Experimental concise report

Identification of mutations in the *COL7A1* gene in a proband with mild recessive dystrophic epidermolysis bullosa and aortic insufficiency

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Summary

We report the clinical and molecular findings in a patient with a mild form of recessive dystrophic epidermolysis bullosa (RDEB) and aortic insufficiency. To our knowledge, this is the first report of association between RDEB and abnormalities of the aortic valve. Analysis of the *COL7A1* gene has revealed two new mutations, a 20-bp duplication and a splice-site mutation. These findings support the recessive inheritance of the moderately severe cases of mild RDEB.

Report

Dystrophic epidermolysis bullosa (DEB) results from mutations in the *COL7A1* gene on chromosome 3p, which encodes type VII collagen, the major component of anchoring fibrils. The site and specific nature of the underlying mutations determine the clinical phenotype, which ranges widely from a relatively mild disorder, typically inherited autosomal dominantly (DDEB), to a severe, mutilating condition, inherited autosomal recessively (RDEB). Over 100 distinct mutations within the two non-collagenous domains, NC1, NC2, and the helical domain of *COL7A1* have been identified in DEB patients,[1] and in the majority of cases, the mutations have been specific to individual families, with only a few reported cases of recurrent mutations.[1 2 3] The correlation between the mutations and the observed clinical phenotypes is still emerging.

Probands with dominant DEB, characteristically have a glycine substitution mutation on one allele, while patients with the most severe type of RDEB have premature termination codon (PTC) mutations in both alleles of *COL7A1* resulting in a complete lack of anchoring fibrils and collagen VII.

Within the dominant and recessive forms of DEB, there is another sub-group of patients in which the affected individuals have a relatively mild phenotype, the parents appear unaffected, and the family history is negative for blistering disease. These so called "sporadic" cases of DEB were previously thought to have a new dominant DEB mutation, however, many patients in this group are now known to have a mild, recessive form of DEB.[4]

In the mild forms of RDEB, at least one of the *COL7A1* alleles typically contains a missense mutation, which renders it capable of producing full-length, albeit functionally imperfect, type VII collagen polypeptides.[5] The other allele can contain a PTC, a second missense mutation, an in-frame deletion or a splice site mutation. As a result of this combination of mutations, mutant full-length type VII collagen molecules may be able to assemble into a small number of poorly functioning anchoring fibrils. The resulting attachment structures, though weak, are present, which accounts for the milder phenotype observed in these forms of RDEB.

RDEB has been associated with several long-term sequelae. Some of the most common complications include nutritional deprivation secondary to severe mucous membrane lesions leading to esophageal strictures and scarring. Dental caries are also frequently observed. Chronic anemia due to increased blood loss from the skin and gastrointestinal tract, coupled with decreased oral intake often results in continuous iron therapy supplemented by blood transfusions. Cutaneous carcinomas, particularly squamous cell carcinoma, have also been associated with RDEB.

Although far less commonly observed, certain cardiac conditions have been reported in patients with RDEB. Several cases of dilated cardiomyopathy in children with RDEB have been described.[6 7 8] In addition, mitral valve prolapse, which has a known association with several connective tissue disorders, has also been reported in two patients with RDEB. [9 10] The common pathological mechanism between the valvular anomaly and RDEB is thought to be due to an abnormality of collagen metabolism. [10]

Herein, we report two new *COL7A1* mutations, a 20-bp duplication, and a splice-site mutation in a proband with clinically significant aortic insufficiency necessitating aortic valve replacement. To our knowledge, this is the first time that abnormalities of the aortic valve have been reported in a patient with RDEB. In addition, these findings extend the body of evidence implicating inheritance of two mutations of intermediate severity in the mild form of RDEB.

The proband reported here is a 25-year-old male who is one of three siblings born to non-consanguineous, clinically unaffected parents. There was no history of dermatological disease in the family. Clinical findings in the patient included erosions and atrophic scarring in sites of previous trauma, such as bilateral knees, shins and heels. Complete nail dystrophy was noted, as was pseudosyndactyly of both hands (Fig. 1). In addition, the patient had a history of chronic, progressive aortic regurgitation secondary to aortic valvular degeneration, with a severely dilated left ventricle, for which he underwent aortic valve replacement surgery in the spring of 2001. The histological findings showed dysplasia of the aortic valve, with focally abnormal architecture with accumulation of myxoid material and focal interruption of the fibrosa. One portion of the tissue appeared to be an elastic artery with extensive fibrosis and loss of elastic tissue.

Previous studies have suggested that an underlying etiology for aortic valvular degeneration may be defective synthesis of collagen or elastic fibers, lending support to a possible link with EB. [11] Furthermore, in a recent case report, a Japanese EB patient with severe mitral regurgitation was shown to have similar microscopic myxomatous changes of the valve to that seen in our patient. These authors suggest that an abnormality in collagen metabolism may play a role in the development of valvular degeneration and EB. [10] Further studies are necessary to determine whether a true relationship between these conditions exists, and if so, what common mechanisms and factors influence their development.

In order to search for mutations in the *COL7A1* gene, we used a combined strategy of heteroduplex analysis and direct PCR sequencing. Genomic DNA was isolated from peripheral blood lymphocytes of the proband using PureGene DNA Isolation Kit (Gentra Systems, Minneapolis, MN) and used as a template for PCR amplification. 8μl of the PCR product was prepared for heteroduplex analysis using conformation sensitive gel electrophoresis (CSGE). Heteroduplexes were visualized by staining with ethidium bromide. Bands of altered mobility detected in the CSGE gel were directly sequenced using the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, Foster City, CA).

Sequencing of the PCR products corresponding to exons 23 and 64 from the patient's DNA resulted in the identification of two novel mutations in the *COL7A1* gene, a 20 bp duplication at the intron 22/exon 23 boundary, and an A-to-G transition at position +4 of the 5' donor splice site of intron 64 (IVS 64 +4 A>G) (Fig. 2). To verify the duplication mutation, we subcloned the PCR product corresponding to exons 22-24 using the TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA). We sequenced a total of 7 clones, corresponding to 2 wild type and 5 mutant alleles. To verify the splice site mutation, we used a mismatched PCR strategy, with the following primers: 5'TGGCCTGAATGGAA AAAAC**C**TG3' (forward) and 5'CTATGTTTCTGGATGCATCTG3' (reverse). The forward primer introduces a *Dde* I restriction site in the wild-type allele.

The screening of 72 control chromosomes from unrelated, healthy individuals failed to disclose the presence of the splice site mutation. The nature of the duplication

mutation, together with the absence of the splice variant in control subjects, suggests that these mutations are not common polymorphisms, but represent true pathogenetic mutations.

Among several possibilities, the most likely explanation for the first mutant allele is a duplication of the sequence cctgcagAAGTGCCTGGGTC, at the intron 22/exon 23 boundary within the amino-terminal non-collagenous NC-1 domain, specifically within the fibronectin type III-like repeat domain FN-9. To our knowledge, this is the first time that this mutation has been reported. This duplication is predicted to lead to a PTC 59-bp downstream in exon 23 and could have several potential consequences: a frame-shift mutation with a PTC resulting in degradation of the mutant transcript by nonsense mediated mRNA decay, in-frame skipping of exon 23 or retention of intron 22.

The majority of the *COL7A1* PTC mutations that have been studied thus far at the mRNA level show marked *COL7A1* mRNA decrease consistent with nonsense-mediated mRNA decay. [12] However, aberrant mRNA splicing around exon 23 is another possible consequence of the mutation found in this patient. In a previous study, a 16-bp intraexonic deletion in exon 87 of *COL7A1* in a family with DDEB was identified, and predicted to generate a frameshift mutation and a downstream PTC. [13] Although the deletion did not disrupt any consensus splicing sequences, the mutation resulted in exon skipping and the subsequent restoration of the reading frame, instead of the expected generation of a PTC.

The second mutation observed in our patient, an A-to-G transition, occurred at position +4 of the 5' donor site of intron 64, within the central collagenous domain of the type VII collagen polypeptide. An A-to-G transition affecting the same position in the 5' splice site has previously been reported in the beta-spectrin gene where it lead to skipping of an exon, a shift in the reading frame and a PTC within one amino acid residue of the novel sequence.[14] More recently, an A-to G transition at position +4 within the 5' splice site has been described in intron 87 of *COL7A1* in a patient with the Hallopeau-Siemans variant of RDEB, where it also lead to a PTC.[15] To our knowledge, this is the first incidence of this mutation in the *COL7A1* gene.

Because this mutation occurs within the 5' donor splice site, its consequence is at the transcriptional level. To compare the splice sites from the wild-type and mutant

sequences, we calculated their consensus values according to Shapiro and Senapathy.[16] In this procedure, splice sites are scored relative to the frequency with which the same bases have been found in 542 normal splice sites of primate genes. The splice-site score for the wild-type 5' donor splice site of intron 64 is 83.21% and for the mutated form of the 5' donor splice site of intron 64 is 72.45% (a sequence identical to the consensus gives a 100% score). This could result in decreased efficiency of the splicing apparatus leading to lower levels of the expected mRNA transcript. This would explain the lack of phenotype in the parent from whom this mutant allele was inherited, as well as the relatively mild phenotype of the proband despite his recessive condition.

On the other hand, the single base pair substitution could potentially result in an aberrant splicing pattern, leading to the complete skipping of exon 64, the retention of intron 64, or the activation of cryptic splice sites in the region.

In conclusion, this case report exemplifies the notion that identification of disease causing mutations can be a valuable tool in the characterization of the functional domains of the encoded proteins, and may have important implications for understanding the complex genotype-to-phenotype correlations of EB and other heritable diseases.

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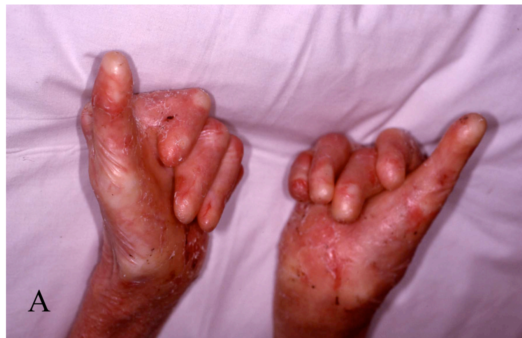
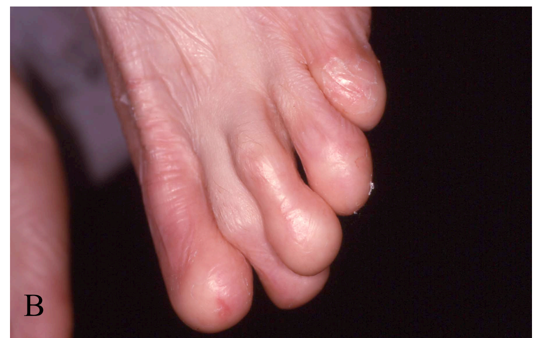
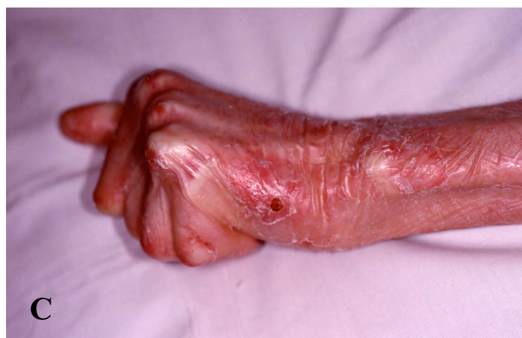
Figure 1 Clinical presentation of the proband with mild RDEB. Note the complete nail dystrophy of both hands (**a**) and feet (**b**), as well as pseudosyndactyly of the hands (**c**) seen in recessive DEB. Healing erosions are also present (**d**).







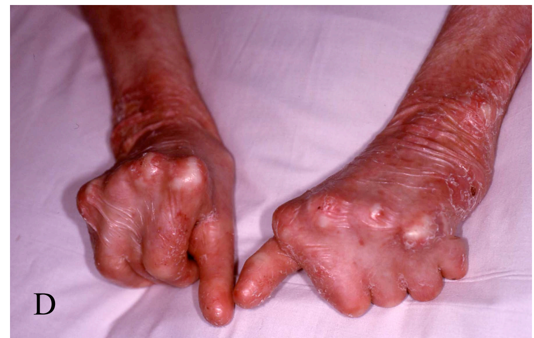
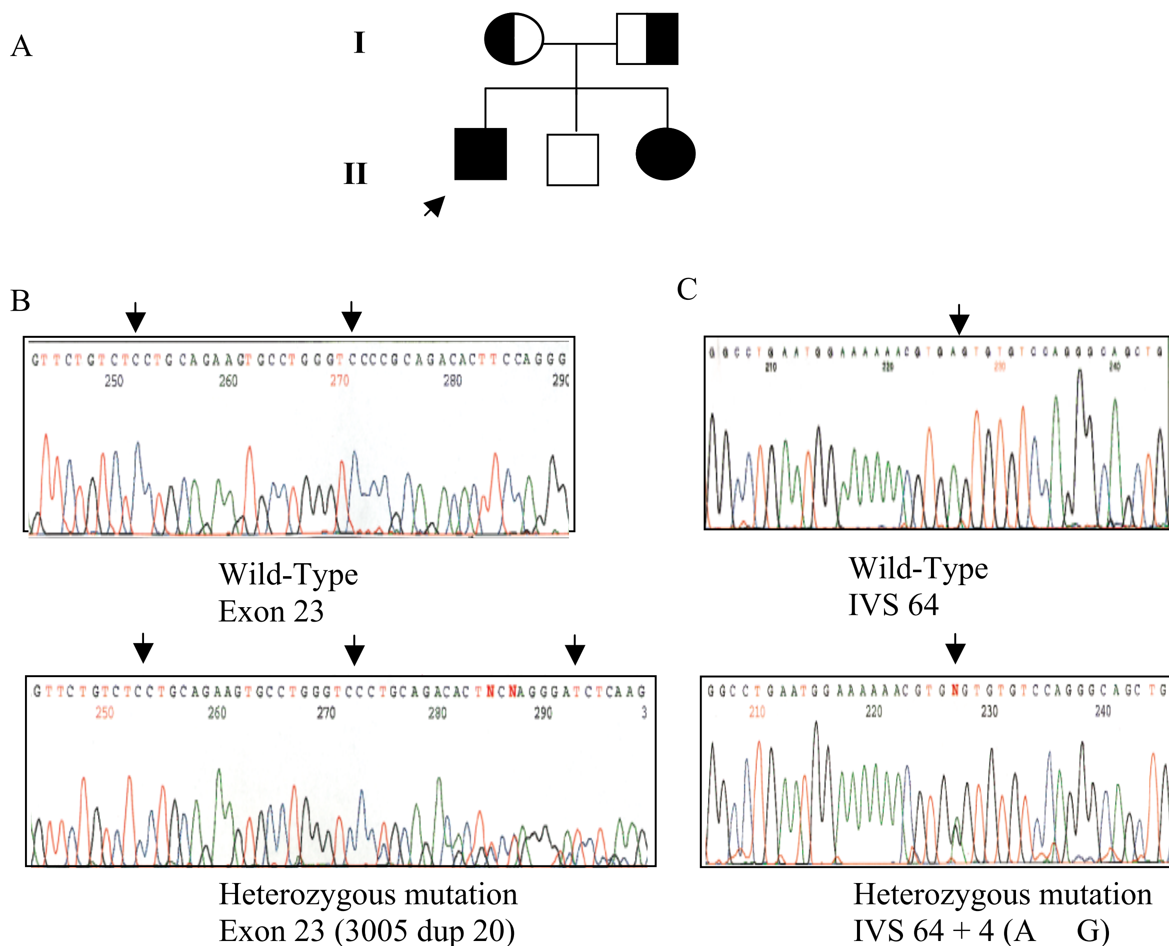


Figure 2 Molecular findings in the *COL7A1* gene (**a**) Pedigree of the proband with mild RDEB. The arrow denotes the proband. (**b**) Sequence of the 20-bp duplication mutation. Automated sequencing of the PCR product corresponding to exon 23 in a control subject (top), as well as the patient (bottom) are shown. Note the 20-bp duplication, indicated by arrows, in the affected individual heterozygous for this insertion in the mutant allele. (**c**) Sequence of the splice-site mutation. Automated sequencing of the PCR product corresponding to IVS 64 in a control subject (top), as well as the patient (bottom) are shown. Note the double peak indicated by an arrow in the affected individual heterozygous for the substitution (A-to-G) in the mutant allele. The corresponding peak in the wild-type sequence (A only) is also denoted by an arrow.



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