GGAGTACAAGATCCTGCTGG-3'<br>5'-CTAGCCAATGCCTAGACCTG-3' | 303 |
| 8 | 5'-TCCTCACCTTCTTGGCCTCC-3'<br>5'-GCTGGGCAGCCTCAGTTCTT-3' | 193 |

## 3.2 Patients

In this study 20 patients with clinically defined EBS were investigated. The patients were referred to the *Epidermolysis bullosa Zentrum* of the University of Freiburg, or material and clinical information were sent by co-operating centers. Following informed consent, EDTA blood specimens were obtained from affected individuals and close relatives. In most cases, skin biopsies were obtained for immunofluorescence staining. The study was performed under the approval of ethical committee of the University of Freiburg, and was conducted according to Declaration of Helsinki Principles.

## 3.3 Mutation detection

### Isolation of gDNA

Genomic DNA (gDNA) was extracted from peripheral blood cells using QiAmp DNA mini kit (Qiagen) according to the manufacturer's protocol. Concentration and purity were determined by spectrophotometry. gDNA quality was verified by 0.8 % agarose gel electrophoresis using 3 µl of each sample and 3 µl of loading buffer. gDNA was diluted to 10 ng/µl in AE-buffer, and was used as a template for PCR.

### Amplification of gDNA fragments by PCR

For amplification of all *KRT5* and *KRT14* exons and exon/intron boundaries, 9 and 8 pairs of specific primers were designed according to Schuilenga-Hut *et al* and Wood *et al* respectively [44;57]. To improve mutation detection, we set up a working strategy based on priority regions. For PCR *Hot start Taq polymerases* (Eppendorf, Genaxxon) were used and Expand Long Template enzyme (Roche Diagnostics, Mannheim, Germany) for specific full-length amplification. For the reaction mix and PCR conditions, see Table 15 and 16.

Table 15. Reaction Mixture for PCR

*KRT5* Exon: 5

| Substance | Vol (µl) | Vol (µl) |
|---|---|---|
| H2O | 28.3 | 13.3 |
| Buffer(x10) | 5 | 5 |
| dNTP (2.5mM) | 4 | 4 |
| Primer F (20pmol) | 1.25 | 1.25 |
| Primer R (20pmol) | 1.25 | 1.25 |
| Taq | 0.2 | 0.2 |
| DNA (10ng/µl) | 10 | 25 |
| Final volume | 50 | 50 |

*KRT14*

| Substance | Vol (µl) |
|---|---|
| H2O | 34.75- x |
| Buffer 1(x10) | 5 |
| dNTP (2.5mM) | 7 |
| Primer F (20pmol) | 1.25 |
| Primer R (20pmol) | 1.25 |
| Expand Long template Enzyme | 0.75 |
| DNA (500ng) | x |
| Final volume | 50 |

Table 16. PCR and sequencing conditions

PCR Program for *KRT5*

| Exon:1,2,5,7,9 | | | Exon 3 | 5'TD | Exon 4 | 2'TD |
|---|---|---|---|---|---|---|
| Initial denaturation | 94° | 5 min | 94° | 5 min | 94° | 2 min |
| Denaturation | 94° | 1 min | 94° | 1 min | 94° | 30 sec |
| Annealing | 55° | 1 min | 61° | 1 min | 60° | 30 sec |
| Extension | 70° | 1 min | 70° | 1 min | 70° | 1 min |
| Cycles | 35 | | 40 | | 35 | |
| Final extension | 70° | 10 min | 70° | 10 min | 70° | 10 min |

| Exon:6 | | 2'TD | Exon 8 | |
|---|---|---|---|---|
| Initial denaturation | 94° | 2 min | 94° | 5 min |
| Denaturation | 94° | 1 min | 94° | 1 min |
| Annealing | 55° | 1 min | 61° | 1 min |
| Extension | 70° | 1 min | 70° | 1 min |
| Cycles | 35 | | 35 | |
| Final extension | 70° | 10 min | 70° | 10 min |

AT: Annealing temperature

TD: *touch down* conditions, first five cycles AT+5°C

Long-Range PCR Program for *KRT14*

| | | |
|---|---|---|
| Initial denaturation | 94° | 2 min |
| Denaturation | 94° | 10 sec |
| Annealing | 62° | 30 sec |
| Extension | 68° | 4 min |
| Cycles | 5 | |
| Denaturation | 94° | 10 sec |
| Annealing | 60° | 30 sec |
| Extension | 68° | 4 min |
| Cycles | 5 | |
| Denaturation | 94° | 10 sec |
| Annealing | 59° | 30 sec |
| Extension | 68° | 4 min |
| Cycles | 25 | |
| Final extension | 68° | 7 min |

Sequencing conditions

| | | |
|---|---|---|
| Denaturation | 96° | 10 sec |
| Annealing | (AT) | 5 sec |
| Extension | 60° | 3 min |
| Cycles | 25 | |

## Agarose gel electrophoresis

1.5 % agarose TBE-buffer gels, stained with 0.5 % ethidiumbromide, were used to evaluate PCR products. 0.5 cm slots were loaded with 3 µl loading dye, and then 5 µl PCR product or 3 µl DNA sample. And 3 µl Ready load 100 bp DNA ladder (Invitrogen). Gels were run at 120V, 400mA for 30 min, visualized and documented using a digital documentation system (Intas GDS).

## DNA sequencing

For sequencing, PCR products were purified either with QIAquick PCR purification kit (Qiagen) according to the manufacturer's protocol, or using the polymeric gel Biogel P100 (Biorad) in Millipore multiscreen 96 well filter plates, to eliminate undesired side products and unused dNTPs. To prepare the Millipore filter plates, each well was loaded with 350 µl of Biogel P100 (Biorad) and fixed on a 96 well plate and then the plates were centrifuged at 1100 rpm for 3 min. The flow through was discarded. The Millipore multiscreen filter plates, containing the Biogel P100, were then fixed on new sterile 96 well plates. They were now ready

for loading of the PCR products. After loading the PCR products to the wells, the plates were centrifuged at 1000 rpm for 4 min. The purified PCR products were obtained in the sterile 96 well plates; the Millipore filter plates were discarded.

The DNA concentration of PCR products was measured by spectrophotometry [30;31].

For the sequencing reaction either upstream primers, for forward sequencing, or downstream primers, for reverse sequencing, were used. The primers used were the same as for PCR (except *KRT14*, cf. Table 13 and 14). For the composition of the reaction mix see Table 17. The sequencing reactions were carried out in the same thermal cyclers as the PCR, the reaction conditions for ABI prism 3100 automated sequencer and are listed in Table 16.

The products of the sequencing reactions were purified using the polymeric gel Sephadex G50 (Amersham) in Millipore multiscreen 96 well filter plates. In this case the procedure was the same as for purifying PCR products, using Sephadex G50 (Amersham) instead of Biogel P100 (Biorad).The centrifugation steps were carried out at 1500 rpm instead of 1100 rpm.

The samples were then submitted to automated sequencing in an ABI prism 3100 sequencer (ABI, Darmstadt, Germany). The sequencers delivered raw data, which were stored digitally. Through base calling of the raw data, DNA electropherogram sequences were obtained.

Table 17. Reaction mix for sequencing of PCR products

| ABI prism 3100 | Vol (µl) |
|---|---|
| H2O | 7- x |
| DMSO(50%) | 1 |
| Big Dye1.1 | 1 |
| Primer F/R (5pmol) | 1 |
| PCR product | x(30-50ng) |
| Final volume | 10 |

DNA electropherogram sequences were compared to the reference sequence from the NCBI Entrez Nucleotide database [NM_000424, NM_000526] manually, either on printout or using standard sequence alignment software. To improve the efficiency of the protocol, automated mutation detection software was used.

## Restriction enzyme digestion

To confirm sequence variants by a second method, restriction enzyme (RE) digestion was performed wherever possible. The database Webcutter or Restriction Mapper [http://rna.lundberg.gu.se/cutter2/; http://www.restrictionmapper.org/] were used to find out whether the variant would lead to new or eliminate existing cutting sites. RE digestion was

performed in a final volume of 20 µl with up to 500 ng of DNA according to the manufacturer's protocols. Digested PCR products were analyzed by agarose gel electrophoresis.
In case of novel mutations, 100 normal control chromosomes were analyzed.

Table 18. Reaction mixture for restriction enzyme digestion

| Substance | Vol (µl) | Substance | Vol (µl) | Substance | Vol (µl) |
|---|---|---|---|---|---|
| H2O | 11.7- x | H2O | 15.8- x | H2O | 17.5- x |
| Buffer 2(x10) | 2 | Buffer 1(x10) | 2 | Buffer React 6(x10) | 2 |
| BSA(x10) | 2 | BSA(x10) | 2 | BSA(x10) | – |
| SAM (100µM) | 4 | | | | |
| Enzyme(Bae I) | 0.3 | Enzyme(Sac I) | 0.2 | Enzyme(Pvu II) | 0.5 |
| PCR product | x (500 ng) | PCR product | x (500 ng) | PCR product | x (500 ng) |
| Final volume | 20 | Final volume | 20 | Final volume | 20 |

## 3.4 Indirect immunofluorescence of skin cryosections

Skin biopsies were frozen in liquid nitrogen. 2-4 µm cryosections were incubated at RT with 50µl of the chosen first antibody (dilution cf. Table 10) over night in a wet chamber. Then they were washed 5 x 4 min with TBS. The incubation time with 50 µl of the respective second antibody was 30min-2h in a dark chamber at RT. Afterward the cryosections were again washed 5 x 4 min with TBS and dried carefully. As a final step, the slides were mounted in Mowiol, or equivalent and dried at 4°C until embedding medium is solid, then stored at -20°C.
Cryosections were visualized with confocal laser scanning microscopy (Zeiss LSM510, 30mW argon laser at 488 nm) or digital immunofluorescence microscopy (Zeiss Axiophot) and stored digitally.

## 3.5 Bioinformatics

The online tools and databases used are listed in Table 19.

Table 19. Online tools and databases

| Tool | Application | Accession |
|---|---|---|
| Online Mendelian Inheritance in Man database (OMIM) | Epidermolysis bullosa phenotype | #131760<br>#131800<br>#131900<br>#131960 |
| NCBI Entrez Nucletide | *KRT5* cDNA<br>*KRT14* cDNA | NM_000424<br>NM_000526 |
| NCBI Entrez Gene | *KRT5*,*KRT14* gene, link | http://www.ncbi.nlm.nih.gov/ |
| Human Gene mutation Database Cardiff | *KRT5*,*KRT14* mutations | http://www.hgmd.cf.ac.uk/ac/index.php |
| SWiss-Prot/TrEMBL | K5<br>K14 | K2C5_HUMAN (P13647)<br>K1C14_HUMAN (P02533) |
| Restriction Mapper<br>Webcutter | Restriction enzyme database | http://www.restrictionmapper.org/<br>http://rna.lundberg.gu.se/cutter2/ |

# 4. Results

## 4.1. Keratin mutations and their consequences

### Clinical features

All 20 patients in this study presented with bullous lesions of the skin, without scarring. The age of onset was different, from birth or during childhood. Based on the family pedigree, 2 patients had recessive epidermolysis bullosa simplex (REBS) with consanguineous unaffected parents. In 7 families the inheritance pattern was dominant, while 8 patients represented spontaneous cases. In one family, two sisters were affected. In 2 cases, no data were available.

### Indirect immunofluorescence

IIF was performed on the skin of 14 out of 20 patients. IIF was the most important diagnostic tool to distinguish EBS from other EB forms. It also allowed a fast first prognosis for the patient. REBS corresponded well with reduced or absent staining of K5 or K14, DEBS with positive or reduced K5 or K14 levels. Nevertheless, IIF is not always perfectly specific or free of artefacts. The quality of the skin cryosections plays an important role. Furthermore, IIF diagnosis needs professional experience, even though much less than the assessment of electron microscopy of the skin.

### Mutation survey

Mutations in 20 patients were investigated. By direct sequencing, we identified *KRT5* mutations in 10 patients and *KRT14* mutations in 10 patients. In both REBS patients, homozygous *KRT14* mutations were found (Table 20). 18 different mutations were identified in *KRT5* or *KRT14*: 12 missense mutations, 1 deletion/insertion, 3 deletion, and 2 duplication mutations (Table 21). The most labor intensive part of our protocol was analysis of the sequences. The workload was significantly reduced with the software *Mutation Surveyor* (www.softgenetics.com); [30]. This program was chosen because it had the best sensitivity of all tested programs as compared to manual printout sequence analysis. It was also the most convenient to use. The improvement of efficiency by this software justified its relatively high price.

Table 20. Phenotypes and genotypes in EBS patients investigated

| Patient No. | Clinical diagnosis | Mutation c.DNA | Protein | Reported in |
|---|---|---|---|---|
| 1 | EBS-K | *KRT14:*c.427delC | K14:p.L143fsX2 | Has et al (2006) |
| 2 | EBS-K | *KRT14:*c.129dupC | K14:p.S44fsX38 | Has et al (2006) |
| 3 | EBS-WC | *KRT5:*c.1362delGGAG insAGCTGGTA | K5:p.Q454fsX118 | this study |
| 4 | EBS-WC | *KRT5:*c.788_799delACAAGCGT ACCA | K5:p.N263_T266del | this study |
| 5 | EBS-MP | *KRT14:*c.1104_1145dup | K14:p.I368_Q382dup | this study |
| 6 | EBS-DM | *KRT5:*c.556G>C | K5:p.V186L | this study |
| 7 | EBS-K | *KRT5:*c.1250C>A | K5:p.A417D | this study |
| 8 | EBS-WC | *KRT5:*c.514A>G | K5:p.I172V | this study |
| 9 | EBS-DM | *KRT14:*c.364C>G | K14:p.L122V | this study |
| 10 | EBS-WC | *KRT14:*c.1162C>G | K14:p.R388G | this study |
| 11 | EBS-MP | *KRT5:*c.74C>T | K5:p.P25L | Uttam et al (1996) |
| 12 | EBS-DM | *KRT5:*c.527A>G | K5:p.N176S | Stephens et al (1995) |
| 13 | EBS-WC | *KRT5:*c.991C>T | K5:p.R331C | Rugg et al (1993) |
| 14 | EBS with circinate erythema | *KRT5:*c.1649delG | K5:p.G550fsX76 | Gu et al (2003) |
| 15 | EBS-MP | *KRT5:*c.1649delG | K5:p.G550fsX76 | Gu et al (2003) |
| 16 | EBS-DM | *KRT14:*c.373C>T | K14:p.R125C | Chen et al (1995) |
| 17 | EBS-DM | *KRT14:*c.374G>A | K14:p.R125H | Coulombe et al (1991) |
| 18 | EBS-WC | *KRT14:*c.808G>A | K14:p.V270M | Rugg et al (1993) |
| 19 | EBS-K | *KRT14:*c.815T>C | K14:p.M272T | Schneider et al (2005) |
| 20 | EBS-K | *KRT14:*c.815T>C | K14:p.M272T | Schneider et al (2005) |

Table 21. Types of mutations detected in this study

| No. | **Deletion/ insertion mutations** | Consequence | Exon | Phenotype | Type |
|---|---|---|---|---|---|
| 1 | *KRT5*:788_799del | in-frame deletion | 3 | EBS-WC | novel |
| 2 | *KRT5*:1362del4ins8 | PTC | 7 | EBS-WC | novel |
| 3 | *KRT5*:1649delG* | PTC | 9 | EBS with circinate erythema; EBS-MP | recurrent |
| 4 | *KRT14*:427delC | PTC | 1 | REBS | novel |
| 5 | *KRT14*:129dupC | PTC | 1 | REBS | novel |
| 6 | *KRT14*:1104_1145dup | in-frame duplication | 6 | EBS-MP | novel |
| | **Missense mutations** | | | | |
| 1 | *KRT5*:74C>T | P25L | 1 | EBS-MP | recurrent |
| 2 | *KRT5*:514A>G | I172V | 1 | EBS-WC | novel |
| 3 | *KRT5*:527A>G | N176S | 1 | EBS-DM | recurrent |
| 4 | *KRT5*:556G>C | V186L | 2 | EBS-DM | novel |
| 5 | *KRT5*:991C>T | R331C | 5 | EBS-WC | recurrent |
| 6 | *KRT5*:1250C>A | A417D | 7 | EBS-K | novel |
| 7 | *KRT14*:364C>G | L122V | 1 | EBS-DM | novel |
| 8 | *KRT14*:373C>T | R125C | 1 | EBS-DM | recurrent |
| 9 | *KRT14*:374G>A | R125H | 1 | EBS-DM | recurrent |
| 10 | *KRT14*:808G>A | V270M | 4 | EBS-WC | recurrent |
| 11 | *KRT14*:815T>C* | M272T | 4 | EBS-K | recurrent |
| 12 | *KRT14*:1162C>G | R388G | 6 | EBS-WC | novel |

* Each of these mutations was detected in two unrelated patients

## Novel and recurrent *KRT5* and *KRT14* mutations

In this study, 10 novel mutations were identified, representing 55.55% of the total mutation number as shown in Table 22. We found 5 novel deletion/insertion mutations: c.1362delGGAG insAGCTGGTA, c.788_799delACAAGCGTACCA in *KRT5* and c.427delC, c.129dupC, c.1104_1145dup in *KRT14* resulting in PTC, or in, in-frame deletion and in-frame duplication (Table 21). Besides, 5 novel missense mutations were revealed: I172V, V186L, A417D in *KRT5* and L122V, R388G in *KRT14*. They lead to reduced levels of K5 or K14 in the skin, as shown with IIF staining. Recurrent mutations were: c.74C>T, c.1649delG in *KRT5* and c.373C>T, c.374G>A in *KRT14*.

Table 22. Total and novel mutations in this study

| Mutations | Novel mutation found |
|---|---|
| 18 | 10 (55.55%) |

Table 23. Summary of the 10 families with *KRT5* mutations.

| Mutation type | Number of published mutations | Novel mutations in this study |
|---|---|---|
| Nonsense | 2 | 0 |
| Missense | 38 | 3 |
| Splice site | 2 | 0 |
| Small deletions | 3 | 1 |
| Small insertions | 0 | 0 |
| Small insdels | 0 | 1 |
| **Total** | 45 | 5 |

*The source for the published mutations was The Human Gene Mutation Database.

Table 24. Summary of the 10 families with *KRT14* mutations.

| Mutation type | Number of published mutations | Novel mutations in this study |
|---|---|---|
| Nonsense | 4 | 0 |
| Missense | 36 | 2 |
| Splice site | 2 | 0 |
| Small deletions | 4 | 1 |
| Small insertions | 0 | 2 |
| Small insdels | 1 | 0 |
| **Total** | 47 | 5 |

*The source for the published mutations was The Human Gene Mutation Database.

## Polymorphisms

In this study, 8 polymorphisms in the *KRT5* gene and 6 in the *KRT14* gene were found. All known *KRT5* and *KRT14* polymorphisms are summarized in Table 25. The polymorphisms identified in this study are indicated with an asterisk (*).

Table 25. Polymorphisms in *KRT5* and *KRT14*

*KRT 5*

| AA co-ordinate | alleles | AA change |
|---|---|---|
| − | A/G | − |
| 34 | C/T | S/F |
| 79* | T/A | S/R |
| 80* | T/A | G |
| 117 | G/A | L |
| 138* | G/A | G/E |
| −* | G/A | − |
| − | C/A | − |
| − | C/T | − |
| 197* | C/A | D/E |
| 198* | C/A | T |
| 210 | T/C | T |
| 232 | G/A | S/N |
| 233 | C/T | I |
| 238 | C/T | G |
| 242 | A/G | S |
| 244 | C/T | L |
| 355* | T/G | T |
| 357 | G/A | A/T |
| − | −/C | |
| − | A/G | − |
| 384 | C/T | H/Y |
| 387* | T/A | S/T |
| 387 | T/A | S |
| 405 | T/C | K |
| − | C/T | − |
| − | T/C | − |
| − | C/A | − |
| − | T/A/G | − |
| 528 | A/G | S/G |

*KRT 14*

| AA co-ordinate | alleles | AA change |
|---|---|---|
| − | G/A | − |
| 2* | C/T | T |
| 27 | G/T | G/V |
| 34 | C/T | V |
| 39 | C/T | S/F |
| 54 | C/T | S/F |
| 63 | C/T | C/Y |
| 63* | C/T | C |
| 65* | G/A | L |
| 77* | G/A | S |
| 80 | A/G | F |
| 94* | C/T | A/T |
| 119 | T/C | M/T |
| 123* | A/G | N |
| 144 | −/T | |
| − | G/C | − |
| 185 | A/G | N |
| 215 | G/A | E/K |
| − | −/T | |
| − | C/T | − |
| 257 | G/A | M/I |
| − | −/G | |
| − | C/G | − |
| − | A/G | − |
| − | T/A | − |
| − | T/C | − |
| 452 | C/T | V/I |
| 454 | C/A | D/Y |
| | | |
| | | |

| 543 | G/A | G/S |
|---|---|---|
| − | C/G | − |
| − | G/A | − |
| − | C/T | − |
| − | C/T | − |
| − | C/T | − |

* Identified in this study

| | | |
|---|---|---|
| | | |
| | | |
| | | |
| | | |
| | | |

## 4.2. Genotype – phenotype correlations

Some patients in this study had interesting novel mutations and/or unusual genotype-phenotype correlations. In the following, their clinical presentation, antigen mapping and genetic findings will be presented to delineate the relationships and to enhance the understanding of genotype-phenotype correlations.

### Recessive EBS

In this study, we investigated two unrelated patients with severe neonatal blistering, both offspring of consanguineous, unaffected parents of Turkish (patient 1) or German (patient 2) origin.

Patient 1, a 2 y-old boy, showed blistering, predominantly on hands and feet, since birth (Figure 5a). In the course of the disease bullae became rarer, occurred mechanically induced also on the head and trunk and healed without scarring. Patient 2, aged 1 y, showed at birth extensive blistering of the hands and feet (Figure 5b) and suffered from congenital pneumonia. Oral blisters were present in the first days. Later, he developed blisters on arms, legs and trunk after mechanical trauma.

Indirect immunofluorescence (IIF) of skin cryosections was performed with monoclonal antibodies against human keratin 5 and keratin 14.In both cases, it revealed a split level through the basal cell layer (Figure 5c and d), and antibodies against keratin 14 yielded a negative staining (Figure 5e), suggesting keratin 14 defects in both cases.

Mutation detection in *KRT14* revealed in patient 1 a homozygous one base pair deletion in exon 1, designated c.427delC (Figure 5g); this results in a frame shift starting with codon 143 and formation of a premature termination codon (PTC), two codons downstream, p.L143fsX2. In patient 2, also in exon 1 of *KRT14*, a homozygous duplication, c.129dupC was detected (Figure 5h), leading to a frame shift starting with codon 44 and PTC 38 codons downstream (p.S44fsX38). The parents were found to be heterozygous carriers of the respective mutations.

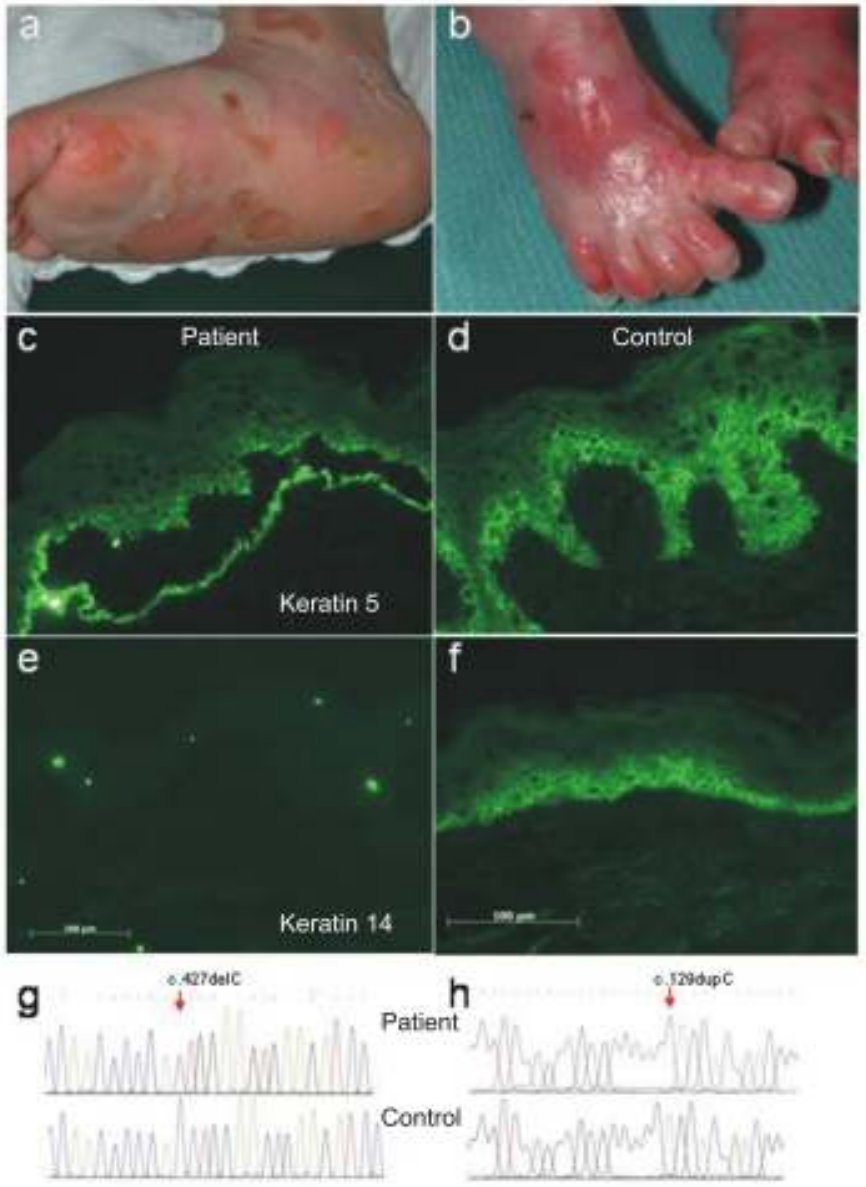


**Figure 5. Patient 1 and 2 with EBS-K.** (a) Blisters and erosions on the feet of patient 1 at the age of 2 years. (b) At 2 weeks of age patient 2 showed severe blistering and absence of skin on feet. (c) IIF with antibodies against keratin 5 showed intra-epidermal splitting in the skin of patient 2. (d) IIF of control skin with antibodies against keratin 5. (e) IIF staining with antibodies against keratin 14 yielded a negative signal in patient's 1 skin. (f) IIF with antibodies against keratin 14 produced a positive signal in the basal keratinocyte layer in control skin. (g) Partial sequence of *KRT14* exon 1, showing in the upper panel the mutation c.427delC (*red arrow*) and in the lower panel the control sequence. (h) Partial sequence of *KRT14* exon 1, showing in the upper panel the mutation c.129dupC *(red arrow*) and in the lower panel the control sequence.

## Novel deletion / insertion mutations in EBS

**Patient 3** was a 31 year-old female. Her mother, maternal grandfather and great-grandmother were also affected; her father and brother were unaffected. Onset of the disease was in the first year of life with blisters on the extremities. The clinical picture was consistent with the diagnosis of EBS-WC with blistering confined exclusively to her hands and feet, without scarring, but with hyperkeratosis of soles, hyperhidrosis of the feet and toe nail dystrophy (Figure 6a and b). IIF of the skin revealed reduced keratin 5 staining with an abnormally shaped basal cell layer and hyperkeratosis (Figure 6c and d).

We found a heterozygous mutation in exon 7 of the *KRT5* as a deletion of GGAG starting from the nucleotide position 1362 and insertion of AGCTGGTA (c.1362delGGAGinsAGCTGGTA) (Figure 6e). The affected mother was also heterozygous for the same deletion/insertion mutation as her daughter. No mutation was found in father of the index patient.

The consequence of this mutation was a frameshift starting from the codon 454 for glutamine and a PTC, 118 codons downstream from this mutation site (p.Q454fsX118). It created a new restriction enzyme site of Sac I. The mutation was absent in 100 control alleles.

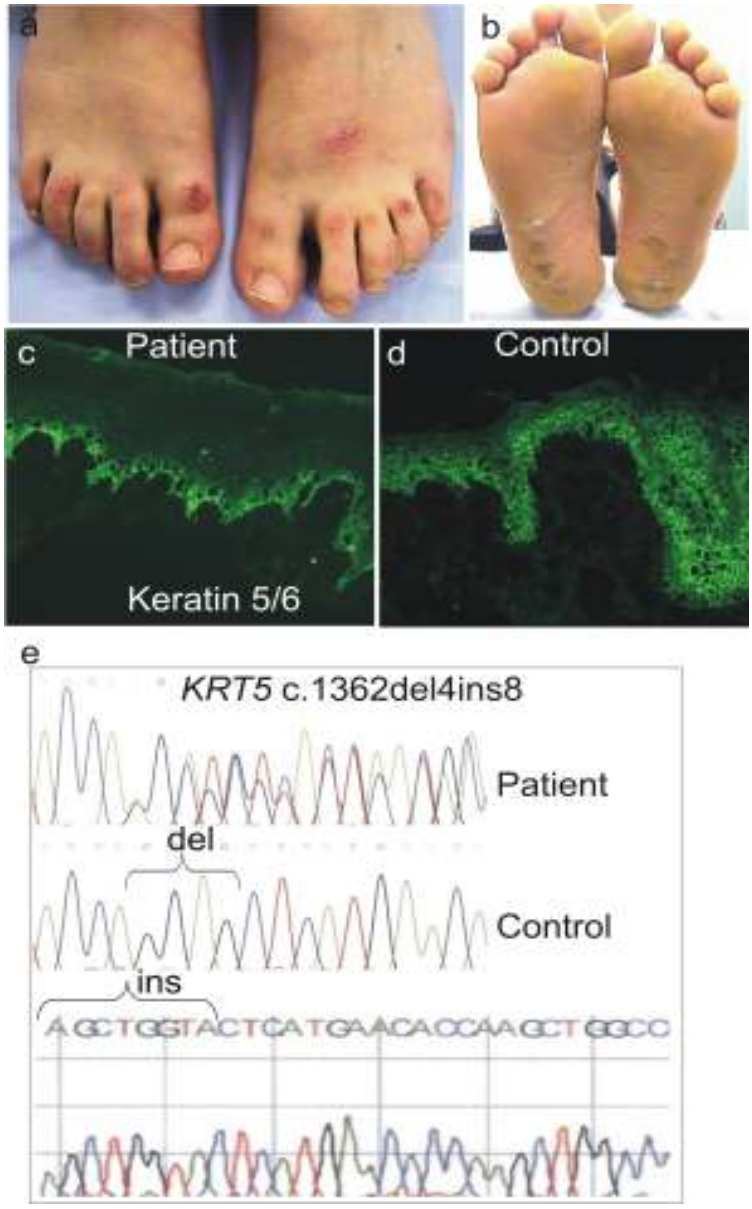


**Figure 6. Patient 3 with EBS-WC**. (a) Skin blistering affecting the dorsal side of the feet. (b) Hyperkeratosis and erosions of soles. (c) IIF with antibodies against keratin 5/6 showed abnormal stratum basale and reduced staining. (d) IIF of control skin with antibodies against keratin 5/6. (e) The sequence analysis of *KRT5* exon 7 PCR fragments found a heterozygous deletion of GGAG starting from the nucleotide position 1362 and insertion of AGCTGGTA. In the upper panel the patient's sequence is shown, in the middle panel the control sequence, and in the lower panel the mutated allele.

**Patient 4** was a 12 year-old girl with no family history of EB. Onset of the disease was at birth with mechanically induced blistering on feet and seldom on hands. There was no nail dystrophy and no scarring. Previous IIF had shown type IV collagen on the base of the blister. A second biopsy analyzed with IIF showed a thick epidermis with a reduced K14 staining (Figure 7a and c)

Mutation analysis showed the presence of a novel heterozygous mutation in exon 3 of *KRT5* as the deletion of 12 nucleotides, at codons 263-266, encoding the amino acids NKRT (Figure 7e). This mutation was designated c.788_799del and causes the discontinuity of the 1B domain. We did not find any potential pathogenic mutations in *KRT14*. To confirm the mutation restriction digestion with Bae I was performed. The mutation was not present in 100 normal chromosomes from unrelated unaffected controls. No DNA samples from the unaffected parents were available for testing, but very probably, the mutation occurred *de novo* in this case.

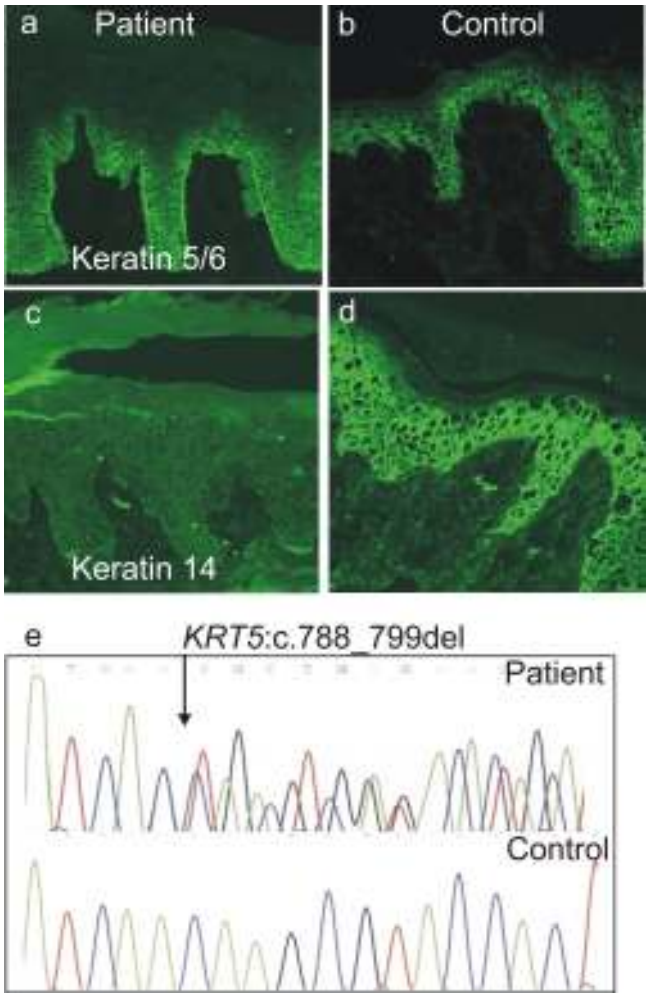


**Figure 7. Patient 4 with EBS-WC.** (a**)** IIF shows a thick epidermis with hyperkeratosis, with normal distribution of keratin 5/6 staining. (b) Control skin stained with keratin 5/6 antibodies. (c) Reduced K14 staining in the skin of the patient. (d) Control skin stained with keratin 14 antibodies. (e) Sequencing analysis revealed an in-frame deletion mutation of 12 bp at codons 263-266.

**Patient 5** was a boy with no family history of a blistering disorder. Clinical and pathological features were consistent with a generalized EBS form. As he grew he developed poikilodermatous changes on his anterior chest and neck and still got easily induced traumatic blisters. With age, the clinical presentation became closer to EBS with mottled pigmentation.

Mutation analysis revealed the presence of a heterozygous duplication within the exon 6 of the *KRT14* gene of 42 nucleotides (c.1104_1145dup); as a consequence at position 368_382, 14 amino acids are duplicated (p.I368_Q382dup) (Figure 8a and b). No other mutation was found in the remaining *KRT14* exons or *KRT5* gene. The mutation was not found in his unaffected father, mother or brother, and therefore was a *de novo* event (p.I368_Q382dup). Presumably, the mutation resulting in a 14 amino acids longer keratin 14 may have a dominant-negative effect.

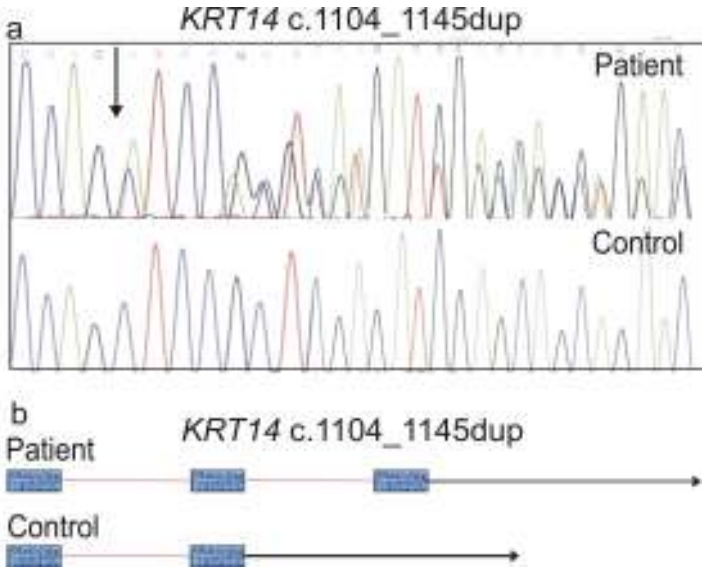


**Figure 8. Patient 5 with EBS-MP.** (a) Part of the sequence analysis of *KRT14* exon 6. In the upper panel: a heterozygous duplication a 42 bp, c.1104_1145dup, was detected in the patient DNA sample. The control sequence is shown in the lower panel. (b) Schematic representation of the duplication and the control sequence.

## Novel missense mutations

**Patient 6** was a 13 y-old girl, who had widespread skin fragility and blistering from birth. There was no family history of blistering. She presented with blisters on large areas of the feet, trunk (Figure 9a and b) and hands, plantar hyperkeratosis, irregular, faint pigmentations around her waist, which had not faded with age, ruptured blisters and erosions on the extremities. IIF of the skin revealed reduced keratin 5/6 staining (Figure 9c).

Sequencing of *KRT5* genomic DNA revealed a point mutation at the first position in codon 186 of one allele, which changes the predicted amino acid coded from a valine (GTG) to a leucine (CTG) (Figure 9e). The mutation was not found in the sample of her unaffected mother. This mutation generated a new Pvu II restriction site. Based on testing with Pvu II digestion, no evidence of a mutation at codon 186 was found in 50 subjects.

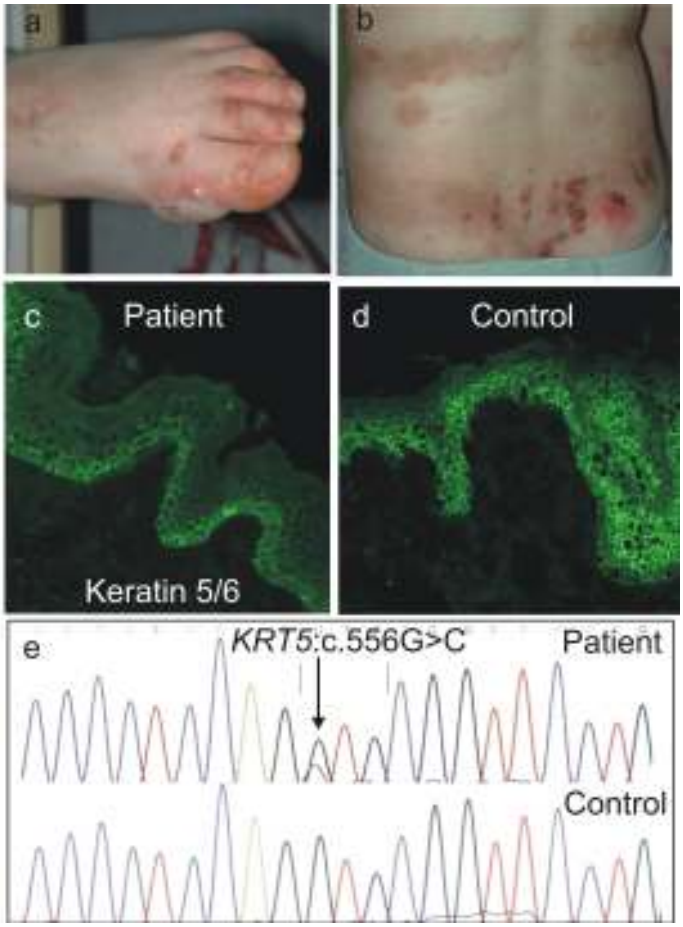


**Figure 9. Patient 6 with EBS-DM.** (a) Multiple blisters on the lateral and dorsal aspects on the left foot. (b) Irregular, faint pigmentations and erosions around her waist. (c) IIF shows reduced keratin 5/6 staining. (d) IIF of normal skin shows positive keratin 5/6 staining. (e) DNA sequence chromatogram of exon 2 of the *KRT5* gene. In the upper panel, a heterozygous point mutation G>C at the first position in codon 186 is shown in the patient's sample. The wild-type sequence is shown in the lower panel.

**Patient 7** was a 14 year-old girl. Her father was also affected with blistering during his childhood and experienced improvement of the clinical picture with age. The patient had blisters predominantly on the extremities, where she also developed milia. IIF of skin demonstrated a thicker than normal epidermis but no difference of keratin 5, 6 staining compared to control (Figure 10a and b).

Sequencing of *KRT5* genomic DNA revealed a point mutation at the second position in codon 417 of one allele, which changes the predicted amino acid coded from an alanine (GCC) to an aspartic acid (GAC) (Figure 10c). No evidence of mutation at the codon 417 was found in 50 subjects by direct sequencing. No DNA samples from the parents were available.

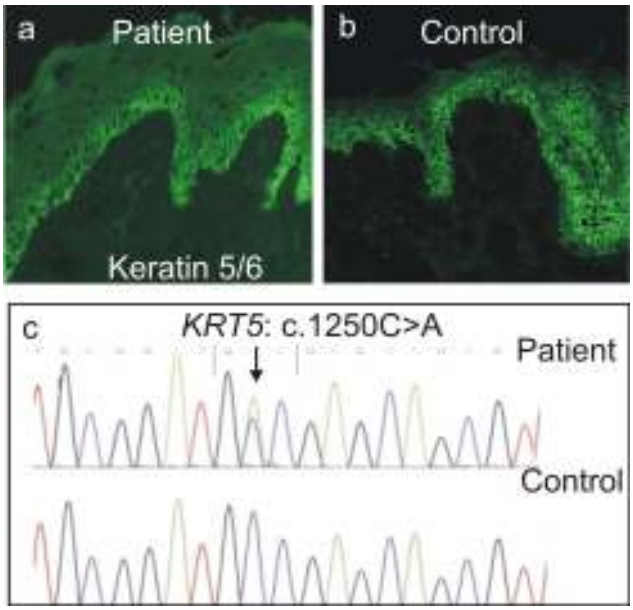


**Figure 10. Patient 7 with EBS-K** (a) IIF staining of the patient skin shows a thicker than normal epidermis with normal distribution of keratin 5/6 staining. (b) Normal control skin demonstrates strong staining with anti-K5/6 antibody. (c) Mutation analysis in exon 7 of *KRT5*: A heterozygous point mutation at the second position in codon 417, c.1250C>A in the upper panel; wild-type sequence in the lower panel.

**Patient 8** was a 22 year-old female. Her mother, maternal grandmother and great-grandfather were also affected. Symptoms started around the age of 17, and consisted of mechanically induced blistering on the feet. Diagnostic antigen mapping showed reduced keratin 14 staining. The other markers including keratin 5 were comparable to the control. Mutation detection found a novel *KRT5* substitution in the patient and her mother, but not in the unaffected father: c.514A>G, leading to the replacement of codon 172 for isoleucine with a codon for valine, p.I172V (Table 20).

**Patient 9** was a 25 year-old female, with no family history of EB. Blistering started within the first days of life. She had generalized herpetiform blisters accentuated on mechanically stressed areas. Grouped blisters were also present on her lips (Figure 11a), perioral areas and oral mucosa. Her toe nails were dystrophic (Figure 11b). She showed hypo- and hyperpigmented mottled skin areas on the legs and palmar keratosis. DNA analysis detected a heterozygous mutation c.364C>G in the exon 1 of *KRT14*, p.L122V (Figure 11c).

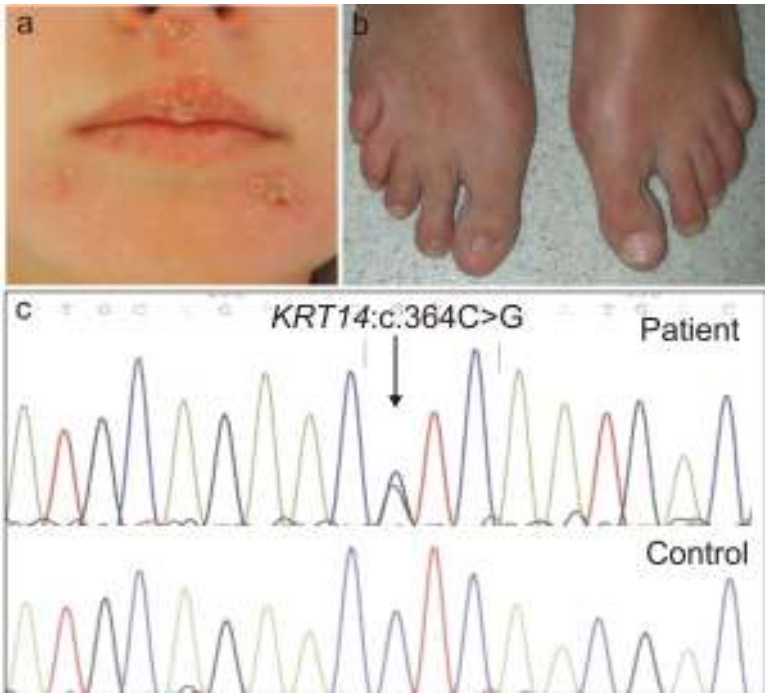


**Figure 11 Patient 9 with EBS-DM.** (a) Some herpetiform blisters on her lip and perioral areas. (b) Toe nail dystrophy. (c) Direct sequencing of genomic DNA of exon 1 of *KRT14* showed a heterozygous C>G transversion at nucleotide position 364, leading to a missense mutation (upper panel); wild-type sequence (lower panel).

**Patient 10** was a 37 y-old female. Also her daughter, sister, mother and grandmother had EB. She had mechanically induced blisters from her first year of life. In the adulthood blistering was restricted to the feet. IIF staining of her skin showed no abnormalities. Direct sequencing of PCR products identified a heterozygous mutation, c.1162 C to G, altering codon 388 of K14 (CGC to GGC) and thus encoding in the affected K14 allele Gly instead of Arg (Table 20).

## EBS with mottled pigmentation and EBS with circinate erythema

**Patient 11** an 8 y-old boy, presented with symptoms of brown pigmentation interspersed with whitish hypo-pigmented spots on his back and extremities (Figure 12a). The clinical appearance was compatible with EBS with mottled pigmentation. He was the first child of non-consanguineous parents. His father also had pigmentation. IIF demonstrated a reduction of K5 and K14 proteins in the epidermis (Figure 12b-e).

Genomic DNA was analyzed for mutations in *KRT5* and a heterozygous point mutation, C > T transition at position 74 causing a p.P25L substitution was found (Figure 12f). The father carried the same mutation.

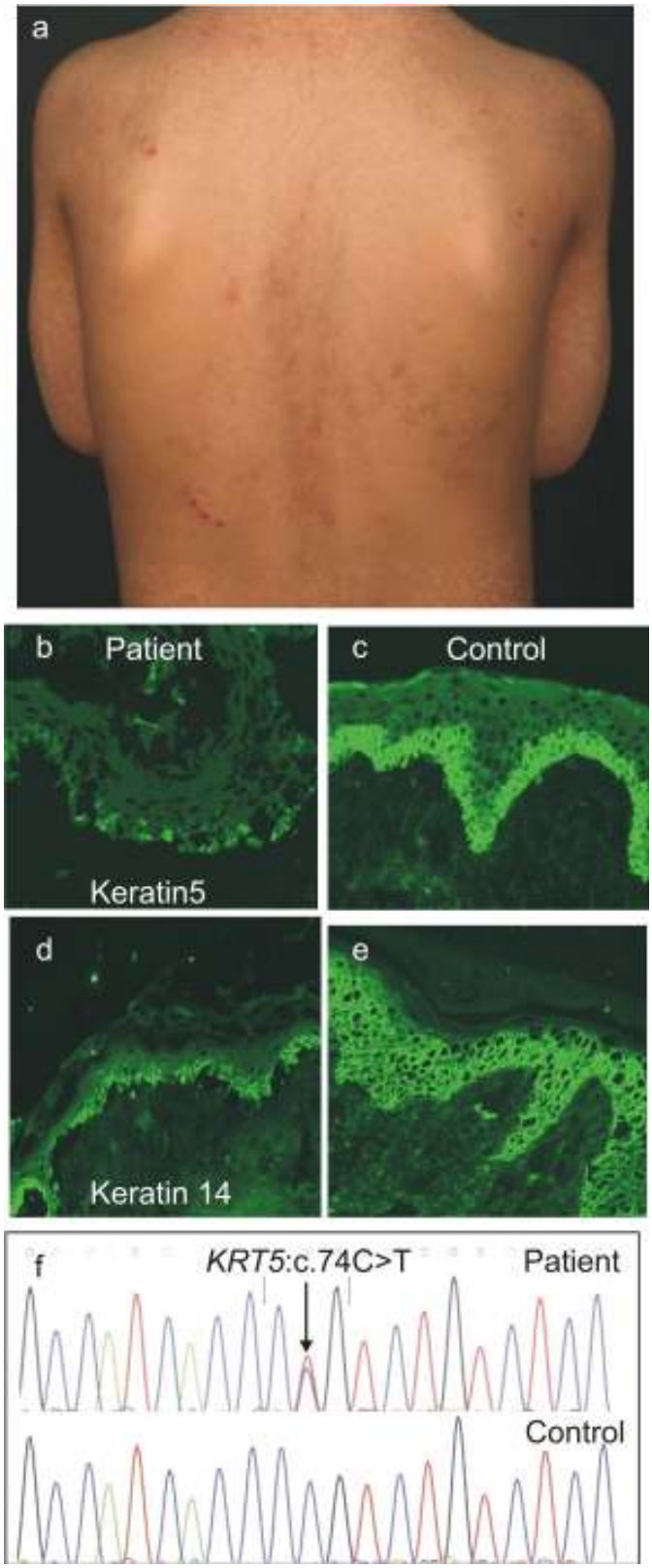


**Figure 12. Patient 11 with EBS with mottled pigmentation** (a) Brown pigmentation interspersed with whitish hypo-pigmented spots on the back and extremities. (b, c) IIF in the skin revealed reduced keratin 5 (b) and keratin 14 (d) staining. (c, e) The normal control showed positive keratin 5 (c) and keratin 14 (e) staining. (f) Direct sequencing of the *KRT5* gene indicated a heterozygous mutation c.74C>T (upper panel, arrow); wild type sequence is shown in the lower panel.

**Patient 14** a boy, currently aged 3y, presented with symptoms of multiple annular erythema on both knees, and toe nail dystrophy since birth (Figure 13a and b). IIF of the skin showed blister formation through the basal cell layer with keratin 5 staining at the blister roof, faint reactivity to anti-K5 antibody and reduced intensity of the pankeratin staining (Figure 13c and e).

**Patient 15** was a girl who had an identically affected sister. They were unrelated to patient 14. She presented with blistering on her extremities and with marked poikiloderma on the trunk but not on the extremities.

Similarly to patient 14, a heterozygous deletion of a guanine nucleotide at position 1649 (1649delG) in exon 9 of the *KRT5* gene was identified (Figure 13g). The deletion is predicted to produce a mutant K5 protein with a frameshift of the terminal 41 amino acids, and mutant protein 35 amino acids longer than the wild-type K5 due to a delayed termination codon. The mutation was absent in both parents of patient 14. No samples were available from the parents of patient 15.

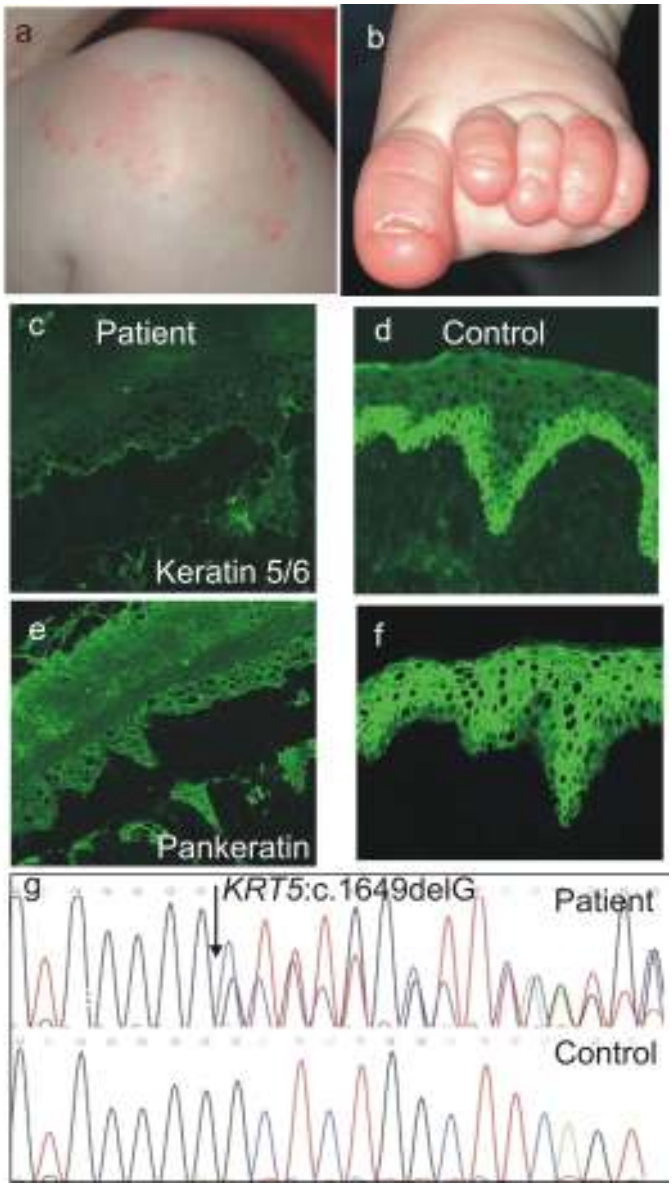


**Figure 13. Patient 14 with EBS with circinate erythema** (a) Annular erythema on the knee. (b) Toe nail dystrophy. (c, e) IIF staining with antibody for K5 (c) and pankeratin (e) showed intra-epidermal splitting and reduced intensity. (d, f) Staining with antibody for K5 (d) and pankeratin (f) in control skin. (g) Part of the sequence analysis of *KRT14*. A single deletion at nucleotide position 1649, leads to a frameshift mutation and delayed termination codon.

Among the 20 patients studied, 10 novel mutations were identified in patients 1-10. Mutations and phenotypes of patients 12, 13 and 16-20 were typical and similar to the previously published, and therefore not described in details in this study (Table 20 section 4.1).

# 5. Discussion

In this study, molecular analysis of 20 EBS patients was performed and 18 different mutations were identified. Together with previous reports we expand the epidermolysis bullosa simplex mutation database. Mutation detection in either *KRT5* or *KRT14* will have to be continued to get a more complete picture of the extensive spectrum of mutations underlying EBS, as well as useful genetic information for families with this genodermatosis.

Epidermolysis bullosa simplex (EBS) represents a large, heterogeneous group of heritable skin-blistering diseases due to fragility of basal layer of epidermal keratinocytes. EBS is a genetic disorder usually with autosomal dominant inheritance. However, in rare families with consanguinity, it can be inherited in an autosomal recessive manner like in our patients 1 and 2. EBS is often regarded as the least severe form of EB; Although usually not life threatening, in the most severe variant, EBS-DM, blistering can be severe enough to be fatal during infancy [21]. Thus far, more than 100 different pathogenic mutations in either *KRT5* or *KRT14* gene have been documented ([41]; http://www.hgmd.cf.ac.uk). Mutations located within the two short (≈10 amino acid residues), highly conserved ends of the helical rod domain are often associated with Dowling-Meara subtype; the milder variants of Weber-Cockayne and Koebner implicate a localization to the L12 linker and central rod domain [10].

The majority of keratin mutations are autosomal dominant and have dominant-negative effects to interfere at the protein level [7;49]. The production of even small amounts of abnormal keratin that polymerizes with normal keratin molecules can disrupt the KIFs framework and lead to tonofilament aggregation and cytoskeletal instability. The truncated polypeptide may not fold or dimerize well and may be degraded. Although undimerized keratins are known to be rapidly degraded [7;35], even short synthetic peptides analogous to the conserved regions of the rod domain can depolymerize keratin filaments [7]. *In vivo,* K14 (type I) is obligatory in pairs with K5 (type II) to form coiled-coil heterodimers which are assembled into higher-order KIFs. K15, a keratin which is similar to K14, is normally expressed in very low levels in basal keratinocytes. Jonkman *et al.* [29] reported patients with severe generalized skin blistering that improved with age and presented hyperkeratosis of the palms and soles. They hypothesized that an up-regulation of K15 compensates for the absence of K14 [29]. This is one way to explain why some affected individuals with EBS had blisters that tend to improve with age, but develop hyperkeratosis.

In our study, the proportion of mutations in *KRT5* (10 of 20; 50%) is equal to *KRT14* mutations (10 of 20; 50%). However, the ratio of *KRT5* and *KRT14* mutations reported in Japanese and Korean EBS patients was 78% to 21% [59]. Whether this equal ratio of *KRT5,* and *KRT14*

mutations is a definite characteristic of a European cohort of patients or not, requires further investigations.

The mutation screening of *KRT14* was facilitated by use of a robust long-range PCR method that specifically amplifies the complete *KRT14* from genomic DNA [57]. The method not only circumvents the need for enzyme digestion of genomic DNA before amplification of each exon, but also is able to detect larger heterozygous genomic deletions. Such deletions might involve one or more exons and would be missed by PCR of individual exons. This *KRT14* mutation detection method is not only efficient, but also rapid and easy.

## Recessive epidermolysis bullosa simplex

In patients 1 and 2, the clinical picture was compatible with the diagnosis of EBS Koebner. Mucosal involvement and other symptoms are difficult to assess because of the very young age and the short time they were available for observation. IIF staining proved to be a very useful diagnostic method, since it showed absence of keratin 14 and thus permitted a rapid identification of the candidate gene. Absence of keratin 14 from the epidermis was caused by homozygous deletion/duplication mutations leading to PTCs. Except for the two novel mutations described here, 11 different *KRT14* mutations associated with recessive epidermolysis bullosa simplex have been published before (Table 26): four nonsense mutations, two missense mutations, one a splice site mutation, one case with compound heterozygozity for nonsense and missense mutations, one a deletion/insertion mutation and two deletion mutations. A less severely affected case reported by Batta et al. (2000) is the recessive case with the shortest expressible *KRT14* sequence to date, predicted to be truncated after only 30 amino acids ([2], patient VI in Table 26). In our patient 1, the truncation of keratin 14 is predicted to start after 143 amino acids, within the very beginning of the rod domain, and in patient 2 after 44 amino acids, within the head domain of keratin 14. Other authors described severely affected keratin 14 “knockout” patients (Table 26). However, no correlation between the position of the PTC and the severity of the phenotype is obvious. Rather, these mutations are more likely to be associated with nonsense-mediated messenger RNA decay. Chan et al (1994) [3] suggested that complete absence of a keratin is less detrimental than the disrupted filament assembly and aggregate formation caused by dominant negative missense mutations [3] The present findings and other reports in the literature agree with this hypothesis in that homozygous missense mutations lead to milder phenotypes with blistering only in the extremities (patients I, X and XI in Table 26), while nonsense mutations are associated with more generalized blistering (patients II, III, IV, V, VII, VIII and IX in Table 26).

Table 26. Review of *KRT14* mutations in recessive EBS

| No. | Phenotype, age (y, years; m, months) | Mutations | Reference |
|---|---|---|---|
| I | Blistering affecting the lateral and dorsal aspects of the feet and plants, two patients aged 20 and 12y | p.E144A/p.E144A | [22] |
| II | Severe generalized blistering, 5y | c.313_314delGC/c.313_314delGC | [42] |
| III | Generalized blistering, 29m | p.Y204X/p.Y204X | [3] |
| IV | Severe generalized blistering, mucous membranes occasionally affected; family with 4 patients aged 74, 67, 47,34y | c.1842-2A>C/c.1842-2A>C | [29] |
| V | Generalized blistering, occasional oral blisters, siblings of 7y and 6y | p.W305X/p.W305X | [7] |
| VI | Mild generalized blistering, 18 m | c.92delT/c.92delT<br>p.I31fs86X | [2] |
| VII | Generalized skin blistering and mild nail involvement and involvement of mucous membranes, 8y | c.744delC/insAG/ c.744delC/insAG<br>p.Y248X | [33] |
| VIII | Widespread blistering and oral mucosal involvement, anemia and failure to thrive in the first year of life, NA | p.Q396X/p.Q396X | [6] |
| IX | Widespread blistering over palms and soles and oral and genital mucosae, NA | p.W305X/p.W305X | [6] |
| X | Blisters over palms and soles, NA | p.Q396X/p.R388H | [6] |
| XI | Blisters over hands and feet, 4y | p.R134C/p.R134C | [24] |
| XII (our patient 1) | Blisters on hands and feet, later generalized, 2y | c.427delC/c.427delC<br>p.L143fsX2 | this study<br>[19] |
| XIII (our patient 2) | Severe blistering of hands and feet, oral erosions, widespread induced blisters, 1y | c.129dupC/c.129dupC<br>p.S44fsX38 | this study<br>[19] |

**Novel deletion / insertion mutations in EBS**

Patient 3 and her mother were affected with EBS-WC and heterozygous for the deletion/insertion mutation in *KRT5*- deletion of GGAG starting from the nucleotide position 1362 and insertion of AGCTGGTA. The father of patient 3 was clinically normal, and not a carrier of the sequence variant. The mutation inherited from the maternal allele, lead to a frameshift and downstream PTC, and might result in premature RNA decay. As shown with IF staining (Figure 6c), reduced levels of K5/6 with breakdown of basal cells was evident in the skin of patient 3. To the best of our knowledge, this is the first deletion/insertion mutation described in *KRT5*.

Mutation analysis of patient 4 showed the presence of a novel heterozygous mutation in *KRT5*, an in frame-deletion of 12 nucleotides, at codons 263-266, encoding the amino acids NKRT in the 1B helical region of K5. This in-frame deletion causes the discontinuity of the keratin 5 polypeptide. The deletion is located in the highly conserved helical rod domain of K5, probably resulting in defective pairing with K14, and thereby disrupting keratin tonofibril integrity. This is likely to lead to aberrant clinical phenotypes, which resemble EBS-WC. This might explain why IIF disclosed reduced K14 staining.

In patient 5, because of the poikilodermatous lesions, the diagnosis of Kindler syndrome was first suspected. Mutation analysis of the *KIND1* gene did not identify any pathogenic mutations and thus did not support the diagnosis. We, therefore, favoured the diagnosis of EBS with mottled pigmentation and tested the *KRT5* and *KRT14* genes. The heterozygous in-frame duplication (c.1104_1145dup) identified in the *KRT14* gene. A recombination could occur between two 13 bp repeats, leading to the 42 bp duplication. It is predicted to lead to a 14 amino acid longer K14 polypeptide (p.I368_Q382dup), and might have a dominant-negative effect. Analysis the *KRT14* exon 6 in the DNA samples of parents and brother of patient 5 revealed normal sequences. The fact, that the mutation was not present in the parents' samples supports the hypothesis of a *de novo* mutation causing a dominant form of EBS. This elongated mutant K14 protein might prohibit normal functions of unaffected K14 or interfere with other protein interactions. It is very probably pathogenic and caused a phenotype which became typical of EBS-MP with age. To the best of our knowledge, this is the first in-frame duplication mutation described in the *KRT14* gene.

**Novel missense mutations in EBS**

The amino acid residues N176 and V186 in 1A domain of K5 are highly conserved among not only type II keratins but also other intermediate filaments, while I172 is conserved among type II keratins (Fig. 14), suggesting that these amino acids play important roles in KIF assembly, stability or function. Mutations which affect important conserved IF amino acids, may interfere with the keratin heterodimer formation and protein-protein interactions [59]. For these reasons, they are very probably disease causing.

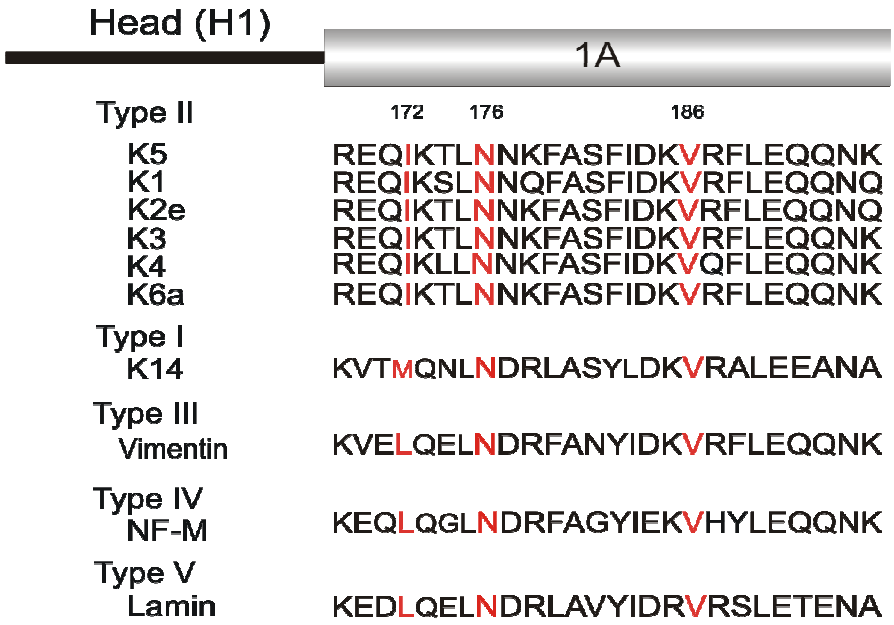


**Figure 14 Schematic representation of a part of the 1A domain of keratin 5.** The α-helical domain of the central rod, 1A, and evolutionary conservation of amino acids sequences along with other type II keratins or IFs. Adopted with modifications from (18).

The mutations V186L and L122V represent conservative amino acid changes and involve no net change of charge; nevertheless the two residues have different sizes, leucine is larger than valine by one $CH_2$ group. An alteration at residue 186 in K5, at position 18 of the 1A helix (c.556G>T; p.V186L) was previously described in association with generalized blistering, diagnosed as EBS-K [34]. However, our patient 6, who was heterozygous for the mutation c.556G>C; p.V186L, had a more severe phenotype and symptoms of EBS-DM indicating phenotypic variability.

The mutated valine residue at position 172 in K5 is the fourth residue within the 1A domain located at the amino-terminal end of the central rod domain [52]. Although this mutation involves the substitution of isoleucine with valine, and yields no change in polarity or charge, the replacement of a larger Ile residue by a smaller Val would be expected to leave a larger cavity in the K5 and K14 heterodimer. A mutation of an adjacent amino acid in K5 (c.519G>C; p.K173N) produces an EBS-K phenotype [52]. Unlike previous heterozygous mutations located within the initial segment of 1A domain of keratin molecules, heterozygous K173N did not result in severe disease or clumping of keratin filaments. Similarly, in patient 8, the heterozygous missense mutation (c.514A>G; p.I172V) caused the presence of localized blisters, diagnosed as EBS-WC.

L122 is a highly conserved hydrophobic amino acid residue in the helix initiation motif of K14. Another mutation at the same amino acid position of *KRT14*, c.364C>T; p.L122F, was described previously in association with EBS-K [58], and c.368 A>G; p.N123S, in association with EBS-DM [50]. In patient 9, the heterozygous missense mutation (c.364C>G; p.L122V) caused the presence of generalized herpetiform blisters, diagnosed as EBS-DM.

R388 is conserved in type I keratins [6]. Replacement of this large and basic residue by a small nonpolar residue in codon 388, residue 83 of the helix 1B region of K14, changes a charged amino acid (Arg) to an uncharged one (Gly). This could give rise to steric hindrance causing incorrect fitting of the K5/K14 coiled-coil [34], and result in a conformational change in the binding sites and impair the association of keratins. In this study, patient 10 with this mutation had localized blistering; similarly to patients with other mutations at the same amino acid position in *KRT14* (c.1162C>T; p.R388C and c.1163G>A; p.R388H) who had been reported previously in association with EBS-WC [1;5;6].

The mutation A417D changed a small apolar amino acid into an acidic one. It altered a conserved residue in the 2B domain of K5 protein, suggesting that this mutation affects the protein folding and protein-protein interactions. Therefore, this missense mutation was likely to be pathogenic. As previously reported, an alteration of codon 418 of K5 (c.1252G>A; p.E418K) located in the stutter region, lead to an interruption in the heptad repeat regularity and was associated with EBS-K [60]. Accordingly, patient 7 showed clinical manifestations of generalized blistering, diagnosed as EBS-K.

**Recurrent mutations in EBS**

The R125 mutation in *KRT14* was one of the recurrent mutations found in this study (2 of 20; 10%). This Arg residue is highly conserved in many intermediate filament proteins and is located in the α-helix initiation motif [8;40;41;55]. Substitution mutations at this site (R125C or R125H) have been identified as 'hotspot' mutations and are causal for EBS-DM. Therefore, this recurrent mutation site is important for the diagnosis of EBS-DM [51]. Our patients 16 and 17 also manifested the clinical features of the Dowling–Meara variant of EBS.

Another recurrent mutation in *KRT5*, P25L, accounts for most of EBS-MP mutations to date [16;17;26;28;37;48]. The P25L occurs within an 18-amino acid motif within the V1 region of the non-helical head domain of K5. *In vitro* assembly studies for K5 showed that the head of K5 is important in formation of the K5/K14 heterodimer and subsequent higher-order assembly [28]. The head of K5 also has a role in desmoplakin I binding, an interaction that binds keratin intermediate filaments to desmosomes [16]. These data suggest that the P25L has an effect on filament elongation and integrity. Patient 11 was heterozygous for the *KRT5* mutation P25L; the clinical appearance was that of EBS associated with mottled pigmentation. His father also exhibited pigmentation and was heterozygous for the same mutation.

The *KRT5* mutation 1649delG, is predicted to add an aberrant sequence of 76 amino acids very rich in alanine and proline residues to the carboxyl-terminal domain of K5. The mutant is 35 amino acids longer than the wild-type K5 [20]. Patients harbouring the identical mutation showed different clinical features as described by Gu *et al* [15] and Horiguchi *et al* [20]. They had either EBS with migratory circinate erythema, or EBS with mottled pigmentation. Patient 14 also presented with symptoms of annular multiple erythema on since birth, while patient 15 present with marked poikiloderma on the trunk. Since the heterozygous mutation was not detected in parents of patient 14, a *de novo* mutation causing a dominant EBS is very probable. Gu *et al* [15] suggested that this mutation in the far 3' end, which creates a delayed termination codon, was likely to cause clinical symptoms by interfering with the functional interactions between K5 and its associated proteins. The aberrant elongated region may interfere with normal K5 folding or mask the epitope in normal K5 [15], which arise from the normal second allele and thus explain the negative IIF staining for K5, K14.

The identical location of a gene mutation might cause several phenotypes of EBS, e.g. EBS-WC, EBS-K, EBS-DM or EBS-MP type. For instance, mutations in the same codon 467 of K5: Ile467Thr was described in a patient with EBS-DM [25], whereas Ile467Leu was described in a patient who had a EBS-WC phenotype [41]. Similarly, different phenotypes were identified in patients with mutations at codon 119 of keratin 14 [5;10;47]. Furthermore, our findings of two

mutations at nucleotide position 1649 of *KRT5* (c.1649delG), leading to different phenotypic expression, further implies that the relationship between the mutation and the phenotype may be not so clear and definite.

## Outlook

The present investigation provides a contribution for expanding the EB mutation database, and to better understanding of the pathogenic mechanisms underlying EBS. Consequently, genetic counselling is available for the couples affected with EBS, and there is hope for future molecular therapies to restore the epidermis to normal function.

There is currently no cure for EBS. The treatment of EBS is typically supportive and preventive, consisting of wound management, adequate nutrition and infection control. The care should focus on prevention and treatment of secondary infections. However, as the genetic defects in EBS, are known, genetic counselling can be performed. Knowledge of genetic defects and molecular pathogenesis will be prerequisites for development of molecular therapies. Concerning gene therapy for EBS, current practices and proposed approaches comprise use of ribozymes or RNAi (http://www.geneclinics.org/profiles/ebs/; http://www.debra-international.org/a_mclean8.htm). Addition of other functional proteins like desmin [11], and induction of a compensating mutation [49], have also been carried out in experimental models. However, as demonstrated in this study, further knowledge on genotype-phenotype correlations and epigenetic factors is needed, before individually designed biological therapies can become clinical reality.

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