Genotyping of unusual phenotypes of epidermolysis bullosa
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A very mild form of non-Herlitz junctional epidermolysis bullosa: BP180 rescue by outsplicing of mutated exon 30 coding for the COL15 domain

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Abstract

Mutations in the gene COL17A1 cause non-Herlitz junctional epidermolysis bullosa. Here, we describe patient EB 089-01 who, despite two heterozygous mutations in COL17A1, has an extremely mild form of the disease missing most of the characteristic clinical features. DNA analysis revealed a frameshift mutation 3432delT and a nonsense mutation 2356C→T (Q751X). cDNA analysis showed that the deleterious effect of the latter mutation was skirted by deleting the premature termination codon containing exon 30. In this way, the reading frame was restored, resulting in a 36 nucleotides shorter mRNA transcript. Immunoblot analysis showed expression of the 180-kDa bullous pemphigoid antigen (BP180 or type XVII collagen) with a slightly higher SDS-PAGE mobility, in line with the deletion of 12 amino acids from the COL15 domain. Immunofluorescence of skin sections showed diminished, but correctly localized expression of type XVII collagen, and this, in concert with the mild clinical phenotype, suggests that this COL15 mutated type XVII collagen is still partly functional.
Introduction

The 180-kDa bullous pemphigoid antigen (BP180, type XVII collagen) is a transmembrane protein of the hemidesmosome [1]. The extracellular carboxy-terminal tail of the molecule contains 15 collagenous domains (COL1-COL15), from which COL15 with 242 amino acids is by far the largest [2]. Apart from the full-length protein a second 120-kDa soluble form, named LAD-1, exists. Mutations in the gene for type XVII collagen (COL17A1) [3,4] result in non-Herlitz junctional epidermolysis bullosa (nH-JEB), a recessively inherited bullous disease of skin and mucosae. Complete deficiency of type XVII collagen is clinically characterized by congenital generalized blistering with mild atrophic scarring, mild mucosal involvement, incomplete universal atrophic alopecia, pigment changes, as well as rudimentary nails and dental abnormalities [5].

Methods

All methods have been extensively described by us before. For immunofluorescence analysis, we used unfixed cryostat sections (4 µm) of snap frozen skin biopsies [6]. For analyses of cDNA and protein, we used extracts of keratinocytes grown from 4-mm skin biopsies in serum-free medium [7]. For immunoblotting extracts were prepared by solubilizing cells with Laemmli sample buffer [8]. RNA was isolated by harvesting cells with 1 ml/25 cm² TRIZOL (Invitrogen, Breda, The Netherlands) and from this first strand cDNA was prepared using the M-MLV reverse transcriptase cDNAse synthesis system with oligo dT priming (Invitrogen). Primers used to amplify mutated regions were as follows:

1. nt 2017-2387, downstream 5’-CCTGGATCTGGAGAGAAAGG-3’ upstream 5’-CCAGTAAGACCTTGTTCACC-3’.
2. nt 3321-3710, downstream 5’-GTACGGTGTCAGCTTGTTCT-3’ upstream 5’-GAGAGGTCCTCCACGCTGAT-3’

PCR conditions were 35 cycles of 30 s at 94°C, 30 s at 60°C, and 90 s at 72°C. Genomic DNA was extracted from peripheral blood using 6 M NaCl and chloroform [9]. Two intron primers at the borders of exon 30 were used to amplify exon 30 [3]. All cloning for sequencing was performed using the pCR4-TOPO vector (TOPO II TA Cloning kit, Invitrogen).
Results

Here, we describe a 36-year-old Dutch male patient (EB 098-01) with an unusually mild form of blistering. He was the child of healthy nonconsanguineous parents and the first and only case of epidermolysis bullosa in the family. A few days after birth, blisters developed under the nails. After puberty, the blister frequency slightly reduced. The blister formation was mild without scarring or milia and mostly confined to palms, back of the hands, and the lower legs. There was depigmentation on the wrists and the lower part of the back. On the left lower leg, there were lichenoid pigmented papules and excoriations. Eyelashes and scalp hairs were absent. One of the eyebrows showed partial alopecia. The teeth were abnormal.

Immunofluorescence antigen mapping of intact skin showed abnormal expression of type XVII collagen. Staining of the 1A8C epitope specific for the intracellular domain was reduced (+) compared to healthy control skin (4+) (fig. 1A and 1B). In contrast, 1D1 staining for the extracellular domain was only slightly reduced (fig. 1C and 1D). Also monoclonals 123 and 97-1, specific to the LAD-1 molecule, stained with slightly reduced intensity (data not shown). Integrin α6β4, laminin-332, type VII collagen and keratin 5 were normal. Ultrastructural examination of lesional skin revealed that the general plane of cleavage was through the lamina lucida fitting a diagnosis of junctional epidermolysis bullosa.

Figure 1 Type XVII collagen expression is reduced in patient skin. Immunofluorescence microscopy of the epidermal basement membrane zone using monoclonal antibodies 1A8C (A, B) and 1D1 (C, D), respectively, to the endo- and ecto-domain of type XVII collagen on skin specimens of the patient (A, C) and healthy control skin (B, D). Expression of the 1A8C epitope is reduced, whereas the 1D1 epitope is only slightly reduced. While both mutations are in the extracellular domain, the staining for the intracellular domain is more heavily reduced than that of the extracellular domain. This might be explained by the observation that 1A8C only stains the full length 180-kDa polypeptide, whereas 1D1 in addition stains the LAD-1 molecule [20]. Note the loss of intercellular 1D1 staining in the patient (C, D).
Mild non-Herlitz junctional epidermolysis bullosa

As immunofluorescence suggested a defective type XVII collagen expression, the patient’s DNA and mRNA were analyzed for mutations in the \textit{COL17A1} gene. One of the alleles contained the known Dutch 3432delT mutation. This exon 48 mutation results in out-of-frame reading, thereby generating a premature termination codon (PTC) and likely premature RNA decay. This mutation generates two flanking \textit{Sfi}I sites and digestion analysis of genomic PCR amplimers confirmed the heterozygous presence of 3432delT. To identify the second mutation, cDNA was PCR-analyzed using 10 overlapping primer sets. This revealed two amplimers in the region nt 2017 (exon 23) to nt 2387 (exon 31), a normal size amplimer and a smaller amplimer that lacked the 36 nt exon 30 (fig. 2A). Sequencing of genomic DNA gave a heterozygous point mutation, 2356C→T in exon 30 (fig. 2B). This mutation would result in a PTC, but obviously the reading frame is restored by skipping exon 30. This skipping of exon 30 was, even after prolonged cycling, not found in control keratinocyte mRNA. Sequencing also revealed a second heterozygous nucleotide, 2367+13T→G in intron 30 (fig. 2B). Cloning of genomic DNA showed it to be on the same allele as 2356C→T. Computer analyses of splice junction sequences showed this position to be not important for splicing; therefore, this substitution is unlikely to interfere with the splicing of exon 30 [10].

Immunoblot analysis showed the running distance of the patient’s type XVII collagen to be slightly higher than that of wild-type type XVII collagen with a difference of approximately 1 kDa (fig. 2C). No other protein products were observed, implying that the exon 48 mutation did not result in truncated type XVII collagen products. Based on immunoblot staining intensity measurements, a type XVII collagen expression level of about 15% of normal was calculated.
Figure 2 Detection of the nonsense mutation 2356C→T and the resulting reduced-size type XVII collagen protein. (A) Agarose gel analysis of RT-PCR amplified patient mRNA (lane P) with primers F2017 and R2387 shows two products of different size. The higher molecular weight product corresponds with the control product (lane C). A 100-bp ladder (lane M) was used. (B) Sequencing genomic DNA revealed a heterozygous point mutation in exon 30 at position 2356 and a substitution in intron 30, both indicated with arrows. The wild-type sequence is shown above the nucleotide sequence of the patient. The exon/intron border is indicated by |. (C) Patient and control keratinocyte extracts were run in alternate lanes on a 5% SDS-PAGE slab gel. After transferring the protein pattern to nitrocellulose, type XVII collagen was stained with monoclonal 1A8C. Patient type XVII collagen (lanes P) runs slightly faster than control type XVII collagen (lanes C), indicating that the mutated protein is of lower molecular weight. Note that the marker protein (arrow) above type XVII collagen runs at identical speed in both patient and control lanes confirming that the observed difference in running is an intrinsic feature of the mutated protein. To correct for the lower amount of type XVII collagen in the patient cell extract, the control cell extract was diluted 1:9 with a cell extract from type XVII collagen-negative nH-JEB keratinocytes.
Discussion

Skipping of mutated PTC-containing exons resulting in a milder phenotype is a rescue mechanism that has been described before in other genetic disorders [11-13]. In our patient the skipping of mutated exon 30 also results in a less severe phenotype. A similar observation was done by Ruzzi et al [14] involving deletion of exon 33. However, in their case exon 33 skipping was also present as low-level alternative splicing in normal keratinocytes.

Not all COL15 exon deletions lead to milder phenotypes. A patient with a deletion of exon 32 showed the classical severe phenotype [15]. In contrast with the above two mild patients no residual type XVII collagen was present. Exon 32 deletion, therefore, must have a more profound effect on the expression level of type XVII collagen than the two other deletions. All three exon deletions do not interfere with the reading frame or the Gly-X-Y motif, and remove only short 9-12 amino acid peptide segments. It has been suggested that loss of possible proline hydroxylation sites might account for loss of type XVII collagen protein stability. Hydroxylation of proline to 4-hydroxyproline occurs in vertebrate collagens preferably on the Y-position and these 4-hydroxyprolines stabilize the triple-helix by forming interstrand hydrogen bonds [16]. Comparing the number of lost X- and Y-position prolines between the three different patients does not give any correlation with, and therefore cannot explain the phenotypical differences. What so far has not been considered is the possibility that these exon-encoded protein segments are involved in ligand binding sites or signal transduction. If so, a more important function might be attributed to the exon 32 encoded segment of the COL15 domain.

The natural occurring partial rescue of type XVII collagen, by which the nH-JEB phenotype is reverted into a much milder phenotype, are encouraging for future strategies using exon skipping as possible gene therapy in epidermolysis bullosa.

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References


