Post-transcriptional mechanisms in type XVII collagen synthesis
Zalen, Sebastiaan van

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CHAPTER 5

EFFECT OF NONSENSE MUTATIONS ON \textit{COL17A1} mRNA TRANSCRIPT LEVELS IN KERATINOCYTES OF HEMIDESMOSOMAL EPIDERMOLYSIS BULLOSA PATIENTS

Sebastiaan van Zalen, Anna M.G. Pasmooij, Miranda Nijenhuis, Marcel F. Jonkman, and Hendri H. Pas

Submitted
ABSTRACT

Hemidesmosomal Epidermolysis Bullosa (HEB) is an inherited bullous skin disorder characterized by diminished epidermal-dermal adhesion. The clinical phenotype includes generalized skin blistering, dental anomalies, universal alopecia, and nail dystrophy but the severity may vary between individual patients. The underlying defect is reduced expression of either type XVII collagen or laminin 5 due to mutations in their coding genes. Most of the reported mutations in the COL17A1 gene result in premature termination codons (PTCs). These mutations lead to reductions of the COL17A1 mRNA transcripts probably due to nonsense mediated mRNA decay (NMD).

In this study we investigated the actual amount of decay of the two major alternative type COL17A1 mRNA transcripts in keratinocytes of seven HEB patients that had different mutations and compared this to the expression level of type XVII collagen protein and the clinical phenotype. All mutations, irrespective their nature or the presence of in-frame skipping of the mutated exon, caused a severe reduction of mRNA expression that was lower than in general observed in other genetic diseases. No correlation between the phenotype and the amount of decay was detected. Cycloheximide blocking of translation resulted in increased expression of COL17A1 mRNA transcripts in line with decay due to NMD. Moreover, we found in vivo evidence for known in vitro NMD mechanisms. Interestingly in two patients that had one PTC containing exon removed through alternative splicing we did not find increased mRNA levels. We conclude that other than NMD mechanisms must also be active in keratinocytes of these patients.
INTRODUCTION

Mutations in the COL17A1 gene lead to Hemidesmosomal Epidermolysis Bullosa (HEB), a subset of bullous genodermatoses characterized by generalized blistering from birth, alopecia of scalp, absence of body and secondary hair, nail dystrophy, and tooth enamel hypoplasia. COL17A1 codes for type XVII collagen: a transmembrane hemidesmosomal adhesion molecule. Hemidesmosomes are essential structures of the basal epithelial cells that anchor these to the underlying basement membrane matrix.

The type XVII collagen protein consists of 155 kDa of amino acid sequence, contains extracellular carbohydrate moieties and can be phosphorylated. It has a type II transmembrane orientation, thus with a COOH-terminal ectodomain and an NH2-terminal cytoplasmic domain. The ectodomain contains 15 collagenous subdomains (COL1-COL15) by which the molecular tail folds into a triple-helical structure. In addition, a second soluble form of 120 kDa is found, which consists of the cleaved ectodomain.

The COL17A1 gene is located on the long arm of chromosome 10, in band 10q24.3. It spans 52 kb of genomic DNA and contains 56 exons that finally lead to a coding sequence of 4491 nucleotides. Differential splicing of exon 56 results in two alternative transcripts that differ 610 nucleotides in length in the 3' Untranslated Region (3'UTR). At the 5' end six different 5'UTRs are found of which two are major (Van Zalen et al., in press).

Most of the reported mutations in COL17A1 are either nonsense or indel mutations that both lead to premature termination codons (PTCs). These mutations lead to different phenotypes varying between severe and very mild. The severe phenotype includes generalized blistering with alopecia (generalized atrophic benign epidermolysis bullosa, GABEB), whereas the mild phenotype (localized atrophic benign epidermolysis bullosa, LABEB) is limited to blistering of hands, feet, and face and does not display alopecia (Pasmooij et al., submitted). The milder phenotypes (LABEB) are especially seen when PTCs are removed by in-frame skipping of the mutated exon, which then results in the rescue of a shorter, but still partly functional type XVII collagen protein.

Missense mutations may lead to different degrees of mild phenotypic effects depending on the functional importance of the changed amino acid. Examples of less milder phenotypes are amino acid substitutions in the collagenous domain that interfere with correct folding of the triple helix. Incorrect folding of the helix leads to non-specific degradation of the mutant type XVII collagen and abnormal dentition.

PTCs in COL17A1 lead to lower levels of COL17A1 mRNA. These lower levels of COL17A1 mRNA are thought to be the result of nonsense-mediated mRNA decay (NMD). This is emphasized by the up-regulation expression of NMD involved genes in HEB keratinocytes.
However, hardly any data are available on the actual contribution of NMD to the decay of *COL17A1* mRNA in relation to the specific mutation and the phenotype of the patient. NMD is an mRNA-degradation process that specifically allows for the removal of PTC-containing mRNA transcripts. This prevents truncated proteins from being formed, and precludes possible dominant-negative effects of such truncated proteins.

NMD is both a splicing and a translation-dependent event since the NMD pathway is linked to the splicing dependent deposition of a protein complex (exon junction complex; EJC) at 20-24 nucleotides 5' of each exon-exon junction on the coding mRNA\textsuperscript{208-210}. This EJC consists of several proteins that are loaded onto the mRNA in the nucleus, and then removed in the cytoplasm by ribosomes during the pioneer round of translating\textsuperscript{211}. However, if a PTC is located more than 55 nucleotides upstream of the last EJC this results in the retention of the EJC which then triggers the NMD response\textsuperscript{212-214}.

In this study we investigated in HEB patients the decay of the *COL17A1* mRNA transcripts by real-time PCR and Northern blotting, and correlated the results with the clinical phenotypes and the expression of the type XVII collagen protein.

**MATERIAL AND METHODS**

**Patients**

We used cultured keratinocytes of seven HEB patients that all had mutations leading to PTCs on both alleles (Table 1). For details we refer to the same patient code in Pasmooij et al (submitted).

Patient EB 086-01 was an 11-week-old child with LABEB, who was compound heterozygote for the mutations 1877-2A>C and 3432delT\textsuperscript{106}. The 1877-2A>C mutated allele was rescued by outsplicing of the whole exon 22 and of part of exon 22 that both restored the reading frame. The resultant proteins were slightly shorter and Western blot demonstrated in cultured cells a protein level of 4-5% compared with normal cells. Electron microscopy showed intra-epidermal split-level very low in the basal keratinocytes.

Patient EB 098-01 was a 36-year-old male with LABEB, who was compound heterozygote for the mutations 2356C>T and 3432delT\textsuperscript{105}. The 2356C>T allele was rescued by outsplicing exon 30 resulting in a 1 kDa shorter protein. Protein level in cultured cells was 15% of normal healthy keratinocytes.

Patient EB 035-01 was a 35-year-old male with GABEB, who was compound heterozygote for the mutations 2342delG and 3781>T\textsuperscript{165}. The protein level in keratinocytes was below detection limits.
Table 1. Patient mutations

<table>
<thead>
<tr>
<th>#</th>
<th>Sex</th>
<th>Phenotype</th>
<th>Mutation at DNA level</th>
<th>Resulting mRNA</th>
<th>Refs</th>
</tr>
</thead>
<tbody>
<tr>
<td>EB 086-01</td>
<td>F</td>
<td>LABEB*</td>
<td>1877-2A&gt;C/3432delT</td>
<td>in-frame exon skip/PTC</td>
<td>Pasmooij et al, 2004 a</td>
</tr>
<tr>
<td>EB 098-01</td>
<td>M</td>
<td>LABEB</td>
<td>2356C&gt;T/3432delT</td>
<td>in-frame exon skip/PTC</td>
<td>Pasmooij et al, 2004 b</td>
</tr>
<tr>
<td>EB 035-01</td>
<td>M</td>
<td>GABEB**</td>
<td>2342delG/3781C&gt;T</td>
<td>PTC/PTC</td>
<td>Scheffer et al, 1997</td>
</tr>
<tr>
<td>EB 011-01</td>
<td>M</td>
<td>GABEB</td>
<td>2342delG/2342delG</td>
<td>PTC/PTC</td>
<td>Scheffer et al, 1997</td>
</tr>
<tr>
<td>EB 117-01</td>
<td>F</td>
<td>GABEB</td>
<td>3236delC/3236delC</td>
<td>PTC/PTC</td>
<td>Pasmooij, submitted</td>
</tr>
<tr>
<td>EB 093-01A***</td>
<td>M</td>
<td>GABEB</td>
<td>3781C&gt;T/4425insC</td>
<td>PTC/PTC</td>
<td>Pasmooij et al, 2005</td>
</tr>
<tr>
<td>EB 026-01A</td>
<td>F</td>
<td>GABEB</td>
<td>1706delA/3781C&gt;T</td>
<td>PTC/PTC</td>
<td>Jonkman et al, 1997</td>
</tr>
<tr>
<td>EB 026-01B</td>
<td>F</td>
<td>revertant Gene conversion</td>
<td>wild type/PTC</td>
<td>Jonkman et al, 1997</td>
<td></td>
</tr>
</tbody>
</table>

* localized atrophic benign epidermolysis bullosa
** generalized atrophic benign epidermolysis bullosa
*** no revertant cells were available for cultures.

Patient EB 011-01 was a 40-year-old male with GABEB, who was homozygous for the 2342delG mutation. As patient No. 1 in the original paper on type XVII collagen deficiency he had severely reduced long and short COL17A1 RNA variants by Northern blot, and no detectable type XVII collagen protein by Western blot in extracts of cultured keratinocytes\(^\text{132}\).

Patient EB 117-01 was a 42-year-old female with GABEB, who was homozygous for the 3236delC mutation (Pasmooij et al, submitted). She had the most severe phenotype of this series with universal alopecia and cutaneous squamous cell carcinomas in blistering areas.

Patient EB 093-01 was a 75-year-old male and compound heterozygote for 3781C>T and 4425insC. This patient appeared to be mosaic for type XVII collagen expression having the GABEB phenotype with in addition one little patch of sturdy healthy skin on the middle finger. Immunofluorescence demonstrated restored protein expression in several biopsies.

The molecular basis for the repair of the different patches in this particular patient has recently been described by Pasmooij et al\(^\text{112}\). We obtained keratinocytes cultured from negative skin and these did not produce detectable type XVII collagen.

Patient EB 026-01 was a 27-year-old female with GABEB, and compound heterozygote for the mutations 1707delA and 3781C>T. This patient also was a mosaic patient with larger patches of normal looking skin on the arms and ankle. In such patches the type XVII collagen expression was restored and molecular analysis revealed that different patches had undergone repair by different mechanisms\(^\text{112}\). We have used two types of cultured cells for analysis, the negative cells and the cells that had the 1706delA mutation repaired by gene conversion\(^\text{110}\).
Skin analysis by immunofluorescence demonstrated that patients EB 086-01 and EB 098-01 were positive, although reduced, for the intracellular 1A8C epitope, whereas patients EB 035-01, EB 011-01, EB 117-01, EB 093-01, and EB 026-01 were negative, with exception of the revertant patches in patients EB 093-01 and EB 026-01. The extracellular 233 and NCC-Lu-227 epitopes were present in all patients with the exception of patient EB 117-01.

**Cell culture**

The squamous cell carcinoma line UMSCC-22B, originally derived from a tumour of the hypopharynx, was cultured in DMEM (Gibco, Paisley, UK) supplemented with 5% fetal calf serum (Cambrex, Verviers, Belgium), 2 mM L-glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin (Gibco) at 37°C with 5% CO₂. Keratinocytes were cultured from punch biopsies from clinically unaffected skin. The epidermis was separated from the dermis after overnight incubation with 0.03% trypsin, 0.02% EDTA at 37°C for 10 minutes. The harvested keratinocytes were cultured under serum-free conditions using Keratinocyte-Serum Free Medium (SFM) (Gibco) according to Mitra and Nickoloff. This medium was supplemented with 25 µg/ml Bovine Pituitary Extract (BPE) and 0.1 ng/ml Recombinant Epidermal Growth Factor (rEGF) (all from Gibco).

For translation inhibition experiments keratinocytes were treated with 10 µg/ml cycloheximide (Sigma, St. Louis, MO) for six hours. Thereafter, cells were harvested by extraction with 1 ml Trizol/25 cm² (Invitrogen, Carlsbad, CA).

**mRNA isolation**

Cells were grown to sub-confluence and then harvested by extraction with 1 ml/25 cm² Trizol reagent (Invitrogen). Chloroform was added and after centrifugation the total RNA was precipitated from the aqueous phase with isopropyl alcohol. Then the poly(A⁺) mRNA was purified from this with the mRNA isolation kit from Roche (Mannheim, Germany) that works with poly(dT)-conjugated magnetic beads. This last step was performed twice to ensure that the mRNA was extremely pure for use in Northern blotting experiments.

**Real time PCR**

Two-hundred nanogram total RNA was used for synthesis of first-strand cDNA with SuperScript III RNase H minus reverse transcriptase (Invitrogen) in a 20 µl final volume containing 300 ng of random hexamers (Invitrogen) and 40 units of RNase OUT (Invitrogen).

Combinations of unlabeled PCR primers and Taqman® minor groove binder (MGB) probes (FAM™ dye-labeled) for use in real-time RT-PCR were purchased as Assay-on-Demand from Applied Biosystems (Nieuwekerk a/d IJssel, The Netherlands). The targets included the housekeeping gene β-actin (assay ID: Hs9999903), all COL17A1 transcripts (Hs00166711), the short variant COL17A1 transcript (Hs00996062), and the long variant COL17A transcript. This latter set was specially developed by Assay-on-Design of Applied Biosystems against the unique long variant 3’UTR sequence.

For each gene the final concentration of primers and MGB probes in TaqMan PCR MasterMix (Applied Biosystems) was respectively 900 and 250 nM, and 1 µl of cDNA was
added to the PCR-mix. TaqMan real-time RT-PCR was performed in an ABI PRISM 7900HT Sequence Detector (Applied Biosystems). Amplification was performed with the following cycling conditions: 2 min at 50°C, 10 min at 95°C, and 40 two-step cycles of 15s at 95°C and 60s at 60°C. Triplicate real-time RT-PCR analyses were executed for each sample. Threshold cycle values (C\text{t}) were determined with the help of SDS2.2.2 software and were averaged for each sample. The two COL17A1 mRNA variant specific primers sets were tested for non-specific annealing and amplifying by real-time RT-PCR analyses of pCR4-vectors (TOPO™ TA cloning kit, Invitrogen) in which either one of the two 3'UTR variants of type XVII collagen was cloned. The primer set specific for the longer 3’UTR variant was not able to detect any plasmid containing the shorter 3’UTR variant and visa versa, indicating the specificity of the two primer sets.

Vandenbroecke et al proposed to establish standard curves based on plasmids containing appropriate inserts in order to validate the absolute mRNA concentrations when comparing expression splice variants\textsuperscript{215}. To establish standard curves for the type XVII specific primer sets we cloned three type XVII collagen PCR-products, that contained the appropriate target sequences, into separate pCR4-vectors. Real-time RT-PCR of ten-fold serial dilutions of these vectors with the respective primer sets resulted in reliable standard curves. With the help of these standard curves we were able to calculate absolute mRNA concentrations in keratinocytes of HEB patients. mRNA concentrations in keratinocytes of three different healthy donors were averaged and used as reference for COL17A1 mRNA expression in each real-time RT-PCR experiment.

An alternative method to determine relative expression of mRNA transcripts is to use the \Delta\text{Ct}: \Delta\text{Ct}= {C\text{t}(\text{type XVII collagen variant})- C\text{t}(\beta\text{-actin})}. Relative expression of each variant is determined by the inverse of the quotation: E= 2^{-\Delta C\text{t}}. Expression levels calculated in this way did not significantly differ from values interpolated with standard curves (data not shown).

RNA probes

Digoxigenin (DIG)-labeled antisense RNA probes were synthesized using the DIG-Labeling mix (Roche) according to the manufacturer’s protocol with some minor modifications as described below. DNA fragments were generated by PCR on SCC cDNA that was produced by transcribing SCC mRNA with Superscript reverse transcriptase (Invitrogen). PCR products were cloned into vector pCR4 (Invitrogen). Plasmids were linearized with restriction enzymes NotI or BcuI (MBI Fermentas, Vilnius, Lithuania), depending on the RNA polymerase, respectively T3 or T7 (Fermentas), that was used in the subsequent transcription reaction. Probes were then generated by \textit{in vitro} run-off transcription on linearized plasmid. For detection of COL17A1 mRNA two antisense probes were used (location 230x626 and 3679x4131; numbering according to Giudice\textsuperscript{4}) and for detection of β-actin one probe was used (location 915-1336; numbering on basis of Genbank accession number NM101001)

Northern blotting

Four-hundred ng samples of mRNA were electrophoresed on 6,6% formaldehyde-1,2% agarose gel and capillary transferred to a positively charged Nylon Membrane membrane
(Amersham Biosciences, Braunschweig, Germany) in 10xSCC (1.5 M NaCl, 150 mM NaCitrate, pH 7.0). The mRNA was covalently immobilized to the membrane by 5 minutes 304 nm UV-cross-linking. Next, the membrane was hybridized overnight with the DIG-labeled RNA probes at 68°C in DIG Easy Hyb buffer. The membrane was washed two times with 2xSCC+0,1% SDS at room temperature followed by two more stringency washes with 0,5xSCC+0,1% at 68°C. Next the membrane was incubated with anti-DIG-AP F_{ab} fragments 1:20,000 in blocking solution. Chemiluminescence was developed by CPD-star 1:100 in detection buffer (100 mM Tris-HCl, 100 mM NaCl, pH 9.5) and luminescence was detected by exposing the membranes to Lumi-Film Chemiluminescent Detection film. To check for equal loading of mRNA a probe against β-actin was co-hybridized. All reagents used in the incubation and signal development were purchased from Roche.

RESULTS

mRNA expression in HEB keratinocytes

Keratinocytes of all HEB patients had strongly reduced COL17A1 mRNA levels with the exception of revertant keratinocytes of patient EB 026-01 (Fig. 1A). The reduction was over twenty-fold in HEB keratinocytes compared to wild type COL17A1 mRNA expression with the exception of patient EB 093-01. Keratinocytes that had nonsense mutations located further upstream of the last exon-border tended to have stronger reductions of mRNA expression (Fig. 2A). However, this correlation was not statistically significant. Surprisingly, the keratinocytes of patients EB 086-01 and EB 098-01 with LABEB only had slightly higher mRNA expression levels than the type XVII collagen negative cells of the other patients, although still significant (P<0.05 for all primer sets). We had expected much higher values considering the in-frame skipping of the mutated exon, and the production of shortened, but partial functional type XVII collagen in cultured cells and in skin in vivo.
Effect of PTCs on *COL17A1* mRNA transcript levels in keratinocytes of HEB patients

**Figure 1. COL17A mRNA expression in patients’ keratinocytes**

(A) mRNA expression of the two major *COL17A* transcripts in HEB cells is severely lower than *COL17A1* mRNA expression in normal keratinocytes. Expression of *COL17A1* mRNA is determined with real time RT-PCR and is corrected for β-actin mRNA expression and is expressed as percentage compared to normal human keratinocytes for each primer set that is set as 100%. Data are the mean and standard deviation of at least two independent experiments and in each experiment keratinocytes of three healthy donors were used. Real time RT-PCR experiments were performed on total RNA, since control experiments showed no difference in expression when total RNA was used in stead of mRNA.

(B) Northern blotting of *COL17A1* mRNA of HEB keratinocytes confirms the decay observed in the real-time RT-PCR techniques. Anti-sense β-actin probe was co-hybridized to control for mRNA loading (not shown). *In lanes NHKs and EB 026-01B 100 ng mRNA is loaded while all other lanes contain 400 ng mRNA. One representative experiment out of three is shown and in this experiment keratinocytes of one healthy donor were used.

The type XVII collagen negative cells of patient EB 093-01 showed a relatively higher level mutated *COL17A1* mRNA, although a 14-fold reduction in contrast with NHKs was still observed. As this patient was mosaic we took special care to assure that no contamination with revertant cells had occurred. Neither immunoblot analysis of cells, nor immunofluorescence staining of biopsies, nor sequence analysis of the cDNA batch used in the real time PCR experiments did give any evidence of contamination.
Figure 2. NMD response in GABEB patients cells tend to be stronger as the distance between the PTC and the 3’most exon increases.  
(A) Patient mRNA levels plotted against the distance to the last exon-border for the most downstream PTC of the respective patient. Diamonds represent expression level of all \textit{COL17A1} variants, squares the long variant level and circles the short variant levels. Lines are trend lines.  
(B) Raise of transcript levels after cycloheximide treatment as function of the distance between the most downstream PTC and the last exon-border. Symbols are similar as in A. Lines are trend lines.  

As an independent control on our quantitative PCR data we also analyzed the mRNA level by Northern blotting, which confirmed the relative differences in expression levels between the different patient keratinocytes (Fig. 1B). The short variant \textit{COL17A1} mRNA transcript was relatively more reduced in patient cells than the long variant mRNA (mean value 3.7 versus 5.4). Student’s t-test revealed that this difference was significant (P<0.01). This was also demonstrated by the fact that the ratio, between the longer and the smaller variant, was significantly higher in keratinocytes of HEB patients compared to normal keratinocytes (Fig. 3).
Effect of PTCs on *COL17A1* mRNA transcript levels in keratinocytes of HEB patients

A prerequisite for the execution of NMD is that PTCs must be located more than 50 nucleotides upstream of the last exon border\(^{213,216}\). In all patients’ keratinocytes mutations were located more than fifty nucleotides 5′ of the last exon border of the both *COL17A1* mRNA transcripts (Table 2).

**COL17A1** mRNA decay is caused by nonsense mediated mRNA decay

To investigate whether mutant *COL17A1* mRNA decay is under NMD control we treated control and HEB keratinocytes with cycloheximide. This inhibits the NMD machinery by blocking general protein translation and thereby it suppresses NMD decay. Consequently those transcripts under NMD control will demonstrate increased levels.

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**Figure 3. Different decay of the long and short variant *COL17A* mRNA.**

Ratio of expression between the larger and the shorter *COL17A1* mRNA transcript is larger in keratinocytes of HEB patients in comparison with NHKs. *P*<0.05, **P*<0.01, ***P*<0.001 compared to NHKs with the help of Student’s t-test for independent samples, two-tailed. Expression is determined on basis of real time RT-PCR experiments and is corrected for β-actin mRNA expression. Data are the mean of at least two independent experiments and in each of these experiments keratinocytes of three healthy donors were used.
Table 2. Distance of each PTC to last 3'exon-border and COL17A1 mRNA expression levels.

mRNA concentrations of patients were compared to the expression in normal healthy keratinocytes for both primer sets and expression was presented as a percentage compared to healthy keratinocytes, which was set at 100% for both primer sets. Expression data are the mean of at least two independent experiments.

<table>
<thead>
<tr>
<th>Pat#</th>
<th>distance in nucleotides 5' to the last exon-border</th>
<th>% relative expression long transcript</th>
<th>% relative expression short transcript</th>
<th>n</th>
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</thead>
<tbody>
<tr>
<td>EB 086-01</td>
<td>1164</td>
<td>6.44</td>
<td>4.29</td>
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</tr>
<tr>
<td>EB 098-01</td>
<td>1164</td>
<td>7.93</td>
<td>5.83</td>
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<tr>
<td>EB 035-01</td>
<td>2245/815</td>
<td>2.95</td>
<td>1.87</td>
<td>3</td>
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<tr>
<td>EB 011-01</td>
<td>2245/2245</td>
<td>3.18</td>
<td>1.74</td>
<td>2</td>
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<td>7.26</td>
<td>3.50</td>
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<td>2890/815</td>
<td>4.91</td>
<td>4.98</td>
<td>3</td>
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<tr>
<td></td>
<td>mean ± 2sd</td>
<td>5.37 ± 3.86</td>
<td>3.65* ± 3.05</td>
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<tr>
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<td>24.89</td>
<td>13.42</td>
<td>1</td>
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<tr>
<td>NHK</td>
<td></td>
<td>100</td>
<td>100</td>
<td>4</td>
</tr>
</tbody>
</table>

*P< 0.01 compared to the relative expression of the long transcript with the help of Student’s t-test for paired samples, two-tailed

Treatment of HEB keratinocytes with cycloheximide indeed led to an increase of COL17A1 mRNA transcripts in HEB keratinocytes, indicating that COL17A1 mRNA transcripts containing a nonsense mutation are under control of NMD (Fig. 4). In contrast, COL17A1 mRNA transcript levels in normal keratinocytes slightly decreased after cycloheximide treatment.

This increase was observed in all HEB keratinocytes and for four patients the increase of expression of the short variant was statistically significant. For two patients we could not determine whether the increase was significant due to shortage of patient material. For no obvious reason, in patient EB 026-01 hardly any increase was seen.

Overall the extent of increase tended to be higher in keratinocytes that had nonsense mutations further upstream of the last exon-border (Fig. 2B). Also this correlation was not statistically significant. When comparing the effect of the cycloheximide on the long and short variant mRNA the average increase of the smaller transcript was significantly (P= 0.05) higher (2.3) than that of the larger transcript (1.7).
Effect of PTCs on *COL17A1* mRNA transcript levels in keratinocytes of HEB patients

**DISCUSSION**

In this study we demonstrate that nonsense mutations in the *COL17A1* gene lead to severely low levels of *COL17A1* mRNA transcripts. Surprisingly, reading frame rescue through outsplicing of the PTC-containing exons does not result in much higher levels. Most mRNAs bearing PTCs are degraded by nonsense mediated mRNA decay (NMD), although some PTCs can escape NMD\(^{217}\). NMD is both a splicing and translation-dependent process, which involves a cascade of proteins\(^ {208}\). Inhibition of the translation turns of the NMD pathway, which results in an elevated expression of those mRNA transcripts subject to NMD. When we treated cells with cycloheximide we indeed observed in all HEB keratinocytes raised *COL17A1* transcript levels, while transcript levels of normal human keratinocytes slightly decreased.

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**Figure 4.** *COL17A1* mRNA levels are elevated after cycloheximide treatment.

Induction of expression is observed in HEB keratinocytes after blocking of protein translation with cycloheximide. Induction is expressed by *COL17A1* mRNA expression after treatment divided by *COL17A1* mRNA expression in keratinocytes of the same patients that were not treated, for each primerset. mRNA expression was determined with real-time PCR experiments on total RNA and is corrected for β-actin mRNA expression. *P*<0.05, **P**<0.01 compared to untreated keratinocytes with the help of Student’s t-test for paired samples, one-tailed.
This latter effect may be due to the fact that cycloheximide is a broad-spectrum inhibitor of protein translation, which not only blocks the NMD machinery, but also may influence the transcription of mRNA or regulation of mRNA stability along treatment. So far, elevated expression of PTC bearing transcripts after treatment with non-specific inhibitors of protein translation have been considered as proof of principle for NMD. However, specific inhibition of the main components of the NMD machinery will provide better insight in the actual contribution of NMD on the decay of PTC bearing transcripts.

The data in this study thus establish that the COL17A1 transcripts are subject to NMD. The reduction in the mRNA levels is remarkably lower than those described for PTC bearing transcripts of most other proteins. So far, only a minority of nonsense mutations with severely low mRNA levels as our patient’s COL17A1 mRNA levels have been reported. These were observed for genes with programmed DNA gene rearrangements, such as TCR-β and immunoglobulin genes and also few mutations in other genes showed comparable severe decay. In the TCR-β gene a uncharacterized down-regulatory-promoting element (DPE) is hypothesized to be responsible for this strong down-regulation of PTC-containing transcripts. PTCs in the COL10A1 gene cause, as expected, NMD in cartilage tissue. However, in non-cartilage tissue no NMD was observed, indicating that NMD can be under tissue control. The severe decay of PTC-bearing COL17A1 transcripts may suggest that NMD is especially strongly elicited in skin. However, in contrast, other PTC-containing transcripts of skin expressed proteins showed only moderate mRNA decay denying this suggestion.

Among the seven HEB patients, two patients with the LABEB phenotype had the reading frame restored through removal of the PTC containing exon in one allele. These patients cells produced type XVII collagen, as witnessed by IF antigen mapping of the skin (Pasmooij et al, submitted), and by Western blot, albeit at a considerable lower level than normal. Nevertheless, this was not reflected in much higher mRNA expression levels compared to the GABEB patients since only slightly, although significant, higher values were found. We expected expression levels up to 50%, since NMD should only effect the mutated allele here. Only in the reverted cells of patient EB 026-01 we did observe such a higher level, but this patient had a repaired allele that contained again the wild-type sequence. However, the correction of one allele only restores 25% of mRNA production of two wild type alleles. This meagre result agrees with the observation of Pasmooij et al, that an hemizygous COL17A1 status such as in LABEB patients, heterozygous carriers, and in revertant mosaicism, effects the distribution of type XVII collagen in the skin: the apical-lateral type XVII collagen staining of basal cells is lost (Pasmooij et al, submitted).

We do not know what mechanism is responsible for the exon-splicing in patients EB 086-01 and EB 098-01 with LABEB, although we think nonsense associated altered splicing (NAS) is the most probable candidate. The presence of a mutation, which may effect splicing in both patients, is in favor of this hypothesis. Similar
to NMD, also NAS is elicited by PTCs. Both mechanisms are supposed to operate independent of each other\textsuperscript{224,225}. The low \textit{COL17A1} mRNA expression in these patients is in line with the independence of NAS and NMD with each other, since despite the strong NMD response, expression of type XVII collagen is seen, as shown by expression of the epitopes 1A8C and 233\textsuperscript{105,106}.

Both alternative 3'UTR transcripts, the long and the short variant, were subject to breakdown, with the short variant decaying to a lower relative level than the long variant. On the average the long variant was present at 5.4% of the normal keratinocytes value while the short variant decayed to an average of 3.7% of normal. In addition, the cycloheximide effect on the \textit{COL17A1} mRNA expression was significantly stronger on the shorter transcript, thus indicating that it is more subject to NMD. The reason for this remains unclear but we suggest that sequences of the \textit{COL17A1} 3'UTR may be involved in the NMD process, since this is the only difference between the long and the short \textit{COL17A1} transcripts.

When we look at GABEB patients’ steady state mRNA levels and the increase in level after cycloheximide treatment we see that these levels and the distance of the most downstream PTC to the last exon-border tend to correlate. As the distance upstream to the last exon-border increases the steady state levels decrease while the effect of the cycloheximide blocking increases. A possible cause is a phenomenon known as polar effect: nonsense mutations close to the last 5' exon border cause less decay of the resulting mRNA in comparison with PTC further upstream of this last exon border\textsuperscript{226}. In support of a polar effect, in sequence analysis as part of mutation screening in patient EB 093-01 was observed that the mRNA transcript containing the more upstream PTC was more decayed than the other transcript. Thus, a polar effect on NMD decay of mutated \textit{COL17A1} mRNA may be suggested although our patient group is too small to draw a final conclusion.

We did not find a correlation between the location of the mutations and the severity of the symptoms in our patients, since in keratinocytes of patient EB 117-01, who had the most severe symptoms, no lower \textit{COL17A1} mRNA levels were observed. Such a location dependency has been observed in other genetic deficiencies. The location of nonsense mutations in the cystic fibrosis gene, CFTR, influenced the severity of the disease\textsuperscript{227,228}. Similar findings were found for the \textit{COL7A1} gene by Tamai \textit{et al} who observed a correlation between mutation location and severity in Japanese Dystrophic Epidermolysis Bullosa (DEB) patients. In the three cases described the symptoms seemed to depend on the distance between the mutation and the last 3'exon border\textsuperscript{229}. Other authors suggested that NMD could also be dependent on locations of individual PTCs. Ishiko \textit{et al} described a Japanese milder DEB case that did not fit the suggestion made by Tamai \textit{et al}\textsuperscript{230}. And thus is suggested that the sequence surrounding the PTC may also be of importance\textsuperscript{219,227,231}.

We conclude here that in HEB the best correlation with the clinical phenotype is found with the expression of the type XVII collagen protein as witnessed by immunofluorescence antigen mapping. Our two LABEB cases had a residual staining of the 1A8C epitope, which is proof of presence of the full-length molecule,
since the 1A8C epitope is found on the intracellular domain. In the four patients with the GABEB phenotype the 1A8C epitope could not be demonstrated probably due to the concentrations of the full-length molecule being below the detection limit. They however must have a low synthesis level since in contrast with the 1A8C epitope the 233 epitope was clearly present. The 233 epitope is found on the extracellular domain (amino acids 1118-1143\textsuperscript{232}) and thereby reflects the sum of the full-length and the soluble 120 kDa form. In these patients most of the type XVII collagen is apparently present as the soluble 120 kDa molecule. If the synthesis level drops further and becomes that low that also the 233 epitope is lost we find the more severe phenotype as in patient EB 117-01.

In summary, PTCs in \textit{COL17A1} mRNA lead to lower transcript levels than observed in most other genetic diseases. Although we confirmed NMD to be involved in mRNA decay, more important we also demonstrated that other mechanisms must be active. The mRNA level is no indication of actual protein synthesis as no correlation between transcript level and protein level can be demonstrated. These data are of interest for \textit{COL17A1} aimed gene therapy of HEB patients, and for further research on mechanisms of mRNA decay.