SHORT COMMUNICATION

A Silent *COL17A1* Variant Alters Splicing and Causes Junctional Epidermolysis Bullosa

Julia HOFFMANN[1], Federica CASETTI[1], Antonia REIMER[1,2], Juna LEPPERT[1], Gabriele GRÜNINGER[1] and Cristina HAS[1]*
[1]*Department of Dermatology, and* [2]*Berta-Ottenstein-Programme, Faculty of Medicine, Medical Center – University of Freiburg, Hauptstrasse 7, DE-79104 Freiburg, Germany. *E-mail: cristina.has@uniklinik-freiburg.de*
Accepted Jan 23, 2019; E-published Jan 23, 2019

Type XVII collagen is a type II transmembrane protein present in specialized adhesion structures, the hemidesmosomes, which anchor the intermediate filaments to the cell membrane and to the underlying basement membrane. Mutations in *COL17A1*, the gene encoding type XVII collagen cause a spectrum of disorders, being associated with junctional epidermolysis bullosa (EB, MIM 226650), amelogenesis imperfecta and epithelial recurrent erosion dystrophy (MIM 122400) (1). The phenotype depends on the type and dosage of the pathogenic variants. Biallelic *COL17A1* mutations, which lead to premature termination codons, diminish the adhesive capacity of the hemidesmosomes and lead to generalized skin blistering, extensive wounds, enamel hypoplasia, nail dystrophy and irreversible hair loss in patients with junctional EB generalized intermediate (1). Genotype–phenotype correlations show that residual amounts of type XVII collagen resulting from in-frame splicing errors, or from the substitution of specific key amino acids, lead to milder clinical manifestations in localized or late-onset junctional EB (2–6).

METHODS

COL17A1 mutation analysis was performed in a cohort of 68 patients with junctional EB. After obtaining informed consent, EDTA-blood samples and skin biopsies were taken from the index cases, and if possible from parents. The study was approved by the ethics committee of the University of Freiburg, and conducted according to the principles of the Declaration of Helsinki. Mutational analysis of the coding region and exon-intron boundaries of the *COL17A1* gene was performed either by Sanger sequencing or by a next-generation sequencing (NGS) multigene panel containing genes known to be associated with EB (7, 8). Immunofluorescence mapping was done as reported, with a panel of antibodies to adhesion proteins of the dermal–epidermal junction to determine the subtype of EB (9). For type XVII collagen detection, the polyclonal serum NC16A (10) and the monoclonal antibody NC16A3 (Abcam, Cambridge, UK) were employed.

Keratinocytes were isolated from the skin sample of case 1 using standard methods, and cultured in keratinocyte growth medium (Invitrogen, KGM, MA, USA), supplemented with epidermal growth factor and bovine pituitary extract. Total RNA was isolated from subconfluent keratinocyte cultures using the RNAeasy® FFPE kit (QIAGEN, Hilden, Germany), reverse transcribed into cDNA (Fermentas, St Leon-Rot, Germany) and PCR amplified with primers spanning *COL17A1* exon 46 (Table SI[1]). Amplicons were cloned into the TopoTA vector, and DNA isolated from single clones submitted to Sanger sequencing. Immunoblot analysis was performed with keratinocyte lysate of case 1 and from a normal control and detected with the affinity purified NC16A polyclonal sera. *In silico* predictions of the consequences of the variant were performed with Mutation Taster and Human Splicing Finder (http://www.umd.be/HSF3/).

RESULTS AND DISCUSSION

Using a diagnostic approach combining mutation analysis and immunofluorescence mapping, biallelic *COL17A1* pathogenic variants were identified in 64 of 68 junctional EB cases, while in 4 cases, a single *COL17A1* heterozygous pathogenic allele was identified (97.06% detection rate). Careful re-evaluation revealed in all 4 patients the same heterozygous variant of uncertain significance (gnomAD), c.3198C>T, p.Ser1066Ser (Table SI[1], Fig. S1a[1]). Analysis of the DNA from the parents confirmed segregation of the variants in the families and the compound heterozygous state in the index cases. According to ExAC, dbSNP, HGMD professional and 1000Genomes, the variant c.3198C>T, rs369035370, was found in heterozygous state in 2 individuals and has a minor allele frequency of <0.01. *In silico* predictions indicate that c.3198T alters splicing by the following putative mechanisms: (*i*) by marginal increase in the activity of the exonic cryptic donor splice site GTTgtgagt (underlined, Mutation Taster: from 0.2185 to 0.2356; Human Splicing Finder: 2.74% increase), (*ii*) by formation of a new donor splice site TGAgttact (underlined, Human Splicing Finder: 69.34% increase), and (*iii*) by alteration of an exonic splicing enhancer (ESE) site, agctac (Human Splicing Finder) (Fig. S1b[1]).

To verify these predictions and address the pathogenicity of this variant of uncertain significance, total RNA was isolated from keratinocytes of case 1 (compound heterozygous for p.Gly803* and p.Ser1066Ser). *COL17A1* mRNA levels were strongly reduced in the keratinocytes of case 1 compared with control cells (Fig. S1c[1]). Sequencing revealed that the allele carrying the mutation c.2407G>T, p.Gly803* was partially degraded due to mRNA decay (Fig. S1c[1]). Sequencing of cloned amplicons identified the normal nucleotide C, the variant T, or an abnormal sequence at position c.3198 (Fig. S1d[1]). The abnormal sequence consisted in the usage of an upstream donor splice site within exon 46 (prediction (i)), leading to removal of 16 nucleotides c.3193_3208del, frame shift and formation of a premature termination codon, p.Val1065Leufs*35 in the NC5 (non-collagenous 5) domain of type XVII

[1]https://doi.org/10.2340/00015555-3133

doi: 10.2340/00015555-3133
Acta Derm Venereol 2019; 99: 460–461

collagen. These results show that the allele c.3198T induces a splicing error and a leaky cryptic splice site, which is inconstantly used. The splicing consequences could be better appreciated by RNA-seq, which allows precise quantitation of the relative abundance of aberrant transcripts, including those which are minor transcripts. Immunoblot analysis of keratinocyte lysates demonstrated low levels of full-length and shed ectodomain of type XVII collagen in case 1, but no evidence for the presence of the truncated polypeptide p.Val1065Leufs*35. Therefore, we hypothesize that low levels of correctly spliced transcripts generate the residual amount of type XVII collagen (Fig. S1e[1]).

These molecular findings correlate with reduced immunoreactivity for type XVII collagen in the skin samples of the patients (Fig S2[1]). Cases 1 and 2 can be considered functional hemizygous for c.3198C>T because of the strong decay of the mRNA resulting from the c.2407G>T (p.G803*) allele. Indeed, p.G803* was reported as a null-mutation in patients with classical junctional EB generalized intermediate (formerly known as generalized atrophic benign EB; GABEB) (11). The residual amount of type XVII collagen resulting from c.3198T is very low, barely detectable *in vitro* and *in situ*. Case 3 is compound heterozygous for c.2237delG (p.G746Afs*53) and c.3198C>T and demonstrates clearly detectable type XVII collagen in the skin (Fig. S2[1]). The mutation c.2237delG was considered a null-mutation and reported in patients with junctional EB from the Netherlands (12). It is possible that the cryptic splice site activated by c.3198T displays variable degrees of leakage, explaining the variability in type XVII abundancy among our patients. Finally, the highest amount of type XVII collagen was found in the skin of case 4, due to the residual expression from both mutant alleles c.3198C>T and c.4156+1G>A (7).

These genotypes and biochemical phenotypes correlate with clinical manifestations of moderate to mild severity of junctional EB in cases 1 and 4, which were followed-up over several years (Fig. S2[1], Table SI[1])). Cases 2 and 3 were lost from follow-up. Cases 1 and 4 have rather mild acral cutaneous blistering and enamel defects. Case 1 has palmoplantar blistering and genitourinary strictures. Both patients demonstrate ocular involvement. Indeed, 2 recently reported *COL17A1* hypomorphic variants, c.2816C>T and c.3156C>T, affect splicing and lead in a heterozygous state, to inherited recurrent corneal erosions (13–15).

In conclusion, this study proves the pathogenicity of the hypomorphic *COL17A1* variant associated with mild to moderate junctional EB. The *in silico* prediction of splicing alteration was confirmed by sequencing the mRNA; surprisingly the prediction with a relatively low score was experimentally proven. Using this pathogenic sequence variant, the detection rate of mutations in *COL17A1* in our cohort of patients is 100%.

ACKNOWLEDGMENTS

The authors thank the patients and their families for their involvement. CH was supported by a grant from Debra International HAS-1.

The authors have no conflicts of interest to declare.

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