Exon skipping therapy for dystrophic epidermolysis bullosa
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Amelioration of junctional epidermolysis bullosa due to exon skipping in the COL17A1 gene

Amelioration of junctional epidermolysis bullosa due to exon skipping

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Abstract
Mutations in the COL17A1 gene lead to the genetic blistering disorder junctional epidermolysis bullosa generalized intermediate type (JEB-gen-intermed). Antisense oligonucleotide-mediated exon skipping is a strategy that aims to skip the mutation-containing exon and thereby produce a smaller but functional protein. COL17A1 is an interesting candidate, as 53 of the 55 exons (96%) can be skipped without disturbing the reading frame. Information on the functionality of the shortened protein product is important in order to obtain support for this therapeutic strategy. Here we report a patient with JEB-gen-intermed with amelioration of the phenotype due to exon 49 skipping by two distinct mechanisms – premature termination codon-induced exon skipping and revertant mosaicism – both of which induced skipping of the same exon. The patient was compound heterozygous for two inherited COL17A1 mutations, a frameshift mutation in exon 18 (c.1490_1491delinsT, p.Ala497Valfs*23) and a nonsense mutation in exon 49 (c.3487G>T, p.Glu1163Ter). Upon clinical examination, skin patches were found that were resistant to blister formation. In these patches, naturally corrected cells were present that harboured an additional splice-site mutation, c.3419–1G>T, resulting in skipping of the mutation-containing exon 49. This natural gene therapy phenomenon shows that type XVII collagen with residues 1140–1169 deleted is largely functional. In addition, in affected skin cells a low level of exon 49 skipping was observed. Our results support the notion that skipping of a mutated in-frame exon in COL17A1 ameliorates the phenotype.
Amelioration of junctional epidermolysis bullosa due to exon skipping in the COL17A1 gene

Background
Absence of type XVII collagen (C17) protein due to mutations in the COL17A1 gene results in the genetic blistering disorder junctional epidermolysis bullosa generalized intermediate type (JEB-gen-intermed).1 Patients with JEB-gen-intermed have fragile skin and mucous membranes from birth. Blistering develops with little or no trauma. The transmembrane protein C17 plays a key role in adhesion in the dermoepidermal junction.

Currently there is no cure for this devastating disease, although several therapies are under investigation, among which is antisense oligonucleotide (AON)-mediated exon skipping.2, 3 This new therapeutic strategy is based on the use of AONs that bind to complementary sequences of the pre-mRNA. The aim is to induce skipping of the mutation-harbouring exon by modulation of pre-mRNA splicing, and thereby produce a slightly smaller, although functional, protein. In Duchenne muscular dystrophy this therapy is very promising and at the forefront of research with ongoing clinical phase III studies.4 The COL17A1 gene is an interesting candidate, as it has many small exons that can be skipped without disturbing the reading frame: 53 of the 55 coding exons (96%). It is important to have information on the functionality of the shortened C17 product, and on the amount that is needed to result in correction of the phenotype. In this case we show functionality of C17 after exon 49 skipping, which was caused by two mechanisms: (i) premature termination codon-induced exon skipping and (ii) correction of the inherited mutation by a spontaneous somatic mutation, also referred to as revertant mosaicism.

Case report
A 21-year-old woman with JEB-gen-intermed presented clinically with total alopecia, rudimentary nails, enamel dental problems, oral erosions and disseminated tense blisters on the skin healing with atrophic scars. On the lower arm several nonblistering skin patches were observed, both with slight hyperpigmentation and ‘different sensation’, as compared with surrounding areas (Figure 1). Analysis of blood genomic DNA identified compound heterozygosity for a frameshift mutation in exon 18 (c.1490_1491delinsT, p.Ala497Valfs*23) and a nonsense mutation in exon 49 (c.3487G>T, p.Glu1163Ter) of the COL17A1 gene. The patient provided informed consent for the study.

To test for skin functionality the ballpoint test was used.5 In this rub test the tip of a retracted ballpoint pen is pushed over the skin for a distance of 2–4 mm with a quick movement, and subsequently the presence or absence of blister formation can be observed under a dermatoscope. Punch biopsies of 4 mm were obtained for immunofluorescence microscopy from affected skin and clinically healthy skin without blister formation after the ballpoint test. Stainings were performed with three monoclonal antibodies for C17: VK4 (endodomain 381–399, exon 7), Lu-226 (epitope 1080–1107, exons 47–48) and 233 (epitope 1118–1143, exons 48–49).6, 7 The biopsy taken from affected skin fitted with a diagnosis of JEB-gen-intermed. The staining was negative for 233, and reduced for Lu-226
and VK4 (Figure 2a). The epitopes of 233 and Lu-226 are close to each other, and it was therefore unexpected that the staining of these two antibodies was different.

In the biopsy taken from clinically healthy skin a mosaic pattern was observed. Along 95% of the basement membrane zone the staining resembled the staining of the biopsy taken from affected skin. Interestingly, approximately 5% of the biopsy showed a stronger staining more comparable with normal human control skin for VK4 and Lu-226. However, 233 was still negative. The epitope of 233 consists of the amino acids of exons 48–49 (1118–1143). As the Lu-226 and 233 epitopes showed a different pattern, it was expected that a somatic mutation had occurred in exons 48–49 resulting in loss of the 233 epitope.

Discussion
The ameliorated phenotype in revertant skin in our patient is due to the presence of C17 that is slightly smaller (30 amino acids missing from 1140–1169), although functional, as demonstrated by the ballpoint test performed in revertant skin of the patient's forearm. Knowing the threshold for C17 is essential for therapy options. Kiritsi et al. showed that about 12–14% of the physiological C17 levels are sufficient to have a major influence on the clinical phenotype leading to only mild cutaneous involvement. Moreover, Ruzzi et al. showed that as little as 3–4% can result in a mild phenotype. That the amount of C17 is important can also been seen in our patient. Despite the fact that the slightly smaller C17 is functional, the production in affected skin was too low to result in absence of blister formation (Figure 1).

Premature termination codons can result in exon skipping. One of the proposed mechanisms is due to interference with exonic splicing enhancer sequences (ESEs). ESEs are DNA sequence motifs consisting of six nucleotides that enhance accurate splicing.
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Serine/arginine-rich proteins bind to ESEs and thereby promote exon splicing. When using Human Splicing Finder Version 2·4·1 (http://www.umd.be/HSF/), which is a bioinformatics tool to predict splicing signals, the programme predicts two RESCUE-ESEs in exon 49: GGATCA (nucleotides 3425–3430) and GAGGAG (nucleotides 3484–3489, Figure 3). When introducing the nonsense mutation c.3487G>T the second RESCUE-ESE is lost, which could account for the skipping of exon 49. The disturbance of an ESE site crucial for exon definition was also proposed by Covaci et al. for exon 87 skipping in the COL7A1 gene by the silent exonic c.6846G>C, p.Leu2282Leu mutation, which was localized 15 bp from the acceptor splice site.

Recent studies have indicated that the majority of patients with COL17A1 mutations have naturally corrected revertant keratinocytes. From this natural form of
gene therapy, lessons can be learned regarding the function of C17, as besides exon 49 skipping, deletion of exon 30 also leads to normally functioning protein (Table 1). Furthermore, the c.2237delG, p.Gly746AlafsTer53 COL17A1 mutation in exon 30 was corrected by multiple mechanisms within one patient. Also, the deletion of both exons 30 and 31 together led to correction. Finally, skipping of exon 33 by a constitutive process occurring at a very low level in all keratinocytes led to an unusually mild JEB phenotype.\textsuperscript{11}

![Figure 3. Schematic display of pre-mRNA splicing of exon 49.](image)

**Figure 3. Schematic display of pre-mRNA splicing of exon 49.** Wild-type exon 48–50 pre-mRNA splicing results in the joining of exons 48, 49 and 50. The small blue boxes in exon 49 represent predicted exonic splicing enhancer sequences (ESEs). Due to the germline nonsense mutation c.3487G>T (red rectangle), one of the predicted ESEs is lost. Consequently, next to the transcript bearing a premature termination codon, an exon 49-deleted mRNA variant is produced that is lacking the nonsense mutation, which is translated into a shortened type XVII collagen protein (C17). In the revertant keratinocytes the additional somatic splice-site mutation (c.3419–1G>T) indicated by the green rectangle is present. This additional splice-site mutation results in only exon 49-deleted mRNA. The exon 49-deleted mRNAs are translated into C17 lacking amino acids 1140–1169.

However, not all exon deletions result in a milder phenotype, as was shown by the deletion of exon 32 in patients with a more classical severe JEB-gen-intermed phenotype.\textsuperscript{14} The amino acids encoded by exon 32 are predicted to have a more important function. Generation and characterization of recombinant C17 lacking amino acids encoded by specific exons would provide more insight into the functionality of internally truncated C17 variants. Assays that would be helpful are trypsin digestion and binding-affinity measurements to binding partners of C17, such as laminin-332 and α6 integrin.

In conclusion, this is the first reported patient in whom two different mechanisms are observed – premature termination codon-induced exon skipping and revertant mosaicism – both of which lead to skipping of the same exon. The amount of C17 produced is crucial. The revertant areas resisted blister formation, whereas the amount of C17 produced in affected keratinocytes was too low to result in absence of blistering. Our observation supports the development of exon skipping therapies for JEB, such as AON-mediated exon skipping, by showing that skipping of a mutated in-frame exon in COL17A1 ameliorates the phenotype.
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Table 1. Review of published and unpublished studies of exon skipping in COL17A1.

<table>
<thead>
<tr>
<th>Exon</th>
<th>Mutation</th>
<th>Clinical Phenotype</th>
<th>Cause of milder phenotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 (36 bp)</td>
<td>c.2251C&gt;T; p.Gln751Ter</td>
<td>Mild form of JEB-gen-intermed</td>
<td>Exon skipping most likely induced by premature termination codon. An additional mutation (c.2262 + 13T&gt;G) was found in the intron 30 border but was predicted to have no effect on splicing. Protein production estimated to be 15% of normal</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>c.2237delG; p.Gly746AlafsTer53</td>
<td>Revertant area with clinically healthy phenotype</td>
<td>Correcting mutation c.2263 + 2T&gt;C at DNA level</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Correcting mutation c.2228-101_2263 + 70delins15 at DNA level</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Correcting mutation c.2259_2263 + 9del at DNA level</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Correcting mutation c.2238C&gt;T at DNA level</td>
<td>13</td>
</tr>
<tr>
<td>30 + 31 (108 bp)</td>
<td>c.2237delG; p.Gly746AlafsTer53</td>
<td>Revertant area with clinically healthy phenotype</td>
<td>Correcting mutation c.2227 + 153_2336–318del resulting in deletion of 2165 bp at DNA level</td>
<td>13</td>
</tr>
<tr>
<td>32 (27 bp)</td>
<td>c.2336–2A&gt;G</td>
<td>Classical JEB-gen-intermed phenotype</td>
<td>Not applicable</td>
<td>14</td>
</tr>
<tr>
<td>33 (36 bp)</td>
<td>c.2383C&gt;T; p.R795Ter</td>
<td>Mild form of JEB-gen-intermed</td>
<td>Constitutive exon skipping occurring at a very low level. Protein production estimated to be 3–4% of normal</td>
<td>10</td>
</tr>
<tr>
<td>49 (90 bp)</td>
<td>c.3487G&gt;T; p.Glu1163Ter</td>
<td>Revertant area with clinically healthy phenotype</td>
<td>Correcting mutation c.3419–1G&gt;T at DNA level</td>
<td>This thesis</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Exon skipping at a very low level possibly because of disruption of an ESE site due to the c.3487G&gt;T mutation</td>
<td>This thesis</td>
</tr>
</tbody>
</table>

JEB-gen-intermed, junctional epidermolysis bullosa generalized intermediate type; ESE, exonic splicing enhancer sequence.
Chapter 7

References


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