Recurrent Mutations in the Type VII Collagen Gene (COL7A1) in Patients with Recessive Dystrophic Epidermolysis Bullosa

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Mutations in the type VII collagen gene (COL7A1) are known to underlie different forms of the inherited blistering skin disease dystrophic epidermolysis bullosa (DEB). Most COL7A1 mutations are unique to individual families, and therefore it is usually necessary to screen all 118 exons of the gene to determine the molecular pathology in a patient with DEB. This study aimed to identify any recurrent mutations in COL7A1 that might be applicable to mutation-detection strategies in these patients. Mutational analysis was undertaken in 23 British patients with autosomal recessive DEB using PCR amplification of genomic DNA followed by heteroduplex analysis, nucleotide sequencing, and restriction site analysis. Two recurrent mutations were identified: R578X (6 of 46 alleles) and 7786delG (7 of 46 alleles). Haplotype analysis revealed that the mutations existed on similar allelic backgrounds in different patients, consistent with propagation of common British ancestral haplotypes, although R578X and 7786delG also have been described in DEB patients from other ethnic backgrounds. Given the high relative frequency of these two COL7A1 mutations, British patients with recessive DEB should be screened initially for these nucleotide changes by PCR amplification of genomic DNA and restriction analysis before more exhaustive screening of COL7A1. Key words: basement membrane zone/blistering disorder/DNA-based prenatal testing/genetic skin disease. J Invest Dermatol 109:246-249, 1997

Mutations in the gene encoding type VII collagen (COL7A1), the major component of anchoring fibrils at the dermoepidermal junction (Keene et al., 1987; Burgeson, 1993), are known to underlie both autosomal dominant and autosomal recessive forms of the inherited blistering skin disease dystrophic epidermolysis bullosa (DEB) (Christiano and Uitto, 1996b). Although more than 100 pathogenetic COL7A1 mutations in DEB have been reported, most are unique to individual families, with no mutational hot spots so far reported in this gene (Christiano and Uitto, 1996a, 1996b). COL7A1 consists of 118 exons, the highest number of exons in any gene published to date (Christiano et al., 1994). Screening all exons of this gene for mutations in patients with DEB is therefore time-consuming, labor-intensive, and expensive. The purpose of this study was to identify any recurrent mutations in COL7A1 in British patients with recessive DEB that might be applied to future mutation-detection strategies. Haplotype analysis in the families studied was also performed to establish whether recurrent mutations were propagated on mutant ancestral alleles within the British population or whether they had arisen as mutational hot spots in COL7A1.

MATERIALS AND METHODS

Clinical Analysis Twenty-three patients with recessive DEB (10 male, 13 female; ages 2–38 y) were included in this study. All were of British Anglo-Saxon ancestry and were not known to be related, and there was no history of consanguinity in any of the families. All patients were examined in the epidermolysis bullosa clinic at St. John’s Institute of Dermatology. The diagnosis of recessive DEB was determined in each case by the family constellation and by skin biopsy specimens showing absent or attenuated immunostaining for type VII collagen at the dermoepidermal junction (Heagerty et al., 1986), as well as absent or poorly formed anchoring fibrils beneath the lamina densa on electron microscopy (Tidman and Eady, 1985). Genomic DNA was extracted from peripheral blood lymphocytes of the patients and immediate family members by standard methods (Sambrook et al., 1989). In two families, genomic DNA was also extracted from chorionic villi in two pregnancies at risk for recurrence of recessive DEB, as described previously (Dunnill et al., 1995).

Molecular Analysis Total DNA was used as a template for amplification of genomic sequences of COL7A1. Pairs of oligonucleotide primers spanning all 118 exons of the gene were synthesized on the basis of intronic sequences to generate polymerase chain reaction (PCR) products (GenBank no. L23982). Specifically, exon 13 and exons 103/104 (see Results) were amplified using the following primers: for exon 13, sense primer 5’CCTTCTCCTCTGCTGCTGCT’ and antisense primer 5’ACCGAGACCAAGTGGAGC’; for exons 103/104, sense primer 5’CGGGCTCGTGGTATCTCTAAG’ and antisense primer 5’CAGGCACTACCATGTTG’. For PCR amplification, approximately 250 ng of genomic DNA...
was used as the template under standard conditions, including 10X buffer (Perkin-Elmer Cetus, Norwalk, CT), 4% dimethylsulfoxide, and 1 U Taq polymerase (Perkin-Elmer Cetus) in a total volume of 25 μl. The amplification conditions for both sets of primers were 94°C for 5 min, followed by 38 cycles of 94°C for 45 s, 55°C for 45 s, and 72°C for 45 s in an OmniGene thermal cycler (Hybaid Ltd., Teddington, Middlesex, UK). Five-microliter aliquots of PCR products were analyzed by 2% agarose gel electrophoresis. Aliquots of 3–8 μl of each sample were prepared for heteroduplex analysis using conformation-sensitive gel electrophoresis (Ganguly et al, 1993) and were visualized with ethidium bromide staining. Samples containing heteroduplexes were subjected to direct sequencing by an ABI automated sequencer (Warrington, UK). The exon 13 and exon 104 mutations (see Results) abolished an Xhol site and a Bsal restriction enzyme site, respectively. Aliquots of 5–12 μl of the appropriate PCR products were used for restriction endonuclease digestion with the corresponding enzyme, according to the manufacturer’s instructions (New England Biolabs, Beverly, MA). Digestion products were examined by 2% agarose gel electrophoresis and were visualized with ethidium bromide.

**Haplotype Analysis**

Three intragenic COL7A1 polymorphisms and two flanking polymorphisms near the COL7A1 locus at 3p21 were studied. For the intragenic markers, genomic DNA was used to amplify with PCR exon 21, exons 28–31, and exons 83–85, and for the flanking markers, DNA sequences incorporating D3S2 and D3F22 were amplified. PCR conditions were identical to those used to amplify exon 13 and exons 103/104. Annealing temperatures were 55°C for all PCR reactions except for D3F22 (60°C). Aliquots of 5 μl of PCR products were analyzed by electrophoresis on 2% agarose gels, after which 5–12 μl of each sample was digested with the following restriction endonucleases, according to the manufacturer’s recommendations (New England Biolabs): exon 21 with ProlI, exons 28–31 and D3S2 with MspI, exons 83–85 with EcoR109, and D3F22 with HindIII (Christiano et al, 1996). The resulting digestion products were examined on 2% agarose gels stained with ethidium bromide.

To provide further information about the haplotypes of the families studied, we amplified the following five sets of microsatellite markers close to the COL7A1 locus at 3p21: D3S1029, D3S1076, D3S1235, D3S1573, and D3S1581 (Research Genetics Inc., Huntsville, AL). The marker D3S1235 was examined by end-labeling the sense primer with [γ-32P]dATP by standard methods (Sambrook et al, 1989). The PCR amplification conditions were: 94°C for 7 min, followed by 27 cycles of 94°C for 1 min, 59°C for 1 min, and 72°C for 1 min. Samples for analysis were prepared by adding 6 μl of PCR product to 4 μl of stop solution (Amersham, Little Chalfont, UK) and heating to 95°C for 5 min. Five microliters of each sample was then run on 6% acrylamide and visualized by exposure to Hyperfilm (Amersham). The other four microsatellite markers were PCR amplified from genomic DNA using the following conditions: 94°C for 5 min, followed by 38 cycles of 94°C for 45 s, annealing temperature for 45 s, and 72°C for 45 s. Annealing temperatures were 55°C for D3S1029, D3S1076, and D3S1573 and 57°C for D3S1581. Eight microliters of each sample was then run on 6% non-denaturing acrylamide gels and visualized using ethidium bromide staining.

**RESULTS**

Identification of Two Recurrent COL7A1 Mutations

Nucleotide sequencing of PCR products spanning exon 13 and displaying a heteroduplex on conformation-sensitive gel electrophoresis analysis revealed a C-to-T transition at position 1732 in exon 13 of COL7A1 (GenBank no. L02870), which converts an arginine residue to a stop codon (CGA to TGA). This mutation, R578X, abolishes a restriction enzyme site for Xhol (Fig 1a). After demonstrating this mutation by nucleotide sequencing in two unrelated patients, we screened the remainder of the patients by restriction analysis with Xhol to assess the presence or absence of R578X. Screening of the 23 British patients with recessive DEB revealed this mutation on 6 of 46 alleles (13%). In all cases, patients were heterozygous for the mutation.

Patients displaying heteroduplex bands in the PCR products spanning exons 103/104 were found to have deletion of a G residue at position 7786 on nucleotide sequencing. This mutation, 7786delG, results in a frame shift and a downstream premature termination codon in exon 106 and causes loss of a restriction enzyme site for Bsal (Fig 1b). After initial identification of the mutation by sequencing in two patients, this enzyme was used to screen all patients in the study. We identified 7786delG on 7 of 46 (15%) alleles studied, with all patients being heterozygous for the mutation.

**Evidence for Propagation of Common British Ancestral Mutant Alleles**

Haplotype analysis of patients and family members with the R578X mutation are shown in Fig 2 (families A to D). R578X was present on an identical haplotype background in all affected families for the three intragenic and two flanking markers studied.

Haplotype analysis in affected families with 7786delG demonstrated that this mutation was present on two haplotype backgrounds, which differed from each other only at the flanking COL7A1 marker, D3S2 (Fig 2; families and patients D to J). Microsatellite markers D3S1235 and D3S1581 were informative in these families, whereas the remaining three microsatellite markers were noninformative.

**Evidence for Recombination in the Flanking Marker D3F22 in Two Families**

Evidence was found for recombination in the COL7A1 flanking marker D3F22 in two families with the R578X

Figure 1. The R578X and 7786delG mutations can be identified by restriction endonuclease analysis. (a) The mutation R578X abolishes an Xhol site. In the father, unaffected sibling, and unrelated healthy control (lanes 1,3,5), the 294-bp PCR product is digested to 186-bp and 108-bp fragments. The mother and proband (lanes 2,4) have both of these fragments in addition to the 294-bp product, indicating that they are heterozygous for this mutation. (b) The mutation 7786delG causes loss of a Bsal site. The mother, unaffected sibling, and unrelated healthy control (lanes 2,3,5) have <60-bp fragments from the 512-bp PCR product. The father and proband (lanes 1,4) have these digestion products in addition to a 120-bp fragment, indicating that they are heterozygous for the mutation. MW, dX174 HaeIII molecular-weight marker.
Figure 2. Evidence for propagation of common ancestral alleles bearing the R578X and 7786delG mutations. Haplotype analysis in affected families is shown using COL7A1 intragenic markers in exon 21, exons 28–31, and exons 83–85, and flanking markers at D3S2 and D3F22. R578X occurs on identical haplotypes in families A, B, C, and D. Mutation 7786delG is present on two haplotypes that differ only at the D3S2 locus: "-" in families D, E, and F and in patients G and H, and "+" in families I and J. Microsatellite markers D3S1235 and D3S1581 are informative for 7786delG, but not for R578X. The other three microsatellite markers examined (see text) were noninformative for both mutations. Recombination at the D3F22 locus is noted in families B and C (*), and recombination at the D3S1581 locus has occurred in family D (*).
mutation, occurring in 3 of 34 (9%) alleles of offspring in the families studied (Fig 2; families B and C). Recombination was also observed in the microsatellite marker D3S1581 in one individual with 7786delG (Fig 2; family D).

**DISCUSSION**

In this study, we have demonstrated the presence of two recurrent mutations in COL7A1 in British patients with recessive DEB, both of which result in premature termination codons of translation on the affected alleles. The mutations, R578X and 7786delG, were found with a relatively high frequency in the population studied, with allelic frequencies of 13% and 15%, respectively.

The mutation R578X results from a C-to-T transition at a CpG dinucleotide site in exon 13 of COL7A1. As with other CpG dinucleotides, this site may be susceptible to methylation-induced deamination of 5-methylcytosine to cause transition to a T residue, which may be the mechanism that generates this particular premature termination codon (Cooper and Youssoufian, 1988). Similar mutational mechanisms exist widely throughout the genome, and the recurrence of R578X in COL7A1 may represent a further example of a mutational hot spot. In support of this, R578X has been demonstrated previously in DEB patients from a different ethnic background (Dunnill et al., 1994). Our haplotype analysis demonstrated, however, that R578X was present on identical allelic backgrounds in the four families studied, consistent with its existence on a common British mutant ancestral allele. Although the mutation may have arisen originally at a mutational hot spot, the haplotypes in these families support its propagation through the British gene pool.

The 7786delG mutation results in a premature termination codon in exon 106 and would also be expected to result in greatly diminished synthesis of type VII collagen through reduced steady-state mRNA levels (Baserga and Benz, 1988; Urlaub et al., 1989; Cheng et al., 1990). This mutation arises in the vicinity of an adjacent G residue and two neighboring repeated triplet sequences of C residues. Deletion of one of the G residues, as occurs in this mutation, may therefore have resulted from slipped mispairing during DNA replication (Krawczak and Cooper, 1991). This sequence-directed mechanism for the generation of 7786delG is supported by the identification of the same mutation in an individual of a different ethnic background (Christiano and Utito, 1996b). Nevertheless, in this study, 7786delG was found in the families studied on two similar allelic backgrounds, which differed only at the site of the COL7A1 flanking marker, D3S2. This is also consistent with the mutations having arisen on a common allele in this population, with recombination at the D3S2 locus at some stage during its propagation through the British population. The analysis of microsatellite markers D3S1235 and D3S1581 gives further support to the existence of a common 7786delG mutant allele in these families.

Overall review of the haplotype analysis showed evidence of recombination at the telomeric D3F22 locus in 3 of 34 alleles of the offspring studied. Although occasional recombination at the centromeric D3S2 locus has been described previously in other DEB patients (Hovnanian et al., 1995), to our knowledge this is the first report of recombination of D3F22. This has implications for studies that use this marker for COL7A1 linkage analysis. In particular, this has relevance for prenatal diagnosis of recessive DEB, in which D3F22 may be used as an informative marker for the pathogenic mutation(s) (Christiano et al., 1996).

The high relative frequencies of R578X and 7786delG found in this study represent the first recurrent mutations of COL7A1 in British patients with recessive DEB. This has significance for future mutation-detection strategies. Specifically, British patients with this disease should be screened initially by PCR amplification and restriction analysis for the presence of these two COL7A1 mutations. Such analysis may avoid the need for more exhaustive screening of the remaining exons of this gene in a substantial number of patients with DEB.

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