A Glycine-to-Arginine Substitution in the Triple-Helical Domain of Type VII Collagen in a Family with Dominant Dystrophic Epidermolysis Bullosa

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We recently demonstrated strong genetic linkage between the type VII collagen gene (COL7A1) and both the dominant and recessive forms of dystrophic epidermolysis bullosa. In this study, we searched for mutations in dominant dystrophic epidermolysis bullosa using polymerase chain reaction amplification of segments of COL7A1, followed by heteroduplex analysis. Examination of the polymerase chain reaction corresponding to exon 73 revealed a heteroduplex resulting from a G-to-A transition at nucleotide 6127 in the triple-helical domain of COL7A1, which converted a glycine residue to an arginine (G2043R). The dominant dystrophic epidermolysis bullosa phenotype in this family probably arose because of a dominant negative effect of this mutation in COL7A1, resulting in the formation of structurally abnormal anchoring fibrils. *Key words: anchoring fibrils/COL7A1 mutations. J Invest Dermatol 104:438–440, 1995*

Epidermolysis bullosa (EB) is a group of mechano-bullous diseases characterized by blistering of the skin and the mucous membranes as a result of minor trauma [1–3]. The diagnostic hallmark of the dystrophic forms of EB is abnormalities in the anchoring fibrils, attachment structures at the dermo-epidermal junction, including morphologic alterations, scarcity, or even complete absence [4]. Type VII collagen is the major, if not the exclusive, component of the anchoring fibrils [5]. We recently cloned the entire human type VII collagen cDNA [6] and gene [7] and mapped COL7A1 to chromosomal locus 3p21 [8]. Using intragenic and flanking restriction fragment length polymorphisms, we and others demonstrated strong genetic linkage between COL7A1 and both the dominantly and recessively inherited forms of dystrophic EB, without evidence for locus heterogeneity (for review, see [9]). In fact, in dominant dystrophic EB (DDEB), the cumulative LOD score (Z_{max}) was greater than 44 in 14 unrelated families, providing overwhelming genetic linkage data in support of COL7A1 as the candidate gene for mutations in DDEB [9]. We recently reported the first identification of a glycine substitution in COL7A1 in a large Finnish family with DDEB [10]. In this study, we report the identification of a distinct glycine substitution in COL7A1 in an Italian family with DDEB.

MATERIALS AND METHODS

PCR Amplification and Heteroduplex Analyses We studied an Italian family with clinical, histopathologic, and ultrastructural features of DDEB (**Fig 1**). Electron microscopy revealed sub-lamina densa blister separation with a significantly reduced number of anchoring fibrils, which were sparse and hypoplastic. DNA isolated from peripheral blood lymphocytes of two affected and two unaffected family members was used as a template for amplification of genomic sequences within COL7A1. For this purpose, oligonucleotide primers were synthesized on the basis of intronic sequences to generate approximately 300 base pair (bp) products, which cover the entire coding sequence of COL7A1. This sequence information has been deposited in the GenBank data base under accession no. L23982. The primers used to amplify the 287-bp fragment containing exon 73 were: upstream primer, 5′GGGTGTAGCTGTACAGCCAC3′; and downstream primer, 5′CCCTCTTCCCTCACTCTCCT3′.

For polymerase chain reaction (PCR) amplification, approximately 500 ng of genomic DNA was used as template. The amplification conditions were 94°C for 7 min, followed by 94°C for 45 seconds, 55°C for 45 seconds, and 72°C for 45 seconds, for 40 cycles, in an OmniGene thermal cycler (Marsh Scientific, Inc.). Amplification buffer contained 1.5 mM $MgCl_2$ and 2 U of Taq DNA polymerase (Gibco, BRL), in a total volume of 50 μl. Aliquots of 5 μl were analyzed on 1.0% agarose gel electrophoresis, and 10 μl of the sample was prepared for heteroduplex analysis [11] according to the manufacturer's recommendations (MDE, AT Biochemicals). Heteroduplexes were visualized by staining with ethidium bromide. Bands of altered mobility detected in heteroduplex analysis were sequenced directly using the ABI automated sequencing system.

Verification of the Mutation The mutation detected in the PCR product containing exon 73 was verified at the genomic level. For this purpose, we searched for a potential change in restriction endonuclease sites as a result of the mutation and identified a deleted restriction site for SmaI. DNA in the region of the mutation was amplified by PCR using the same primers indicated above, and the products were cleaved with SmaI according to the manufacturer's recommendations (New England Biolabs) and electrophoresed on 4% NuSieve agarose. The individuals bearing the G2043R mutation were heterozygous for a 138-bp fragment (resulting from the loss of the 93-bp and 45-bp fragments) and for the 93- and 45-bp cleaved fragments, whereas unaffected individuals showed only the 93- and 45-bp fragments. A constant 149-bp band was observed in all individuals (see *Results*).
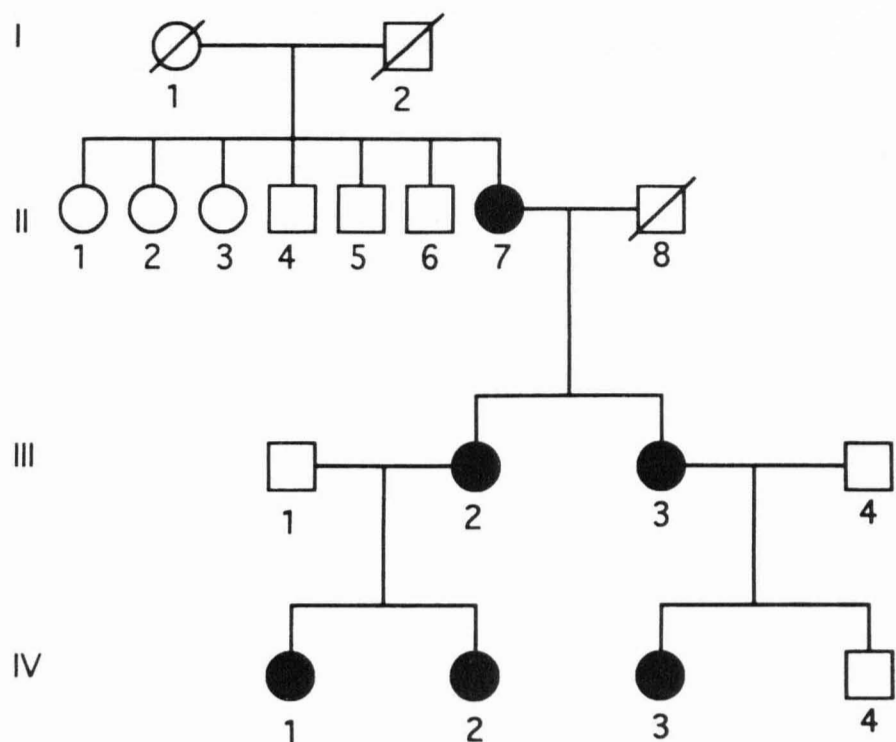
Figure 1. Pedigree of the family with DDEB.

RESULTS

We studied an Italian pedigree with DDEB, consisting of six affected and three unaffected living individuals in three generations **(Fig 1)**. To identify the mutation in this family, we amplified genomic DNA segments by PCR and subjected the products to heteroduplex analysis. Examination of a PCR product spanning exon 73 of COL7A1 demonstrated a band of altered mobility in affected individuals, as compared with unaffected family members or unrelated healthy individuals **(Fig 2A)**. Automated sequencing of PCR products from normal individuals of the family demonstrated the presence of a G in nucleotide position 6127 in all cases. In contrast, sequencing of PCR products containing the mutant allele from affected individuals of the family demonstrated a heterozygous G-to-A transition **(Fig 3)**. This nucleotide change resulted in substitution of a glycine (GGG) by an arginine (AGG) at amino acid position 2043 in one of the COL7A1 alleles. All affected individuals were heterozygous for this mutation, designated G2043R.

Substitution of the first G in the recognition sequence for the endonuclease SmaI, CCC<u>G</u>GG, by an A abolished this restriction enzyme site. To examine the cosegregation of this mutation in the family and to verify its presence in genomic DNA, we performed SmaI digestions on the family members. The results indicated that individuals with altered mobility of this PCR product in heteroduplex analysis were heterozygous for an allele undigestible with SmaI, bearing the G-to-A transition **(Fig 2B)**. In addition, SmaI digestion of PCR-amplified DNA from 22 COL7A1 alleles from unrelated, ethnically matched individuals demonstrated the absence of the mutation G2043R. Furthermore, this mutation was not present in over 80 unrelated patients with different forms of dystrophic EB from varying ethnic backgrounds. These observations suggest that G2043R is not a common polymorphism and is not a prevalent mutation in other EB families.

DISCUSSION

The results of this study demonstrate that DNA from affected individuals in a pedigree with DDEB contains a heterozygous G2043R mutation in COL7A1, the locus to which DDEB previously had been linked [9,12,13]. It was detected only in the affected family members, whereas unaffected family members, 11 ethnically matched healthy individuals, and more than 80 unrelated dystrophic EB patients of varying ethnic backgrounds did not demonstrate this mutation. These observations suggest that the mutation G2043R is the underlying cause of DDEB in this family.
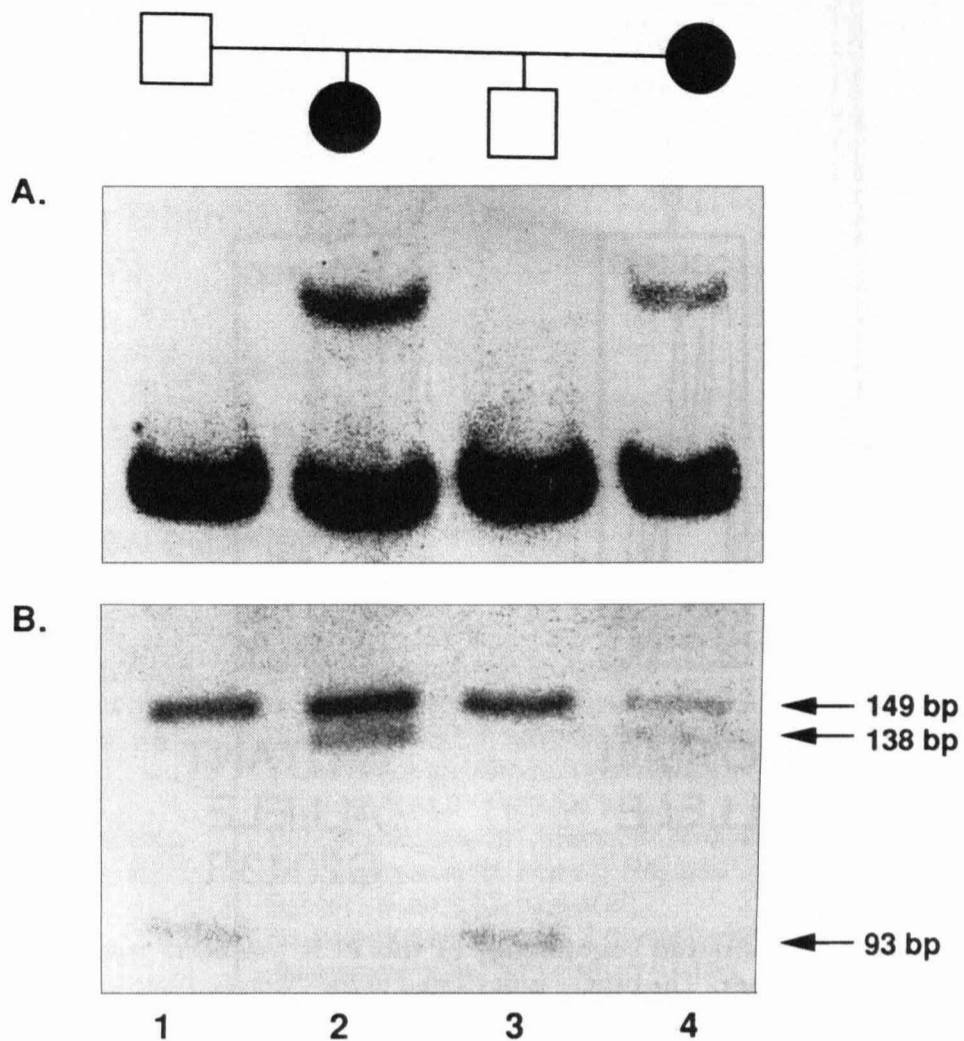

Figure 2. Heteroduplex analysis and verification of the mutation by restriction enzyme analysis. DNA sequences containing exon 73 were amplified by PCR, as described in the text, and the PCR products were examined by heteroduplex analysis. Note a heteroduplex in the affected individuals IV-3 and III-3 (*lanes 2* and *4*), whereas the unaffected individuals IV-4 and III-4 (*lanes 1* and *3*), as shown in the nuclear pedigree (*top*), did not demonstrate the heteroduplex (*A*). The same PCR products were digested with restriction enzyme SmaI (*B*). In affected individuals, a 138-bp fragment is seen, which reflects a loss of the SmaI restriction enzyme site due to the G-to-A transition. Thus, the affected individuals are heterozygous for the uncleaved 138-bp band, which in the allele not containing the mutation is digested in heterozygotes to 93-bp and 45-bp bands (not shown). All individuals also contain a constant 149-bp band.

The collagenous domain of type VII collagen normally contains 19 imperfections or interruptions in the characteristic collagenous Gly-X-Y amino acid repeat. The Gly-to-Arg substitution in this family occurs 65 amino acids downstream from the major nonhelical 39 amino acid interruption (interruption 10) within the collagenous domain, in the Gly-X-Y repeat adjacent to the previously reported glycine substitution, G2040S [10]. We speculate that the stability of the triple-helical domains, although interspersed with nonhelical interruptions, is critical for the function of type VII collagen. In support of this suggestion are preliminary observations that the amino acid sequences of type VII collagen are not well conserved through evolution, yet the positions of the imperfections and interruptions are well conserved [14]. Therefore, we believe that a specific domain organization is necessary for the functional integrity of type VII collagen molecules in anchoring fibrils.
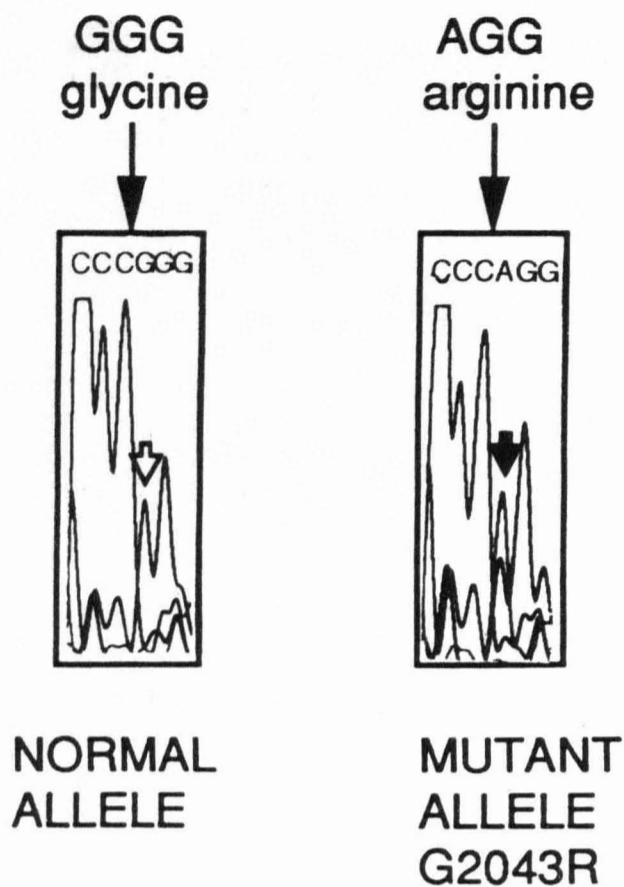
Figure 3. Automated sequencing of the PCR products using the upstream primer. The primer is described in the *Methods*. Note a double peak (G+A, *solid arrow*) in an individual heterozygous for the substitution of G to A in the mutant allele, resulting in the mutation G2043R. The peak corresponding to the normal allele (G only) is also indicated (*open arrow*).

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