A Homozygous Nonsense Mutation in Type XVII Collagen Gene (COL17A1) Uncovers an Alternatively Spliced mRNA Accounting for an Unusually Mild Form of Non-Herlitz Junctional Epidermolysis Bullosa

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In this study, we describe six Italian patients presenting an unusually mild variant of non-Herlitz junctional epidermolysis bullosa associated with a reduced expression of type XVII collagen. All patients are homozygous for a novel nonsense mutation (R795X) within exon 33 of COL17A1 and show a common haplotype, attesting propagation of an ancestral allele within the Italian population. Analysis of patients’ COL17A1 transcripts showed the presence of two mRNA species: a normal-sized mRNA carrying mutation R795X that undergoes rapid decay, and a transcript generated by in-frame skipping of exon 33. Patients’ keratinocytes were shown to synthesize minute amounts of type XVII collagen, which appeared correctly localized along the cutaneous basement membrane. We therefore suggest that the exon 33-deleted COL17A1 splice variant encodes for type XVII collagen molecules maintaining a functional role and account for the mild phenotype of our patients. Key words: alternative splicing/COL17A1/non-Herlitz junctional epidermolysis bullosa/nonsense mutation/recurrent mutation. J Invest Dermatol 116:182–187, 2001

The hemidesmosome-anchoring filament complex and anchoring fibrils are the ultrastructural hallmarks of the basement membrane zone (BMZ) of stratified epithelia. These specialized attachment structures bridge cytoplasmic keratin filaments of the basal epithelial cells to the upper dermis and anchor the epithelium to the underlying mesenchyme (Eady, 1998).

Type XVII collagen, also known as BP180, BPAG2, or HD4, is a structural hemidesmosomal component encoded by a single copy gene, COL17A1 (Nishizawa et al., 1993; Gatalica et al., 1997). The ectodomain of this type II transmembrane molecule contains a series of collagen-like repeats (Giudice et al., 1992) that associate in homotrimeric structures (Hirako et al., 1996). The relevance of type XVII collagen in ensuring adhesion of the epidermis to the dermis was first indicated by the presence of circulating autoantibodies against type XVII collagen in patients affected with the acquired autoimmune blistering skin disorders bullous pemphigoid and herpes gestationis (Giudice et al., 1993). Using an experimental animal model such antibodies have also been shown to induce subepidermal blistering in vivo (Liu et al., 1995).

Several studies have since demonstrated that mutations in the gene COL17A1 and in other genes encoding the major structural proteins of the hemidesmosomes and the epithelial adhesion ligand laminin-5 underlie junctional epidermolysis bullosa (JEB), a group of recessively inherited bullous diseases of skin and mucosae in which blister formation results from tissue separation within the lamina lucida of the BMZ (Zambruno et al., 1998). According to the revised classification system for inherited epidermolysis bullosa, three major subtypes can be distinguished within this group of disorders: Herlitz (lethal) JEB, non-Herlitz (nonlethal) JEB, and JEB with pylorica atresia (Fine et al., 2000). In particular, COL17A1 mutations have been associated with a non-Herlitz JEB variant formerly termed generalized atrophic benign epidermolysis bullosa (GABEB) (OMIM 226650), which is characterized by widespread skin blistering healing with atrophy, alopecia, and normal life span (Pulkkinen et al., 1999; references therein).

In this report, we investigated six patients from four Italian families suffering from an unusually mild form of non-Herlitz JEB. In all probands, a novel homozygous nonsense mutation within exon 33 of COL17A1 was identified. Through the analysis of mutation consequences at the mRNA and protein levels, we provide evidence for a minor alternatively spliced COL17A1 mRNA lacking exon 33, and residual synthesis and expression of type XVII collagen at the cutaneous BMZ.

Materials and Methods

Clinical findings Four Italian families with members affected with non-Herlitz JEB were studied.
The clinical and immunofluorescence findings of the probands in families A and B, four individuals from two related families of an isolated village in central Italy (Fig 1a), have been detailed elsewhere (Mazzanti et al., 1998).

The proband in family C (Fig 1c, C-II.1) was a 52-year-old woman who had suffered from generalized cutaneous and occasional oral blistering since birth. Her parents, both deceased, were not consanguineous and had never presented skin lesions. The patient had grown and developed normally and her skin condition had significantly improved after puberty. Physical examination revealed cutaneous blisters and atrophic scarring localized exclusively at trauma sites, i.e., on hands, feet, elbows, and knees, as well as nail dystrophy and dental abnormalities. The eyelashes, eyebrows, and scalp hair were normal, whereas axillary and pubic hair were almost completely absent.

The proband in family D (Fig 1c, D-II.1), a 33-year-old man born from consanguineous healthy parents, had also developed skin blisters at birth. His history and clinical manifestations were similar to those of proband C-II.1. He did not show any alopecia, however.

Family D was from a small village in southern Italy, whereas family C was from a town in central Italy 70 miles away from the village of families A and B. In addition, families A/B, C, and D were not known to have any common lineage.

In all patients, the diagnosis of non-Herlitz JEB was established on the basis of the clinical examination, family history, and electron microscopy analysis of skin specimens, which detected tissue separation within the lamina lucida of the cutaneous BMZ (not shown).

Keratinocyte cultures A biopsy of a nonlesional skin area was obtained from all patients. Keratinocyte cultures were established for four affected individuals, a heterozygous healthy carrier, and healthy consenting controls, as described by Zambra et al. (1995). In addition, keratinocytes from a previously characterized type XVII collagen-deficient GABEB patient carrying the frameshift mutation 2342delG at the homozygous state were used (Scheffer et al., 1997).

Immunofluorescence analysis Frozen skin sections 5 μm thick obtained from skin biopsies of the probands C-II.1, D-II.1, and B-IV.1, and a healthy volunteer were processed for immunofluorescence using a three-step biotin-streptavidin–fluorescein procedure as described by Kanitakis et al. (1989). The following monoclonal antibodies (MoAbs) and polyclonal antisera were used: 1A8C (mouse MoAb, which recognizes an epitope in the collagen XVII cytoplasmic part), 233 and 1D1 (mouse MoAbs recognizing epitopes localized in the carboxy-terminal half of type XVII collagen extracellular domain) (Nishizawa et al., 1993; Hirako et al., 1996), G0H3 (rat MoAb to the 06 integrin subunit; gift from A. Sonnenberg, The Netherlands Cancer Institute, Amsterdam, The Netherlands), 3E1 (mouse MoAb to the β4 integrin subunit; Telios Pharmaceuticals, San Diego, CA), and GB3 (mouse MoAb to laminin-5).

Detection of mutation in type XVII collagen cDNA Total RNA, extracted by lysis of the cell cultures in the presence of guanidine isothiocyanate, was reverse-transcribed using the SuperScript RNase H free reverse transcriptase (Life Technologies, San Giuliano Milanese, Milan, Italy). The RNA of patients B-IV.2 and D-II.1 was extracted from a frozen skin biopsy using a Micro-Scale total RNA Separator Kit (Clontech Laboratories, Palo Alto, CA). These cDNAs were used as templates in polymerase chain reactions (PCRs) with specific oligonucleotide primers, designed from the published cDNA sequence (GenBank no. M91669), to

Figure 1. Identification and verification of the homozygous mutation R795X in the probands’ COL17A1 gene and inheritance in the families. (a) Sequence analysis of the patients’ PCR products spanning exons 26–34. Compared with normal cDNA (upper panel), a C→T transition at nucleotide position 2488 is evident in patients’ cDNA (middle panel). In addition, a second transcript carrying the in-frame skipping of exon 33 is present in patients’ cells (lower panel). The 2488C→T transition converts the codon for arginine to a stop codon. (b) Total genomic DNA from all affected individuals was subjected to PCR using primers that amplify a 247 bp fragment encompassing exon 33. Compared with a normal control (upper panel), direct nucleotide sequencing of the amplified DNA from the healthy individual A-V.4 is normal. Patients A-V.1, A-V.2, and A-V.5 are homozygous for the mutation, whereas the healthy mother of A-V.1 and A-V.2 and the son of A-V.5 are heterozygous. The corresponding PCR product from the healthy individual A-V.4 is normally digested.

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produce 14 overlapping cDNA fragments of ~400 bp covering the entire open reading frame of type XVII collagen (Giudice et al., 1992). Specifically, primers used to amplify the 394 bp region of type XVII collagen cDNA comprising exons 26–34 (nucleotides 2143–2536) were sense primer 5′-GGCTCTGAGTACCGTTCAAGG-3′ (Pr 7L) and antisense primer 5′-AAGTCACAGTCCTGGCTGGA-3′ (Pr 7R). The amplimers were analyzed on 2% agarose gel, subcloned into the PCR–TM II vector (Invitrogen, Leek, The Netherlands), and subjected to automated nucleotide sequencing (ABI PRISM, 377 DNA Sequencer, Perkin Elmer, Branchburg, NJ).

**Verification of mutation at the genomic level** Genomic DNA was isolated from peripheral blood by standard procedures. Amplification of exon 33 of COL17A1 and its boundaries (247 bp) was performed using primers placed on flanking introns (GenBank no. U76564) (Gatalica et al., 1997). PCR cycling conditions were 95°C for 8 min, 94°C for 45 s, 55°C for 45 s, 72°C for 45 s (30 cycles), and extension at 72°C for 10 min. The amplified products were subjected to direct automated nucleotide sequencing. As the mutation detected in the COL17A1 gene abolishes a HaeIII restriction site, HaeIII endonuclease digestion (New England Biolabs, Beverly, MA) was used to confirm the homozygous state of the mutation in the patients, and to assess its inheritance in the families.

**Search for alternatively processed COL17A1 transcripts** Reverse transcriptase PCR (RT-PCR) based tests were designed to assess the presence of exon 33 alternatively processed transcripts. Total RNA was obtained from cultured keratinocytes of healthy donors, four patients, the heterozygous healthy carrier A-VI.1, 2342delG GABEB cells, and human peripheral blood lymphocytes. A first set of PCRs was performed using the same primer pair (Pr 7L/Pr 7R) and amplification conditions described above. In a separate set of PCRs, the sense primer Pr 7L was used in combination with an antisense primer spanning the junction between exons 32 and 34 and consisting of the last seven bases of exon 32 and the first 13 bases of exon 34 (5′-GGCTCTGAGTACCGTTCAAGG-3′ (Pr 32-34R). Amplification was carried out under highly stringent conditions to avoid mispriming. For primer Pr 32-34R, with the full-length COL17A1 transcript. PCRs were performed in a final volume of 25 µl containing 1 mM MgCl₂, 25 pmol of each primer, 200 µM dNTP, and 2 U of AmpliTag Gold DNA polymerase (Perkin Elmer). PCR cycling conditions were 94°C for 10 min, followed by 94°C for 30 s, 68°C for 30 s, and 72°C for 30 s (35 cycles). Both sets of PCRs were also performed on a cloned wild-type cDNA fragment spanning exons 26–34, as a control. The amplified products were analyzed on a 2% agarose gel and all were subjected to direct sequencing. To verify cDNA quality, amplification of the housekeeping glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcriptional product was carried out using the sense primer 5′-TGGTATCCTGTAGGTCTCCAAGG-3′ (Pr 7L) and antisense primer 5′-ATGCCAGTGACGCTTGCCCTGACG-3′ (annealing temperature 55°C).

**Northern blot analysis** Poly A + RNA purified from 0.4 mg of total RNA (Fast Track® 2, Invitrogen) was subjected to northern blot analysis as described by Chavanas et al. (1997). Quantification of the hybridization signals was performed by densitometric scanning using a GS–750 densitometer (Biorad, Hercules, CA).

**Immunoblotting analysis** Keratinocytes were grown in serum-free medium until subconfluence, lysed with cold water containing 20 µl per ml benzonase (Boehringer Mannheim, Mannheim, Germany), and then extracted with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer. Protein extracts were separated on 5% acrylamide slabs, electrophoretically transferred to nitrocellulose filters, and stained with MoAb 1A8C. The molecular weight of type XVII collagen was assessed by matching in blot-to-blott matches, as described by Pas et al. (1997).

**Haplotyping analysis** To determine the haplotype of the non-Herlitz JEB patients, polymorphic microsatellites D10S577, D10S603, D10S222, D10S566, D10S534, and D10S597, flanking the COL17A1 locus on chromosome 10 (from the Genome database), and the intragenic microsatellite marker COL17A1-C1A (Scheffer et al., 1997) were used. PCRs were performed using [3H]-dCTP for labeling, followed by electrophoresis on 6% polyacrylamide–8 M urea gel. The intragenic COL17A1 intron 16 BstUI, intron 28 NalIV, intron 54 BfaI restriction fragment length polymorphisms were analyzed by PCR amplification of total genomic DNA from family members, using primers, restriction enzymes, and conditions as described by Gatalica et al. (1997).

**RESULTS**

**Non-Herlitz JEB patients’ skin displays residual collagen XVII immunoreactivity** Immunofluorescence analysis of perilesional skin biopsies from the patients showed that, compared with normal control skin, the anticollagen XVII MoAbs IAB, ID1, and 233 all reacted with greatly reduced intensity to the BMZ (not shown) (Mazzanti et al., 1998).

In all patients mutation analysis shows homozygosity for a nonsense mutation in COL17A1 exon 33, which results in two mRNA forms: a normal length mRNA carrying the mutation and an internally shortened transcript carrying the in-frame skipping of exon 33. As collagen XVII expression was markedly reduced in the skin of all patients, search for mutation was initiated by RT-PCR amplification of the entire COL17A1 open reading frame. In all patients, agarose gel electrophoresis of the amplification products corresponding to the cDNA region encompassing exons 26–34 showed two bands, a 394 bp band also present in the healthy controls, and an additional 358 bp fast-migrating fragment, unique to the patients. Nucleotide sequencing of the 358 bp cDNA fragment identified an in-frame deletion (36 bp) corresponding to the sequence encoded by the entire exon 33 (Fig 1a). Sequence analysis of the 394 bp cDNA fragment identified a C→T substitution at position 2488 of COL17A1 cDNA sequence (GenBank no. M91669) (Giudice et al., 1992) (Fig 1b). The 2488C→T transition converts a codon (CGA) for arginine to a premature termination codon (PTC) and predicts a type XVII collagen polypeptide truncated at residue 795 within the fifteenth collagenous subdomain (Col15) of the extracellular region. This mutation was therefore designated R795X (GenBank no. AA851499) (Gatalica et al., 1997). In all the patients, direct sequencing of the genomic DNA fragment encompassing exon 33 and the flanking intronic boundaries revealed homozygosity for transition 2488C→T (GenBank no. U76564) (Gatalica et al., 1997) (Fig 1b). No other nucleotide sequence variation was detected compared with unrelated healthy controls. The nucleotide substitution 2488C→T abolishes an HaeIII endonuclease restriction site. We therefore assessed the inheritance of this mutation in the four non-Herlitz JEB families by HaeIII digestion. As shown in Fig 1(c), electrophoretic analysis of the PCR products confirmed the homozygosity of all patients for mutation 2488C→T and revealed that the mother of patients A-V.1 and A-V.2 and the healthy son of patient A-V.5 were heterozygous carriers.

The COL17A1 exon 33-skipped transcripts are also detectable in normal human keratinocytes. In order to investigate the mechanism leading to the skipping of the exon carrying the nonsense mutation, RT-PCR-based tests were performed. As shown in Fig 2(a), the use of the primers spanning exons 26–34 on a panel of samples revealed the additional 358 bp fragment corresponding to the in-frame exon 33-skipped transcript in our patient (A-V.5) and, to a much lesser extent, in the heterozygous carrier (A-V.1) keratinocytes, but neither in normal cells nor in the type XVII collagen-deficient GABEB cells carrying the homozygous frameshift mutation 2342delG (Scheffer et al., 1997). By using in a separate set of PCR amplifications a reverse primer (Pr 32-34R) that spans the boundaries between exons 32 and 34, however, the exon 33-deleted transcripts were also detected in normal human keratinocytes and in the 2342delG GABEB cells (Fig 2b). These data indicate that the skipping of exon 33 represents a constitutive process occurring at very low level in all keratinocytes.

**Northern analysis shows greatly reduced levels of type XVII collagen mRNA in the patients** Evaluation of the steady-state level of type XVII collagen transcripts was performed by northern blot analysis. Densitometric analysis of the hybridization bands showed that the level of COL17A1 mRNA was reduced by about
In the past few years, defective expression of the hemidesmosomal component type XVII collagen has been shown to correlate to a variant of non-Herlitz (nonlethal) JEB formerly named GABEB, characterized by widespread skin blistering healing with atrophy, mild mucosal lesions, and dystrophic nails and teeth (Jonkman et al., 1995; Pohlka-Gubo et al., 1995). Atrophic alopecia, almost invariably present, has been considered for a long time the distinctive phenotypic manifestation of the disease (Jonkman et al., 1996). Altered collagen XVII expression has also been shown to result in more localized JEB forms, however, with predominantly acral blistering and normal hair (Schumann et al., 1997). So far a number of mutations in the gene encoding type XVII collagen, COL17A1, have been identified in these GABEB patients (Pulkkinen et al., 1999; references therein). In most cases, mutations consist of PTCs affecting both alleles and resulting in the complete absence of collagen XVII (Pulkkinen and Uitto, 1998). Nonsense mediated decay of the abnormal transcripts has been shown to be responsible for the lack of type XVII collagen at both the mRNA and protein level in these patients (Darling et al., 1997; 1998b). On the other hand, a few missense mutations that allow synthesis of mutant type XVII collagen and can be compatible with its deposition along the BMZ have also been identified (Schumann et al., 1997; Floeth et al., 1998).

In this report, we describe the genetic defect underlying an unusually mild clinical non-Herlitz JEB phenotype in six Italian patients. In all probands, search for mutations demonstrated homozygosity for a novel nonsense mutation (R795X) in exon 33 of COL17A1 gene consequent to a C→T base transition. In these patients, the occurrence of the PTC in the coding sequence of COL17A1 correlates with dramatically reduced levels of type XVII collagen transcripts as detected by northern blot, in line with nonsense mediated decay of the mRNA molecules encoding truncated type XVII collagen polypeptides (Maquet, 1995; Darling et al., 1997; 1998b; Hentze and Kulozik, 1999).

Interestingly, RT-PCR experiments evidenced two mRNA species in patients’ keratinocytes: the expected normal-length transcript carrying mutation R795X and an additional transcript carrying the in-frame deletion of exon 33. As using appropriate PCR conditions we could also detect this exon 33-lacking COL17A1 mRNA in normal keratinocytes, we conclude that this shown). Similarly, probands C-II.1 and D-II.1 also displayed the shared haplotype, differing only for the more external flanking markers, providing support for propagation of an ancestral allele within the Italian population (not shown).

DISCUSSION

95% in patients A-V.5 and C-II.1 compared with healthy controls, and even more in patients A-V.1 and A-V.2 (not shown).

Immunoblotting analysis evidences residual synthesis of type XVII collagen molecules. Immunoblot analysis of cultured keratinocyte extracts with MoAb 1A8C, which binds to the intracellular domain of type XVII collagen, showed in our patients (A-V.5 and D-II.1) one band at ~180 kDa that was present, has been considered for a long time the distinctive phenotypic manifestation of the disease (Jonkman et al., 1996). Altered collagen XVII expression has also been shown to result in more localized JEB forms, however, with predominantly acral blistering and normal hair (Schumann et al., 1997). So far a number of mutations in the gene encoding type XVII collagen, COL17A1, have been identified in these GABEB patients (Pulkkinen et al., 1999; references therein). In most cases, mutations consist of PTCs affecting both alleles and resulting in the complete absence of collagen XVII (Pulkkinen and Uitto, 1998). Nonsense mediated decay of the abnormal transcripts has been shown to be responsible for the lack of type XVII collagen at both the mRNA and protein level in these patients (Darling et al., 1997; 1998b). On the other hand, a few missense mutations that allow synthesis of mutant type XVII collagen and can be compatible with its deposition along the BMZ have also been identified (Schumann et al., 1997; Floeth et al., 1998).

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German/Norwegian (Scheffer R795X. demonstrated for recurrent mutations in polymorphisms within and near non-Herlitz JEB families exhibits a common haplotype for 1998a) families. Therefore, Italian patients affected with 1993; McGrath et al consequences of nonsense or frameshift mutations are mitigated by alternatively processed transcripts encoding at least partially (1D1). cytes of healthy donors does not argue in favor of a physiologic COL17A1 ~179 kDa due to the deletion of 12 amino acid residues in the exclude a developmental role and/or a tissue-specific regulation of function for the exon 33-deleted collagen XVII form, we cannot of the exon 33-deleted type XVII collagen as a faint staining was translation product of the minor mRNA transcript, which of our probands’ skin is also in keeping with synthesis and secretion that the transcript carrying the nonsense mutation R795X does not constitutively splices out the exon harboring the mutation, and the transcript carrying the nonsense mutation R795X does not result in any detectable protein. The immunofluorescence analysis of our probands’ skin is also in keeping with synthesis and secretion of the exon 33-deleted type XVII collagen as a faint staining was visualized with both monoclonal antibodies to the cytoplasmic domain (1A8C) and to the carboxy-terminal end of the molecule (1D1). All together, our data indicate that the disease condition is primarily caused by reduction in the level of full-length collagen XVII mRNA carrying mutation R795X, and that the minor form of type XVII collagen transcripts bearing the internal deletion of exon 33 may account for the unusually mild clinical phenotype of our probands. Indeed, genetic diseases in which the deleterious consequences of nonsense or frameshift mutations are mitigated by alternatively processed transcripts encoding at least partially functional polypeptides have already been reported (Moriski et al, 1993; McGrath et al, 1999).

Finally, we show that mutation R795X shared by four Italian non-Herlitz JEB families exhibits a common haplotype for polymorphisms within and near COL17A1 indicating a founder effect. The propagation of ancestral alleles has already been demonstrated for recurrent mutations in COL17A1 in Dutch/German/Norwegian (Scheffer et al, 1997) and in Austrian (Darling et al, 1998a) families. Therefore, Italian patients affected with epidermolysis bullosa and showing a defective expression of type XVII collagen should first be screened for the presence of mutation R795X.


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skipping in hprt mRNA of Chinese hamster ovary cells results from an artifact of RT-PCR. *RNA* 3:660±676, 1997
