Chapter 6

Localized and generalized atrophic benign epidermolysis bullosa due to COL17A1 mutations in the Netherlands

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Mutations in the gene COL17A1 coding for type XVII collagen cause non-Herlitz junctional epidermolysis bullosa (nH-JEB). Here we give an overview of the genotype-phenotype correlation in 11 patients from the Netherlands with type XVII collagen-deficient nH-JEB. Eight patients had features of severe nH-JEB, also known as ‘generalized atrophic benign epidermolysis bullosa’ (GABEB), with generalized blistering, sparse primary and absent secondary hair, nail dystrophy, and enamel hypoplasia. Three patients had more localized blistering confined to hands, lower legs and face, absent or very mild nail dystrophy, normal primary hair and sparse secondary hair. All 11 patients had enamel hypoplasia. We name this milder subtype ‘localized atrophic benign epidermolysis bullosa’ (LABEB). Immunofluorescence (IF) antigen mapping with monoclonal antibodies 1A8C and 1D1 that bind to type XVII collagen, but not to its 97-kDa fragment was completely negative in all GABEB patients, whereas reduced staining was seen in the LABEB patients. DNA analysis identified five novel deletions: 1284delA, 1365delC, 3236delT, 3600-3601delCT and 4425delT. Our data reveal that in patients with COL17A1 mutations a localized phenotype can be differentiated from a generalized phenotype by IF antigen mapping. The data are important for genetic counseling at early age when the clinical phenotype is not yet clear.

Figures 1, 3, 4, 5 and 6 of this chapter can be found in the appendix Color figures on page 185.
Introduction

About ten different genes expressed in the epidermal basement membrane zone (BMZ) have now been disclosed in which mutations lead to the genetic blistering disease epidermolysis bullosa (EB). This diverse group of inherited disorders is characterized by fragility of the skin and mucous membranes. Based on the level of epidermal-dermal separation, EB is traditionally divided into three main categories: simplex, junctional and dystrophic [1]. In EB simplex the cleavage is within the basal keratinocytes of the epidermis, whereas in junctional EB (JEB [MIM 226650, 226700]) the cleavage takes place within the lamina lucida. In dystrophic EB abnormalities in the anchoring fibrils are observed, often caused by mutations in the type VII collagen gene (COL7A1), resulting in tissue separation below the lamina densa. Two subtypes of JEB have been elucidated, the more severe Herlitz type which is frequently lethal during the first two years of life, and the less severe non-Herlitz subset, previously known as generalized atrophic benign epidermolysis bullosa (GABEB), not affecting the lifespan. Mutations in the genes encoding the lamina lucida/densa protein laminin 5 (LM-332: LAMA3, LAMB3, LAMC2), and the hemidesmosomal proteins type XVII collagen (COL17A1) and integrin α6β4 (ITGA6, ITGB4) are associated with JEB. Patients with mutations in COL17A1 are clinically characterized by life-long blistering of skin and mucous membranes, universal alopecia, nail dystrophy, and enamel hypoplasia [2-4]. The disorder does not affect the lifespan, however, recently a patient with the homozygous mutation 4144del4 was reported with a more severe course of his disease [5]. Despite good medical care, the newborn died shortly after birth.

The COL17A1 gene spans 52 kb of the genome on the long arm of chromosome 10 (10q24.3) [6,7]. It comprises 56 exons, ranging in size from 27 to 390 bp, exon 52 being the largest. At least two different mRNA transcripts are formed, differing in their 3’ untranslated region [8]. Although the shorter transcript has a deletion of 610 nt, both variants code for exactly the same type XVII collagen protein, also known as the 180-kd bullous pemphigoid antigen (BP180). This 1,497 amino acids transmembrane protein has a type II orientation in which the N-terminus is cytoplasmic and the carboxyl-terminal end spans the lamina lucida outside the cell. With the 15 collagenous regions (COL1-COL15), present in the ectodomain, three type XVII collagen molecules can form a triple helix on the basis of the Gly-X-Y repeating sequences [9,10]. Through interactions with the hemidesmosome proteins plectin, BP230 and integrin α6β4, this homotrimeric macromolecule connects the keratin intermediate filament network with the basement membrane compartment of
the basal keratinocyte [11,12]. The type XVII collagen ectodomain is proteolytically shed from the cell surface within the membrane proximal 16th noncollagenous A (NC16A) domain (residues 490-566) by zinc-dependent metalloproteinases as a 120-kDa soluble trimer [13,14], designated linear IgA disease antigen 1 (LAD-1). Subsequently, this LAD-1 can be cleaved to a second soluble form of 97-kDa, representing the linear IgA bullous disease antigen of 97 kDa (LABD97) [15]. The full length polypeptide consists of codons 1-1497, whereas the 120-kDa soluble LAD-1 comprises residues 524-1497 [16]. LAD-1 and LABD97 have different N-termini; the N-terminus of LABD97 appears to be seven amino acids downstream compared to that of LAD-1 (Leucine-524 versus Alanine-531) [16]. Furthermore, peptide sequencing studies of LAD-1 did not reveal fragments of the distal 288 amino acids of type XVII collagen, therefore LABD97 was suggested to contain residues 531-1209 [15,16]. However, measurement studies by Hirako et al [17] and the differences in length and mass between LAD-1 and LABD97, suggest LABD97 to be slightly larger with the C-terminus located at the beginning of exon 52, somewhere between residues 1275 and 1310.

Most of the 61 COL17A1 mutations reported thus far are nonsense mutations and insertions or deletions, leading to premature termination codons (PTCs) and causing the GABEB phenotype. In this study we correlated phenotype to genotype in Dutch EB patients with mutations in COL17A1, who were referred to the Center for Blistering Diseases in Groningen. Besides the detection of five novel deletions, we found an IF marker for the milder phenotype that we suggest to name ‘localized atrophic benign epidermolysis bullosa’ (LABEB).

Materials and Methods

Immunomorphological techniques

Punch biopsies were taken from lesional and nonlesional skin, and prepared for immunofluorescence microscopy (IFM) and electron microscopy (EM) as previously described [18]. All monoclonal antibodies have been described previously. Pankeratin stained with CK1 (DAKO), keratin 14 with LL001 (gift from Dr B. Lane, Dundee, UK), plectin with HD121 (gift from Dr K. Owaribe, Nagoya, Japan), integrin α6 with GoH3, integrin β4 ectodomain with 58XB4 (gifts from Dr A. Sonnenberg, Amsterdam, the Netherlands), uncein with 19-DEJ-1 (gift from Dr J.-D. Fine, Nashville, TE), LM-332 with GB3 (gift from Dr G. Meneguzzi, Nice, France), and type VII collagen with LH7:2 (gift from Dr I. Leigh, London, UK). Monoclonal antibodies specific for type XVII collagen were: 1A8C (epitope residues 155-163 in exon 8-9) (gift from Dr K.
Owaribe) for the endodomain on the full length polypeptide, NCC-Lu-226 (gift from S. Hirohashi, Tokyo, Japan) [19] and 233 (epitope residues 1118-1143 in exon 48-49) (gift from Dr K. Owaribe) for the ectodomain on the shed LABD97, LAD-1 and the full length polypeptide, and 1D1 (epitope residues 1357-1387 in exon 52) (gift from Dr K. Owaribe) for the ectodomain on the full length polypeptide and LAD-1 [20]. LAD-1 was specifically stained with 123 (gift from Dr P. Marinkovich, Stanford, CA), 97-1 and 97-2 (gifts from Dr J. Zone, Salt Lake City, UT). In combination with these primary mouse monoclonal antibodies we used Alexa488-conjugated goat anti-mouse IgG (Molecular Probes, Eugene, OR) as secondary step. For the rat antibody GoH3 a goat anti-rat IgG antibody (Southern Biotechnology Associates, Birmingham, AL) was used as secondary step.

Identification of the mutations
Genomic DNA was extracted from peripheral blood. Mutation analysis was performed by amplification of genomic DNA (GenBank accession numbers U76564-U76604) using COL17A1-specific primers as described by Gatalica et al, and followed by sequencing of the exons [7]. For exon 46, we used instead the sense primer 5'-GTGCTTCAGGTCACCTCCGT-3' and the antisense primer 5'-ACGAGGAGATGAGGCTCTGG-3'. Amplification conditions were 5 min at 94°C, followed by 35 cycles at 94°C for 45 s, 60°C or 55°C for 45 s and 72°C for 45 s and a final extension at 72°C for 7 min.

RNA analysis
To determine the effect of the 4425delT mutation on the mRNA, RNA was analyzed from a nonlesional skin sample of the upper arm of patient EB 168-01. Four cryosections of 10 µm thickness were cut and transferred with a sterile needle into lysis buffer (Stratagene Europe). RNA was isolated according to the accompanied Absolutely RNA Microprep Kit protocol (Stratagene Europe) and eluted with 15 µl instead of the advised 30 µl elution buffer to get a more concentrated RNA sample. Afterwards cDNA was synthesized. In short, to 11 µl RNA, 1 µl 10 mM dNTP mix and 1 µl random primers (Invitrogen, Breda, The Netherlands) were added. The mixture was incubated at 65°C for 5 min and then transferred to ice. After cooling down the sample, 4 µl 5 x first strand buffer, 1 µl 0.1 M DTT, 1 µl Superscript III reverse transcriptase and 1 µl RnaseOUT (all Invitrogen) were added. Then, incubation was at 25°C for 5 min, 50°C for 60 min and 70°C for 15 min. From this cDNA sample 10 µl was used in a PCR reaction with primers F4337 5'-GCATCAGCAAGGTCTTCTCT-3' and R4598 5'-CACGGCTTGACAGCAATACT-3' in a final volume of 50 µl. For the nested PCR, also primers were used that annealed to exon 53 and exon 56 for the
forward and reverse respectively. 1 µl of PCR product of the first PCR was used for the second PCR with primers F4367 5’-CGGACCTCATGGACTTCTTC-3’ and R4596 5’-CGGCTTGACAGCAATACTTC-3’ in a final volume of 50 µl. The amplification product was analyzed by 4% agarose gel electrophoresis and sequenced.

Results

Patients included from former studies

The diagnosis was established in each patient on the basis of clinical findings, IF antigen mapping and electron microscopy (for description see Materials and Methods). Clinical characteristics of seven patients have been previously described in several articles (table 1). Patient EB 026-01 with a mosaic pattern of clinically unaffected patches located on the extensor surfaces of arms and hands, as well as a patch on her ankle, was described in the articles of Jonkman et al [21] and Pasmooij et al [22]. Patients EB 011-01, EB 025-01 and EB 035-02 carried all the 2342delG deletion on at least one of their COL17A1 alleles [23]. Microsatellite marker analysis revealed the apparent founder effect of this 2342delG mutation in the eastern part of the Netherlands. As suggested patient EB 035-02 was a compound heterozygote for the 2342delG mutation and the 3781C→T (R1226X) transition [23]. Due to the presence of in-frame exon skipping in patients EB 098-01 [24] and EB 086-01 [25] followed by the production of a slightly smaller type XVII collagen protein, the blistering was less severe and more confined to hands, lower legs and face. Primary hair on scalp, face and body was normal, whereas secondary hair was not yet developed or lacking in axillae and pubis. Electron microscopy in patient EB 098-01 showed a lucidolytic split and in patient EB 086-01 an intracellular ‘pseudojunctional’ cleavage. We suggest to name this subtype displayed by patients EB 086-01 and EB 098-01 LABEB. Remarkably, two epitopes 1A8C and 1D1 were not completely absent in these LABEB patients: epitope 1A8C is specific for the full length, whereas epitope 1D1 recognizes the full length and the 120-kDa LAD-1, but not the 97-kDa fragment. In contrast, these epitopes were absent in all GABEB patients. In patient EB 093-01 also revertant mosaicism had occurred [22]. Since birth, this proband had generalized blistering after minor trauma, except for a small patch of clinically unaffected skin on his right middle finger.

Patient EB 084-01

A 6-year-old boy, patient EB 084-01 (fig. 1A (III/1)), was the child of healthy nonconsanguineous parents and the first case of EB in the family (fig. 1A). The
parents showed normal toenails and teeth. Since birth the patient had blisters after minor trauma. Generalized bulla and erosions mostly without milia were present. Sometimes blisters in the mouth occurred as well. Eyelashes, eyebrows and scalp hair were present, although the latter reduced. Some fingernails were dystrophic, and two were absent. No abnormalities of the eyes and nose were observed. Ultrastructural examination of areas of epidermal-detachment confirmed that the plane of cleavage was in the lamina lucida, with the blister floor covered by the lamina densa. In the blister roof the basal cells lacked hemidesmosomes, whereas in the area with an intact basal membrane the hemidesmosomes appeared hypoplastic. A junctional blister was also seen in the skin specimen used for immunofluorescence microscopy antigen mapping; keratin (CK1) stained the blister roof, whereas LM-332 (GB3) and type VII collagen (LH7:2) stained exclusively the blister floor. The monoclonal antibody 19-DEJ-1, the marker for JEB, was completely absent. Staining with monoclonal antibodies against type XVII collagen, LAD-1 and LABD97 was all completely negative (1A8C, 1D1, NCC-Lu-226, 123 and 97-2), resulting in the diagnosis of GABEB with loss of type XVII collagen. This is remarkable, since in our experience most 1A8C/1D1-negative patients with GABEB have residual binding of the 233 and NCC-Lu-226 antibodies.

Subsequently, sequencing of COL17A1 revealed the compound heterozygous presence for a novel deletion in exon 15, 1284delA (fig. 1B), and the known Dutch mutation, 3432delT in exon 48 in proband EB 084-01. Both parents were carriers, the father of the mutation 1284delA and the mother of 3432delT.

![Pedigree](image.png)

**Figure 1** (A) Pedigree of patient EB 084-01 (III/1). (B) Sequence analysis of exon 15 of the COL17A1 gene shows the heterozygous presence of the 1284delA deletion in patient’s DNA (lower sequence). Sequencing of control DNA revealed wild-type sequence only (upper sequence). The deletion of A at position 1284 results in a novel sequence beginning at amino acid 394 and concluding in a PTC at position 402.
Healthy fathers of patients EB 084-01 and EB 086-01

In addition to the patient’s skin, also biopsy specimens of the upper arm of two fathers were investigated. The father of EB 084-01 did not show any clinical abnormalities of skin, teeth, nails and hair. Staining for the endodomain (1A8C) was reduced in basal and first suprabasal keratinocytes (fig. 2A and 2B). The C-terminal part of the ectodomain of type XVII collagen (1D1) showed slightly reduced linear staining along the epidermal BMZ (fig. 2C and 2D). Also in the normal skin of the father of patient EB 086-01, carrier of the splice-site mutation 1877-2A→C, an aberrant IF staining of type XVII collagen was seen [25]. The results were similar to the IF mapping of the father of patient EB 084-01, slightly reduced staining of monoclonal 1D1 confined to the epidermal BMZ, and reduced staining of 1A8C.

Figure 2 Reduction in brightness and alteration of distribution of type XVII collagen in heterozygous carriers of a recessive COL17A1 mutation. The cytoplasmic staining of basal and first suprabasal cells by monoclonal antibody 1A8C was significantly reduced in the skin of father of patient EB 084-01 (A) compared with the control (B). Staining with monoclonal 1D1 specific for the extracellular tail of type XVII collagen was slightly reduced along the epidermal BMZ (C) compared to normal control skin (D).

Patient EB 134-01

This patient, a 3-year-old boy (fig. 3A (III/1)), was the first and only child of healthy nonconsanguineous parents. In the family was no history of blistering diseases. Since birth, blisters and erosions occurred after minor friction on hands, feet, lower legs, bottom, and face. No milia and congenital localized absence of skin were observed. The nails of the fingers were intact and the hair was normal. Hyperkeratosis was present on the feet. Growth and teeth development were normal. Ultrastructural examination of healthy skin of the upper leg disclosed hemidesmosomes that were strongly reduced in size and number. The tonofilaments
did not project sufficiently to the basal cell periphery. IF antigen mapping showed a junctional blister, with keratin exclusively in the blister roof (CK1) and LM-332 (GB3) and type VII collagen (LH7:2) in the blister floor. Staining with monoclonal antibodies 1A8C and 1D1 was negative in an intact skin sample of the upper leg. Staining with monoclonal antibodies NCC-Lu-226 and 233, binding to all three forms of type XVII collagen i.e. 180-kDa, 120-kDa and 97-kDa, was strongly reduced. Normal staining was observed for LM-332, type VII collagen, plectin (HD121) and integrin $\alpha 6\beta 4$ (58XB4, GoH3). Taken together, the clinical presentation, electron microscopy and IF antigen mapping pointed to GABEB.

DNA analysis of $COL17A1$ showed the compound heterozygosity in this patient's DNA for a paternal mutation in exon 16, 1365delC, and a maternal mutation, 3600-3601delCT in exon 49 (fig. 3B).

Figure 3 (A) Pedigree of patient EB 134-01 (III/1). (B) Automated sequence analysis of exon 16 of $COL17A1$ disclosed the heterozygous frameshift mutation, 1365delC, in paternal and patient's DNA (lower sequence), whereas the mutation in control DNA (upper sequence) was absent. The deletion of this C leads to a frameshift beginning at amino acid 421, and resulting in a missense stretch of 71 residues and finally a PTC at position 492. (C) On the other allele PCR-amplified DNA of exon 49 showed the maternally inherited CT deletion at position 3600. This mutation results in a PTC at position 1171.
Patient EB 117-01

Patient EB 117-01 was a 43-year-old woman (fig. 4A (III/3)), who developed blisters after minor trauma since birth. She had had three brothers from whom two (fig. 4A (III/2 and 4)) had a blistering disease and died during the first year of their lives. Her consanguineous parents were not affected. Physical examination showed erosive areas with blisters and erythema on the lower legs, knees, and the extensor surfaces of the elbows and lower arms (fig. 4B). Sometimes, blisters occurred in the mouth. Locomotion was problematic. The patient had universal alopecia without scarring; vellus hair, eyelashes, eyebrows and secondary hair were all absent. The teeth, already small and brown at the age of 14, had been extracted. A few fingernails were absent, and the small toenails were either dystrophic or absent. In 2001 three squamous cell carcinomas (SCC) developed on the right knee, an area which was prone to blister formation. The lesions were excised, but one year later a metastasis was detected in a lymph node on the right upper leg. The patient refrained from further treatment and died in June 2003.

Electron microscopy showed epidermal-dermal separation at the level of the lamina lucida. The lamina densa covered the blister floor. The biopsy specimen of healthy skin of the right lower leg revealed the almost complete absence of hemidesmosomes, which were smaller in size than in normal human control skin. IF microscopy antigen mapping showed a lamina lucida level of blister formation, keratin (CK1) exclusively present in the blister roof, and LM-332 (GB3) and type VII collagen (LH7:2) exclusively present in the blister floor. In a healthy skin sample of the right leg all epitopes of type XVII collagen, LAD-1 and LABD97 (1A8C, 1D1, 233, NCC-Lu-226, 123, 97-1) were completely absent. Staining of LM-332 and type VII collagen was normal. In conclusion, the diagnosis was GABEB with complete deficiency of type XVII collagen.

PCR-amplification of all COL17A1 exons, followed by sequencing showed the homozygosity of patient EB 117-01 for a novel deletion in exon 46, 3236delC (fig. 4C). The early death of her two brothers may be explained by poor care conditions in history.
Patient EB 168-01

Index patient EB 168-01 (fig. 5A (III/5)), a 38-year-old man, born in a small village in Kurdic area in Turkey, was the child of healthy consanguineous parents. One 21-year-old cousin of his father's family was affected (fig. 5A (III/3)), whereas his children were clinically healthy (fig. 5A (IV/1-2)). Blistering was mild and localized, confined to the hands, feet and face (fig. 5B). It was possible for him to run for seven kilometers without developing blisters on the feet. The blisters healed with hyperpigmentation without milia. Primary hair on scalp, face and body was normal, whereas secondary hair in axillae and pubis was sparse. Mucous membranes were not involved. The teeth enamel was hypoplastic and showed pits. Toenails were slightly dystrophic, whereas his fingernails showed subungual hyperkeratosis.

Ultrastructural examination revealed a subepidermal blister in a skin specimen of the hand with the cleavage plane underneath the basal cell layer, in accordance with a lucidolytic split. In another biopsy specimen of healthy skin of the upper arm all structures of the BMZ were normal. The number and structure of the hemidesmo-
somes including the subbasal dense plate was also normal, although the diameter of the hemidesmosomes was slightly reduced.

IF antigen mapping of healthy rubbed skin showed keratin 14 (LL001) in the blister roof, and type VII collagen (LH7:2) and LM-332 (GB3) in the blister floor. Integrin \( \alpha 6\beta 4 \) (moabs GoH3 and 58XB4) stained both the roof and the floor of the blister, implying that the split level was high in the lamina lucida. IF antigen mapping of healthy nonlesional skin showed reduced expression and loss of lateral staining of the 1A8C and 1D1 epitopes of type XVII collagen. Staining of NCC-Lu-226 and 233 was only slightly reduced, both apical-lateral and along the epidermal BMZ. Plectin (moab HD121) and integrin \( \alpha 6\beta 4 \) (moabs GoH3 and 58XB4) stained with normal pattern and intensity as normal human control skin. The clinical, IF and EM findings fitted the diagnosis LABEB with reduced expression of type XVII collagen.

Since the parents of patient EB 168-01 were second cousins, it was not surprising that \( \text{COL17A1} \) mutation analysis disclosed the same deletion of a T at position 4425.
in exon 54 on both alleles (fig. 5C). This mutation results in a frameshift with the introduction of a terminal missense stretch of 69 amino acids starting after glycine at position 1441. In the wild-type protein only 56 amino acids are present after glycine-1441. To exclude the possibility of skipping of the 62 bp (63 in the wild type) mutation-bearing exon 54, RNA was isolated from cryosections of the nonlesional skin biopsy of the upper arm. cDNA was synthesized, followed by nested PCR with forward primers located in exon 53 and reverse primers in exon 56. On agarose gel no smaller fragments than the expected 220-bp amplicon were present. Sequencing of this amplimer showed the deletion of nucleotide 4425 in the mRNA. The function of the produced type XVII collagen polypeptide was apparently limitedly impaired by the aberrance of the NC1 tail domain.

Discussion

In this study of 11 EB patients with type XVII collagen deficiency due to COL17A1 mutations we could delimit by IF antigen mapping a new, clinically milder, LABEB phenotype from the more severe GABEB phenotype. Clinically, LABEB was distinguishable from GABEB by mild blistering more confined to hands, feet, and face, limited nail dystrophy, and normal primary hair. LABEB was differentiated from GABEB with IF antigen mapping by reduced expression of the 1A8C and 1D1 epitopes of type XVII collagen (table 1). Moreover, this study found for the first time altered IF antigen mapping in asymptomatic heterozygous carriers of COL17A1 mutations. Specifically, the type XVII collagen epitopes 1A8C and 1D1 were reduced and redistributed in the skin of the carriers, similarly but to a lesser extent than in LABEB patients. We suggest that this can be explained by increased concentrations in the skin of respectively the full length type XVII collagen and its processed forms LAD-1 and LABD97. The first sign of reduced expression is decreased staining of the 1A8C epitope, which denotes loss of the full length molecule. As the reduction becomes more severe the 1D1 epitope, synonym to both the full-length molecule and the 120 kDa LAD-1, is also lost. Finally, the 233 epitope, present on all three forms, disappears. Apparently LABD97, which is deposited in the lamina lucida, is present as the major form. Interestingly, the staining of the lateral cell borders is more influenced by the lower polypeptide production than the staining of the epidermal-dermal junction. Due to the splice-site mutation 1877-2A→C two shortened in-frame mRNA transcripts are generated with different deletions of respectively the first 9 amino acids of exon 22 and the complete 21 amino-acids long exon 22 [25]. The reduced lateral staining in the father of patient EB 086-01 might not only be caused by the lower-level of protein production, but also by destabilization of the homotri-
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**Table 1**: Patients with COL17A1 mutations from the Netherlands.
meric structure due to type XVII collagen molecules with different COL15 lengths. Thus, the carrier status of a COL17A1 null mutation appears to eliminate apical-lateral expression of type XVII collagen in basal cells although this has no effect on the clinical phenotype, possibly as a result of haplotype insufficiency.

The LABEB phenotype in patient EB 086-01 was caused by the production of two in-frame transcripts due to the 1877-2A→C mutation [25]. It was the only patient in whom the cleavage was at the intracellular level. In case of the 2356C→T (Q751X) transition in LABEB patient EB 098-01, type XVII collagen was rescued by deleting the PTC-containing exon 30 [24]. Accordingly, in both cases a smaller type XVII collagen protein with a deletion in the 15th collagenous domain could be detected on immunoblot. It should be pointed out that these deletions did not disturb the Gly-X-Y-motif. Patient EB 168-01, the third LABEB patient, was homozygous for a novel deletion in exon 54 -4425delT- in the first collagenous domain. This mutation leads to a terminal missense stretch of 69 amino acids. It is conceivable that the introduction of this missense C-terminal stretch will lead to aberrant trimers as this first collagenous domain is conserved between mouse and human, suggesting an important function for this region [26]. However, the phenotype of patient EB 168-01 was extremely mild indicating that the first collagenous domain is not essential for trimer formation. In accordance, Areida et al [27] already showed that the formation of the triple helix proceeds in an N- to C-terminal direction. Also important to this mild phenotype is that despite the 4425delT mutation wild-type LABD97 can be produced, and although its precise function is unclear, it almost certainly is involved in epidermal-dermal adhesion. Similar, in another patient with a homozygous mutation in exon 54 - 4410_4413dupCATT- also reduced IF staining of type XVII collagen was observed [28]. In contrast to 4425delT, this mutation leads to a shorter collagen XVII protein. More precisely, the insertion resulted in a missense stretch of 18 amino acids after isoleucine 1436 and elimination of the 43 most C-terminal amino acids. Apparently, mRNA transcripts with PTC-mutations in exon 54 are not completely degraded by nonsense mediated RNA decay, the surveillance mechanism that detects and degrades RNA transcripts containing nonsense mutations.

The delineation of the LABEB phenotype, that can have a junctional (patients EB 098-01 and EB 168-01) or intracellular pseudojunctional split level (patient EB 086-01), encourages us to leave the consensus classification on EB behind [1] and to comply to the classification proposed by Uitto [29]. LABEB and GABEB due to COL17A1 mutations are then classified as hemidesmosomal EB, which may have a split on both sites of the plasma membrane, whereas mutations in the genes for LM-332 result in JEB. The split level in collagen XVII deficient GABEB is never in the LM-332 zone, but located in the higher lamina lucida, whereas the split in LM-332
deficient JEB cleaves through the LM-332 zone, and thus is located in the lower lamina lucida. The difference between the upper and lower lamina lucida split can be made by IF antigen mapping, but not by electron microscopy [30].

Figure 6 Schematic representation of type XVII collagen and the COL17A1 gene with the identified mutations. The protein consists of three domains, an intracellular domain of 466 residues (green), a transmembrane domain of 23 amino acids (red) and an extracellular tail of 1008 residues with fifteen interrupted collagenous domains (blue) and sixteen noncollagenous domains (yellow). The COL17A1 gene encoding this protein, depicted above, contains 56 exons. All COL17A1 mutations found so far are indicated. The mutations in red are COL17A1 mutations that were identified in patients with EB in the Netherlands. The novel deletions are boxed. Note: color figure on page 186.

The novel mutations found in this study are located in exons coding for the intracellular domain (1284delA in exon 15 and 1365delC in exon 16) and the ectodomain (3236delC in exon 46, 3600-3601delICT in exon 49 and 4425delIT in exon 54). These five new deletions in the COL17A1 gene bring the total number of described mutations on 66 (fig. 6). The mutations 2342delG, 3432delIT and 3781C→T (R1226X) occur more frequently in the Dutch population. All were present in three unrelated patients (table 1). Microsatellite analysis has shown previously a founder effect for 2342delG [23]. Most likely the mutations 3432delIT and 3781C→T represent also founder effects. Of all mutations reported thus far, a large percentage (68%) comprises nonsense mutations and deletions or insertions resulting in PTCs. Furthermore, it includes nine missense mutations of which four are located in the largest collagenous domain, COL15. These are G609D [31], G612R [31], G627V [32] and G633D [33]. The preservation of Gly in every third position of the amino acid sequence (Gly-X-Y)_n.
is required for the formation of the collagen triple helix structure. The Gly residues are buried at the center of the triple helix, in a position that accommodates no other residue. Destabilization of this motif through a missense mutation leading to the replacement of one Gly will affect the interaction between the \( \alpha \)-chains. If protein is formed with such a mutation a dominant negative effect can be expected. Evidence for this is found in dental abnormalities as enamel hypoplasia and pitting, observed in carriers of missense mutations G609D, G612R and G627V [31,32]. In case of the G633D proband the parents had a history of dental problems, although it could not be proven if this was due to the glycine substitution [33]. In our population, even though the fathers of patients EB 084-01 and EB 086-01 showed an aberrant immunofluorescent pattern, no dental abnormalities were detected in any of the carriers of \( \text{COL17A1} \) mutations. Dental defects however are obligate in LABEB patients with type XVII collagen rescue by exon skipping. We found no enamel hypoplasia in the family members of proband EB 026-01 after careful examination, although Floeth and Bruckner-Tuderman (1999) described enamel hypoplasia in carriers of the same R1226X mutation [34].

In patient EB 117-01, three SCCs on the right leg had developed at the age of 41 over a period of 15 years. In dystrophic EB the development of SCC is a well-known complication, occurring in both recessive and dominant forms [35,36]. By contrast, only eight GABEB patients, including EB 117-01, have been reported with cutaneous SCC [37-40]. The majority, six, were LM-332 deficient [39]. Interestingly the GABEB patients developed their SCCs after the age of 30, whereas in recessive dystrophic EB the increased risk starts after the age of 15. Also of note is that no SCCs were reported in patients with the LABEB phenotype. In case of the reported homozygous male with the \( \text{COL17A1} \) 4003delTC mutation the age of onset was 58 years [38]. The tumours in the two GABEB patients were on the limbs at sites of chronic wound healing. In our female patient EB 117-01 the carcinomas developed on her knee, a spot where frequently blisters occurred. Hence, clinically the risk of developing SCC appears to parallel the severity and extent of ulceration and scarring in the skin. Notably, patient EB 117-01 was quite severely affected. Her skin showed loss of staining with all monoclonal antibodies directed against type XVII collagen, including the absence of the 233 and NCC-Lu-226 epitopes shared by type XVII collagen, LAD-1 and LABD97. A marker for the development of SCC might therefore be the absence of LABD97 [41], and as such might be an indication for regular preventive oncologic screening of the skin. Only GABEB patient EB 084-01 was similarly negative for all epitopes, whereas the other GABEB patients all showed some residual staining with 233 and/or NCC-Lu-226. As a side observation this study also reveals that monoclonal antibodies 233 and NCC-Lu-226 do not
cross-react with other polypeptides, and that the residual staining of 233 and NCC-Lu-226 in absence of staining with other monoclonals indeed reflects type XVII collagen protein product.

In summary, 11 patients were investigated with mutations in COL17A1. On basis of immunofluorescence microscopy with monoclonals 1A8C and 1D1 directed against type XVII collagen, a localized EB phenotype could be distinguished from a generalized EB phenotype, thereby making it possible to predict the clinical phenotype at early age. Our data underscores once more the prognostic importance of IF antigen mapping in predicting the severity of the disease.

Acknowledgments

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