Dystrophic epidermolysis bullosa pruriginosa (DEB-Pr) is a rare variant of DEB due to COL7A1 dominant and recessive mutations, which is characterized by severe itching and lichenoid or nodular prurigo-like lesions, mainly involving the extremities. Less than 30 patients have been described showing variable disease expression, and frequently, delayed age of onset. We report the clinical and molecular characterization of seven Italian DEB patients, three affected with recessive DEB-Pr and four with dominant DEB-Pr. In all the patients, the signs were typical of a mild DEB phenotype, until the onset of pruritus, which was followed by worsening of the clinical picture, with appearance of the distinctive lichenified lesions of DEB-Pr. Nine mutations were found in the COL7A1 gene, three of which were novel and one was de novo. DEB-Pr patients with either dominant or recessive mutations were shown to synthesize a normal or variably reduced amount of type VII collagen, which was correctly deposited at the dermal–epidermal junction. Since six of these mutations have been reported in DEB patients in the absence of intense pruritus, these data implicate a role of yet unidentified phenotype-modifying factors in the pathogenesis of DEB-Pr.

Dystrophic epidermolysis bullosa (DEB) is a rare mechanobullous genodermatosis inherited either with autosomal dominant or recessive pattern and characterized by fragility, blistering and scarring of the skin and mucous membranes (1). Blistering is due to abnormalities in anchoring fibrils (AF), microstructures mainly composed of type VII collagen (COLLVII), which contributes to the maintaining of dermal–epidermal adhesion (2). COLLVII is a homotrimer composed of three identical α1 chains consisting of a central collagenous domain and two flanking amino- and carboxyterminal noncollagenous domains (NC1 and NC2). The proα1(VII) chains are encoded by the 32-kb COL7A1 gene, localized on chromosome 3p21 (1).

DEB is a clinically heterogeneous disorder showing different degrees of cutaneous and extracutaneous involvement, and several variants have...
been described (3). All the DEB forms result from mutations in COL7A1 (1, 4, 5). More than 300 mutations scattered over the gene have been characterized, and some general genotype–phenotype correlations have been drawn (1, 6–8). In DEB, the pathogenic mutations are commonly family specific, although evidence for some recurrent mutant alleles has been reported. Specifically, the mutations c.497_498insA, c.7344G/A, c.425A/G, p.G1664A, c.4783-1G/A, c.8074delG and c.8441-14del21 (9) are the most frequent in the Italian population (8, 10, 11).

A rare variant of DEB with a highly distinctive phenotype is DEB pruriginosa (DEB-Pr) (12) (OMIM 604129). In DEB-Pr, skin fragility, blistering and scar formation are associated with intense and generalized pruritus, lichenified or nodular prigo-like lesions, violaceous linear scarring, milia, nail dystrophy and variable presence of albopapuloid lesions (12–14). Scarring and lichenified lesions are most evident on the limbs, particularly on the shins, and intact blisters are rarely seen. In DEB-Pr patients, autosomal dominant and autosomal recessive inheritance and sporadic inheritance patterns have been recognized (12, 14–19), and 16 distinct sequence variants in COL7A1 have been reported: 12 glycine substitutions in the collagenous domain (14–19), two splice site mutations (14, 17), and two small nucleotide deletions (14). The pathogenic mechanisms that underlie the disease remain unknown. Serum immunoglobulin (Ig) E levels higher than normal were detected in some patients, although no clear correlation with possible immunological causes of the disorder has been drawn (14, 18).

In this study, we report the clinical and molecular characterization of seven Italian DEB-Pr patients.

Materials and methods

Biological materials and COLLVII immunofluorescence analyses

Patients’ skin biopsies were processed for immunofluorescence (IF) analysis, with the monoclonal antibody LH7:2 (Sigma Chemical Co., St Louis, MO, USA) directed against the COLLVII NC1 domain, and for direct IF using fluorescein-conjugated rabbit anti-human IgG, IgM, IgA, and C3 antibodies (Dako, Glostrup, Denmark). Indirect IF of sera of two patients, diluted 1:20, was performed using human control salt-split skin as substrate. In five patients, a skin biopsy specimen was also processed for fibroblast culture. Fibroblasts were grown in vitro at 37°C in a modified Eagle’s medium supplemented with 10% fetal bovine serum, 100 μg/ml penicillin and 100 μg/ml streptomycin. Skin biopsies and blood samples were obtained from all the patients and their parents following written informed consent.

Mutation detection and verification

The search for mutations in the COL7A1 gene was performed by using strategies previously described and based on polymerase chain reaction (PCR) amplification of either COLLVII messenger RNA (mRNA), purified from cultured fibroblasts, or genomic DNA (gDNA), extracted from blood (8). The mRNA-based procedure allows for the direct sequencing of 22 separate complementary DNA (cDNA) fragments encompassing all the COL7A1 exons (20). The gDNA approach is based on denaturing high-performance liquid chromatography (DHPLC) screening of 72 amplicons covering the entire COL7A1 coding region and its splice sites (11). Independently from the method used, mutation detection was first performed by targeting the seven Italian common mutations on gDNA (8, 11). The cDNA was prepared by reverse transcription of 3 μg of total RNA, as described (8, 20). PCR products were sequenced in both orientations using the BigDye terminator cycle sequencing kit and the ABI PRISM 3100 automated sequencer (Applied Biosystems, Foster City, CA, USA). All the mutations identified at the cDNA level were also verified by gDNA sequencing. Whenever possible, restriction enzyme digestion of gDNA was performed to verify the familiar segregation of the mutations.

For the novel p.R51G missense mutation, 200 alleles from healthy Italian donors were analyzed by direct sequencing of exon 2. The c.6900 + 2delTGAT mutation was designated after subcloning of the corresponding genomic PCR amplicon into the pCR2.1 TA vector (Invitrogen, San Diego, CA, USA) and subsequent sequencing of independent alleles from seven subclones.

Results

Patient clinical findings and IF analysis

Seven Italian patients were studied, six women and one man (Fig. 1, Table 1). There was a family history of DEB in three of the seven patients. Interestingly, the father of patient 5 presented nail dystrophy only and referred the
development since birth of blistering lesions limited to trauma-exposed sites, which progressively improved until complete resolution in his adolescence (Fig. 1C).

In six DEB patients, disease onset was at birth or in early childhood. Of note, in these patients, the skin involvement was relatively mild, with blistering lesions localized predominantly at trauma sites. In four of these patients, DEB signs showed a gradual amelioration during childhood or adolescence. Other disease features included nail dystrophy, slight skin atrophy and milia formation, and in a single patient, alpapuloid lesions and occasional erosions in the oral mucosa were found. All these patients were characterized by a marked and progressive worsening of the skin phenotype after the onset of itching. The last DEB patient (patient 3, Fig. 1B) presented exclusively toenail dystrophy until the appearance of itching symptoms. Upon examination, all the patients showed numerous excoriated and lichenified papules, nodules and plaques with associated scarring localized on shins, foot dorsum, and in some cases, elbows, wrists and back. Blistering lesions were rarely observed. Pruritus development presented an age-related distribution, manifesting at puberty in patients 1, 2, 3, and 5, and in adulthood in patients 4, 6, and 7. In all the patients, the pruritus was severe, generalized, invalidating, unresponsive to conventional therapies and could not be related to specific causes. Serum IgE of patients 5 and 7 were higher than normal, i.e. >4000 KU/l (normal value <200 KU/l). In addition to DEB signs, patient 5 showed allergic disease with asthma and rhinoconjunctivitis. Treatment with z-methylprednisolone led to the improvement of the allergic signs and to a temporary improvement of the cutaneous lesions. In the remaining patients, serum IgE levels were normal.

The IF analysis of skin biopsies from the seven DEB-Pr patients was positive for immunostaining with anti-COLLVII LH7:2 monoclonal...
antibody, showing the presence of variable amounts of COL7VII at the dermal–epidermal junction (DEJ) (Table 2). Figure 2 illustrates the IF findings of the skin biopsies from the four DEB-Pr patients, whose clinical signs are shown in Figure 1.

Direct IF of skin biopsies from DEB-Pr patients 1–5 showed the presence of an IgG and C3 microfibrillar discontinuous pseudoband along the DEJ in patient 1 and of IgM deposits in a similar pattern in patient 3, while no continuous linear Igs or C3 deposits could be detected (not shown). Indirect IF analysis of sera of patients 1 and 4 did not show the presence of circulating autoantibodies against the DEJ (not shown).

Molecular characterization

The seven DEB-Pr patients were subjected to mutational screening in COL7A1. Nine pathogenic sequence variations were detected: four splicing, three missense, and two nonsense mutations (Table 2). Five of these mutations have already been reported in DEB patients without pruriginosa variant stigmata, whereas three were
Dystrophic epidermolysis bullosa pruriginosa

Table 2. COL7A1 mutations identified in the Italian DEB-Pr patients and their effects. The novel mutations detected in this study are in bold.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Transmission</th>
<th>Mutation(s)</th>
<th>Position</th>
<th>Effect</th>
<th>COLLVII</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>RDEB</td>
<td>c.425A&gt;G/c.7344G&gt;A</td>
<td>Ex 3/Ex 95</td>
<td>Splice PTC/normal PTC</td>
<td>++</td>
</tr>
<tr>
<td>2</td>
<td>RDEB</td>
<td>p.R1630X/c.7344G&gt;A</td>
<td>Ex 51/Ex 95</td>
<td>PTC/normal PTC</td>
<td>++</td>
</tr>
<tr>
<td>3</td>
<td>RDEB</td>
<td>c.7344G&gt;A</td>
<td>Ex 2/Ex 98</td>
<td>Missense/PTC</td>
<td>+++</td>
</tr>
<tr>
<td>4</td>
<td>DDEB</td>
<td>p.G1755D</td>
<td>Ex 59</td>
<td>Missense</td>
<td>++</td>
</tr>
<tr>
<td>5</td>
<td>DDEB</td>
<td>p.G2073V</td>
<td>Ex 75</td>
<td>Missense</td>
<td>++</td>
</tr>
<tr>
<td>6</td>
<td>DDEB</td>
<td>c.6900 + 4A&gt;G</td>
<td>IVS 87</td>
<td>In-frame Ex 87 skipping</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>DDEB</td>
<td>c.6900 + 2delT-GAT</td>
<td>IVS 87</td>
<td>In-frame Ex 87 skipping</td>
<td>+a</td>
</tr>
</tbody>
</table>

RDEB, recessive dystrophic epidermolysis bullosa; DDEB, dominant DEB; DEB-Pr, DEB pruriginosa.

aMutation nomenclature according to the website www.hgvs.org recommendations.

bCOLL VII staining intensity by immunofluorescence analysis with the LH7:2 monoclonal antibody: +++ , normal; ++ , reduced; +, highly reduced.

cDe novo mutation.

dKeratinocyte cytoplasmic staining.

novel (p.R51G, p.G2073V, and c.6900 + 2delT-GAT) (Fig. 3, Table 2).

Patient 1 was a compound heterozygote for the c.425A>G and c.7344G>A recessive mutations, resulting in aberrant splicing of exon 3 and exon 95, respectively (21). The c.7344G>A mutation was also detected in patient 2 in compound heterozygosity with the p.R1630X known mutation (6). In patient 3, a novel maternal C-to-G transversion at nucleotide 151 of the COL7A1 coding region (exon 2) was found in combination with the known p.R2492X nonsense paternal mutation (8) (Fig. 3A, Table 2). The c.151C>G sequence variant converts an arginine residue of the NC1 domain to a glycine (p.R51G). This sequence change, identified at the cDNA level and confirmed by gDNA sequencing, was not detected in 200 alleles from unaffected control donors by sequence analysis and was the only missense variation found in the COL7A1 coding region of patient 3. The c.5264G>A transition in exon 59 was identified in patient 4 by DHPLC screening. This nucleotide change results in the Gly1755→Asp substitution (p.G1755D) in the COLL VII collagenous domain (Table 2). The c.5264G>A mutation has been recently reported as a de novo dominant mutation in this dominant DEB-Pr (DDEB-Pr) patient (11). The c.6218G>T transversion in exon 75 was identified in heterozygosity in patient 5 (Fig. 3B) and confirmed in her affected father. This mutation leads to the substitution of a valine for a glycine residue at position 2073 in the COLL VII collagenous domain (p.G2073V).

In patients 6 and 7, affected with DDEB-Pr, mutations altering the donor splice site of intron 87 were found. In patient 6, amplification of the cDNA region covering exons 86–88 showed, in addition to the expected fragment, an aberrant cDNA product showing the in-frame skipping of exon 87 (Fig. 3C). Subsequent direct sequencing of exon 87 amplified from the gDNA led to the identification of the heterozygous c.6900 + 4A>G

Fig. 2. Immunofluorescence analysis of uninvolved DEB-Pr skin with monoclonal antibody LH7:2 against COLL VII. Compared to control skin, variable levels of COLL VII expression are detected along the dermal–epidermal junction of the patients: normal in patient 3, reduced in patients 1 and 5, markedly reduced in patient 6. Scale bar: 20 μm.
Fig. 3. Molecular characterization of dystrophic epidermolysis bullosa pruriginosa patients. (A, B, and D) Sequence chromatograms of COL7A1 exons showing the position of the p.R51G, p.G2073V, and c.6900 + 2delTGAT novel mutations detected in patient 3, patient 5 and patient 7, respectively. (C) Identification and characterization of the molecular mechanism of the c.6900 + 4A>G mutation in patient 6. (a) Agarose gel electrophoresis of the complementary DNA (cDNA) product amplified with primers (F) 5’-GCCTGGACAAAGTGAGGGAGAC-3’ and (R) 5’-CTTCTCTCCCTTGCTCCAGGG-3’ spanning exons 86–88 shown in the patient sample (P), the presence of an aberrant fragment (130 bp) in addition to the wild-type fragment (199 bp), also detected in the control sample (C). The sequence of this aberrant cDNA showed the in-frame deletion of exon 87 (b) due to the c.6900 + 4A>G substitution as shown by gDNA sequencing (c).
Discussion

DEB-Pr is a rare variant of DEB due to COL7A1 dominant and recessive mutations, characterized by peculiar clinical signs and symptoms. In this study, we report the clinical and molecular characterization of all Italian DEB-Pr patients known at present.

In these seven patients, molecular analysis of the COL7A1 gene has disclosed three novel and six known mutations, the latter previously identified in DEB patients without stigmata of the pruriginosa variant. In particular, the c.425A>G mutation (Fig. 3C). In patient 7, a heterozygous deletion mutation, c.6900 + 2delTGAT, was disclosed by DHPLC screening (Fig. 3D). Also, this mutation is predicted to cause aberrant splicing of exon 87.

The p.R51G mutation in exon 2 is the most 5' COL7A1 missense variation reported so far, being positioned at the very N-terminus of the NC1 domain, between the signal peptide (residues 1–23) and the first recognizable cartilage matrix protein subdomain (residues 135–227). The mutation was absent in 200 control chromosomes and was, in addition to the p.R2492X, the only putative disease mutation found in patient 3 following sequencing of the entire COLLVII mRNA. Experimentally, the p.R51G sequence variant seems to be a true missense, as amplification of the cDNA region around this mutation did not show any cryptic splicing activation. Therefore, its pathogenic mechanism is difficult to envisage, although the importance of residue R51 is suggested by its strict conservation in the COLLVII of mammals (23, 24). The Arg51→Gly substitution does not interfere with the AF assembly, as no specific function in triple helix formation and procollagen dimerization has been assigned to the NC1 domain. Indeed, normal amounts of immunoreactive COLLVII encoded by the mutant p.R51G allele were detected along the DEJ of patient 3. Rather, the NC1 domain has been shown to interact with several extracellular matrix proteins including type I and type IV collagen, fibronectin and laminin-5 (25, 26). More likely, the substitution of an uncharged glycine for a positively charged arginine causes a subtle local change of protein structure influencing the adhesive function of the NC1 domain. This is supported by a computer-assisted prediction of the secondary structure of the mutant polypeptide (NPS@: Network Protein Sequence Analysis at http://npsa-pbil.ibcp.fr/), which showed a minimal perturbation of the α-helix profile of the amino acid sequence downstream the mutated position (not shown). Indeed, patient 3 did not manifest blistering lesions until the pruritus onset, after which the patient developed the typical signs of DEB-Pr. The two glycine substitution mutations identified in this study were associated with DDEB-Pr. Both the mutations occur within uninterrupted Gly-X-Y repeats of the COLLVII collagenous domain and thus have dominant-negative effects on the assembly of COLLVII triple helix. Mutation p.G1755D has been recently reported as a de novo event in patient 4 (11). The p.G2073V in patient 5 is a novel mutation, though the substitution of the same glycine with an aspartic acid (p.G2073D) has been reported to occur de novo in combination with the p.R578X mutation in a RDEB patient with localized skin lesions and mucosal involvement (27). Therefore, dominant effect and inheritance of the previously known p.G2073D cannot be excluded. Mutation p.G2073V resulted in a mild phenotype in patient 5, with blistering lesions starting at 1 year and
being limited to trauma-exposed sites, until pruritus onset. Indeed, the same mutation in the patient's father, who never developed pruritus, manifested as a very mild cutaneous phenotype in childhood, and residual nail dystrophy in his adulthood.

In patients 6 and 7, heterozygous splice site mutations affecting the donor splice site of intron 87 were identified. Each of these causes a dominant disease with affected members through several generations, presenting mild phenotype. Both the c.6900 + 4A>G and the c.6900 + 2delTGAT are predicted to result in aberrant splicing of exon 87, and indeed, the cDNA analysis performed in patient 6, harboring the c.6900 + 4A>G mutation, has shown the in-frame skipping of exon 87 from the mature mRNA. Deletion of exon 87 eliminates 23 amino acids from the COL7A1 collagenous domain and results in shortened COL7A1 polypeptides having dominant-negative effects on triple helix assembly. Mutation c.6900 + 4A>G was previously presented as recessively inherited in combination with the p.R682X nonsense mutation, resulting in a severe HS-RDEB phenotype and was believed to lead to premature termination codon formation, but in this previous study, mutation effects were not investigated at RNA level (7). Skipping of exon 87 has been reported to be induced not only by intronic splice site mutations but also by single-nucleotide substitutions or small deletions within exon 87, all resulting in DDEB (11, 17, 28). In particular, the c.6862del16 (14, 28), the c.6847del27 (29) and the c.6849del18 (11) mutations appear to eliminate or delete a region containing four overlapped exonic splicing enhancer sequences, as predicted by RESCUE-ESE algorithm (http://genes.mit.edu/burgelab/rescue-ese/) analysis.

In conclusion, in the DEB-Pr patients reported here, the COL7A1 mutations identified were associated with a mild phenotype until the pruritus onset; however, these mutations alone are not responsible for the clinical symptoms and signs typical of this DEB variant, as affected relatives of patients 5, 6, and 7, and other previously described patients with the same genotype of patient 1, developed only the signs of DDEB or RDEB, respectively. In a similar way, the occurrence of DEB-Pr in a single patient and not in other affected members in families with DDEB has been previously reported (14).

The pruritus is the most invalidating and characteristic sign of DEB-Pr. This symptom can also occur in DEB patients and in other forms of epidermolysis bullosa, though being always less marked than in DEB-Pr patients, and it may be related to wound healing and local chronic skin damage—activating mast cells. Severe, unremitting pruritus in DEB-Pr might have different causes, and in turn lead to new cutaneous manifestations, i.e. scratching and lichenoid lesions. We can only speculate on the influence of other factors involved in the modulation of the phenotype in these patients. There was no evidence of specific causes of itching in our DEB-Pr cases, apart from the finding of elevated serum IgE levels in two patients, one of whom also suffered from asthma and rhinitis. Elevated IgE levels and/or atopy have been reported in other DEB-Pr patients (14, 18). However, they are not consistent findings and do not specifically account for the DEB-Pr phenotype. An amelioration of skin lesions and pruritus was observed in patient 5 during z-methylprednisolone treatment for asthma. The described clinical improvement of DEB-Pr patients with other immunomodulatory drugs, such as cyclosporine, thalidomide and tacrolimus, suggests the involvement of additional immune-mediated factors in the pathogenesis of DEB-Pr (30–32). However, in our DEB-Pr patients, direct and indirect IF were negative for the presence of tissue-bound or circulating autoantibodies in the typical linear continuous pattern observed in autoimmune subepidermal blistering disorders. On the contrary, it is intriguing that DEB patients carrying the c.7344G->A splicing mutation (this study) or mutations resulting in the deletion of exon 87 (17, 28, 29, this study) frequently associate to the development of the DEB-Pr phenotype. In some predisposed DEB patients carrying specific COL7A1 mutations that alter either intrinsic portions or the macromolecular configuration of COL7A1, chronic inflammation and reiterated skin damage might result in the formation and/or presentation of new or cryptic epitopes, which could give rise to an abnormal immunological response.

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