

## ARTICLES

# Epidermolysis Bullosa with Congenital Pyloric Atresia: Novel Mutations in the $\beta 4$ Integrin Gene (ITGB4) and Genotype/Phenotype Correlations

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### ABSTRACT

Epidermolysis bullosa with pyloric atresia (EB-PA: OMIM 226730), also known as Carmi syndrome, is a rare autosomal recessive genodermatosis that manifests with neonatal mucocutaneous fragility associated with congenital pyloric atresia. The disease is frequently lethal within the first year, but nonlethal cases have been reported. Mutations in the genes encoding subunit polypeptides of the  $\alpha 6\beta 4$  integrin (ITGA6 and ITGB4) have been demonstrated in EB-PA patients. To extend the repertoire of mutations and to identify genotype-phenotype correlations, we examined seven new EB-PA families, four with lethal and three with nonlethal disease variants. DNA from patients was screened for mutations using heteroduplex analysis followed by nucleotide sequencing of PCR products spanning all  $\beta 4$  integrin-coding sequences. Mutation analysis disclosed 12 distinct mutations, 11 of them novel. Four mutations predicted a premature termination codon as a result of nonsense mutations or small out-of-frame insertions or deletions, whereas seven were missense mutations. This brings the total number of distinct ITGB4 mutations to 33. The mutation database indicates that premature

termination codons are associated predominantly with the lethal EB-PA variants, whereas missense mutations are more prevalent in nonlethal forms. However, the consequences of the missense mutations are position dependent, and substitutions of highly conserved amino acids may have lethal consequences. In general, indirect immunofluorescence studies of affected skin revealed negative staining for  $\beta 4$  integrin in lethal cases and positive, but attenuated, staining in nonlethal cases and correlated with clinical phenotype. The data on specific mutations in EB-PA patients allows prenatal testing and preimplantation genetic diagnosis in families at risk. (*Pediatr Res* 49: 618–626, 2001)

#### Abbreviations

**EB-PA**, epidermolysis bullosa with pyloric atresia  
**BMZ**, basement membrane zone  
**CSGE**, conformation-sensitive gel electrophoresis  
**PTC**, premature termination codon  
**TEM**, transmission electron microscopy  
**ITGB4**,  $\beta 4$  integrin gene

Epidermolysis bullosa (EB) is a group of genodermatoses manifesting with blistering and erosions of the skin and mu-

cous membranes as a result of mechanical trauma (1, 2). The clinical severity of EB is highly variable. In the mildest cases, blistering occurs primarily on the hands and feet with relatively little morbidity, whereas, in the most severe cases, the disease can be lethal perinatally or during the early postnatal period. Traditionally, EB has been divided into three broad categories based on clinical findings and ultrastructural demonstrations of

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the level of tissue separation within the cutaneous BMZ (3). In the simplex forms of EB (EBS), tissue separation is intraepidermal, and mutations in the basal cell keratin genes, KRT5 and KRT14, underlie the classic forms of EBS inherited in an autosomal dominant fashion. In the junctional forms of EB (JEB), blistering occurs within the lamina lucida of the BMZ, and, in the classic Herlitz JEB, mutations in any of the three genes (LAMA3a, LAMB3, and LAMC2) encoding the subunit polypeptides of laminin 5 underlie this form of EB. In the dystrophic forms of EB (DEB), tissue separation occurs below the lamina densa on the dermal side of the BMZ, and mutations in the gene COL7A1 encoding type VII collagen, the major if not the exclusive component of the anchoring fibrils, have been disclosed in this form of EB (4). Adding to the heterogeneity of EB is the fact that there are, in addition to skin involvement, various extracutaneous manifestations associated with distinct subtypes of EB. These include corneal erosions, enamel pitting, nail dystrophy, scarring alopecia, muscle weakness, and detachment of the respiratory epithelium, as well as involvement of the gastrointestinal and vesico-urinary tract (1).

Recently, a new category, the hemidesmosomal variants of EB, has been proposed, and clinically there are three distinct subtypes within this category (5). First, generalized atrophic benign EB (GABEB) is characterized by life-long blistering of the skin associated with hair and tooth abnormalities. Traditionally, this subtype has been classified as a nonlethal variant of junctional EB (6, 7). It is now known that the majority of patients with GABEB harbor mutations in the type XVII collagen/180-kD bullous pemphigoid antigen gene (COL17A1/BPAG2) (5). Secondly, a distinct variant of EB is characterized by relatively mild blistering of the skin noted at birth, associated with late-onset muscular dystrophy (EB-MD) (8). The onset of the muscle involvement in EB-MD is highly variable, so that in some cases the muscle weakness is present within a few years of age, whereas in others the muscle weakness is not noted until the third or fourth decade of life. Nevertheless, this combination of skin and muscle involvement is now known to result from mutations in the gene encoding plectin, a large adhesion molecule with wide tissue distribution including expression in the hemidesmosomes of the skin as well as in the sarcolemma and Z-lines of the muscle (8). Finally, a distinct variant of EB presents with a combination of perinatal or neonatal blistering of the skin associated with congenital pyloric atresia (EB-PA) (1, 5). Diagnosis of pyloric atresia requires immediate surgical intervention, but the severity of the cutaneous and extracutaneous manifestations can be variable. In some cases, the blistering is extremely severe and can result in early demise of the affected individuals, whereas, in others, a blistering tendency has not been noted until several months or even a few years after the birth (9–13). Also, in some cases, the blistering tendency is initially severe but becomes progressively milder and later in life requires considerable trauma for the clinical features to manifest.

Initial clues to the candidate gene/protein systems in EB-PA were obtained from immunofluorescence analysis of skin. Specifically, markedly reduced or absent staining for  $\alpha 6$  and/or  $\beta 4$  integrin has been demonstrated in the skin of these patients, suggesting that the corresponding genes, ITGA6 and ITGB4,

may harbor the mutations underlying this subset of EB (14–16). Subsequently, a number of mutations in these two genes have been demonstrated, the majority of the mutations being found in the  $\beta 4$  integrin gene (10–13, 17–20), whereas only three cases with mutations in the  $\alpha 6$  integrin gene have been reported thus far (9, 21, 22). Two of the  $\alpha 6$  integrin cases were lethal in the early postnatal period, and in one case an affected fetus with abnormalities in the skin and intestine was detected prenatally by ultrasound examination, and the pregnancy was terminated. Subsequent immunochemical analysis of the latter case revealed altered expression of  $\alpha 6\beta 4$  integrin (22). Therefore, neither the  $\alpha 6$  nor the  $\beta 4$  integrin databases provide definitive explanations for the clinical variability, *i.e.* lethal *versus* nonlethal phenotype.

In this study, we have examined seven new families, each with an individual affected with EB-PA, for mutations in the genes encoding the  $\alpha 6\beta 4$  integrin subunits. We have disclosed a total of 11 novel mutations in the ITGB4 gene, and examination of the entire  $\beta 4$  integrin mutation database suggests intriguing genotype/phenotype correlations.

## MATERIALS AND METHODS

**Clinical material and verification of the diagnosis.** Seven families (A–G) with the clinical diagnosis of EB-PA were referred from an international patient base to the DebRA Molecular Diagnostics Laboratory at the Department of Dermatology and Cutaneous Biology, Jefferson Medical College, Philadelphia, PA, U.S.A. All studies from this laboratory have appropriate Internal Review Board approval. In each family, the proband had history of skin blistering, first noted at birth or shortly thereafter, associated with congenital pyloric atresia. Peripheral blood samples were submitted after obtaining informed consent and DNA was isolated by phenol-chloroform extraction using a standard procedure (23). Samples were obtained from the proband and both parents, if available. Control DNA was obtained from 100 unrelated individuals with no evidence of a blistering skin disease. DNA was used as a template for PCR amplification of exons of ITGB4 and ITGA6 for mutation detection analyses (see below).

Diagnostic skin biopsy specimens were obtained from the probands and subjected to routine histopathologic examination, and five specimens were examined by TEM. Immunofluorescence staining of frozen skin sections with antibodies recognizing  $\alpha 6$  (GoH3) and  $\beta 4$  (3E1) integrins was performed. Antibodies recognizing other BMZ components, including type VII collagen (LH7.2 and NP185), laminin 5 (GB3), bullous pemphigoid antigen (patient sera), and plectin (HD121) were used in comparative immunofluorescence staining of some samples (Table 1).

**Characterization and verification of mutations.** Mutation detection strategy used in this study included PCR amplification of each exon and of the flanking intronic sequences of the ITGB4 gene in each family, followed by heteroduplex scanning of the PCR products by CSGE, as described previously (11). The primer sequences used for amplification of each of the 39 coding exons expressed in the skin (numbers 2–32, 34–41) of the ITGB4 gene have been reported elsewhere (11).

**Table 1.** Clinical features of patients with epidermolysis bullosa and pyloric atresia

Family	Proband age/sex	Clinical features*	Disease variant	EM/IF features†	ITGB4 mutations	Consequences
A	Demise at 4.5 mo/F	Mild blistering at birth, anemia, respiratory distress, gastro-esophageal reflux	Lethal	Lamina lucida cleavage, reduced HDs, $\beta 4$ negative, $\alpha 6$ negative, plectin reduced, BPAG reduced	658delC/R252C	PTC/missense
B	Demise at 2 mo/M	Aplasia cutis congenita of all four extremities; multicystic dysplasia of left kidney; hydronephrosis of right kidney; esophageal, tracheal, bladder, and small intestinal involvement	Lethal	$\beta 4$ negative, $\alpha 6$ negative, plectin altered	D131Y/G273D§	Missense/missense
C	Alive at 15 mo/F	Severe blistering at birth; absent at 1 y	Nonlethal	Hemidesmosomal cleavage, $\beta 4$ reduced, $\alpha 6$ reduced, plectin altered	ND‡/R283C§	ND/missense
D	Demise at 1 mo/F	Obstructed sigmoid colon	Lethal	Lamina lucida cleavage, $\beta 4$ negative, $\alpha 6$ reduced, plectin altered, BPAG negative	1874delTCTinsC/V325D	PTC/missense
E	Alive at 13 yrs/F	Urologic obstruction, ocular involvement, scarring on legs, hypoplastic enamel, laryngeal obstruction,	Nonlethal	$\beta 4$ normal, $\alpha 6$ normal, plectin normal/altered	3112-3C→T, L336P/R1225H	Spl(?)‡, missense/missense
F	Demise at 2.5 mo/M	Mild blistering at birth	Lethal	Intraepidermal cleavage	Q1767X/Q1767X	PTC/PTC
G	Alive at 13 mo/F	Mild blistering, severe corneal erosions	Nonlethal	Hypoplastic HDs, absence of subbasal dense plate, lamina lucida cleavage, $\beta 4$ reduced, $\alpha 6$ reduced, plectin altered	4790delTC/ 4790delTC	PTC/PTC

\* All patients demonstrated blistering and erosions at birth, as well as congenital pyloric atresia. Additional clinical features are listed in this column.

† EM, electron microscopy; IF, immunofluorescence; HD, hemidesmosome; BPAG2, bullous pemphigoid antigen; for IF see Figs. 1 and 2

‡ ND, not detected; Spl(?), a putative splice-site mutation

§ Sequencing of the  $\alpha 6$  integrin gene (ITGA6) did not reveal pathogenic mutations.

The PCR reactions were performed in a total volume of 50  $\mu$ L containing 1  $\times$  PCR buffer, 200  $\mu$ M of each nucleotide, and either 1.25 U of Taq polymerase in the presence of 1  $\times$  Q-buffer (Qiagen, Valencia, CA, U.S.A.) or 1.25 U of Ampli-taq polymerase (Perkin Elmer, Foster City, CA, U.S.A.) in the presence of 4% DMSO, 12.8 pmol of each primer, and 200 ng of genomic DNA. The amplification conditions were as follows: 5 min at 94°C for one cycle, followed by 38 cycles of 45 s at 94°C, 45 s at the annealing temperature (11), and 45 s at 72°C. The PCR products were analyzed on 2% agarose gel and subjected to heteroduplex analysis by CSGE, as described elsewhere (24, 25). All PCR products demonstrating heteroduplexes, suggesting the presence of a sequence variant, were directly sequenced by an automated DNA sequencer (ABI 377, Perkin Elmer).

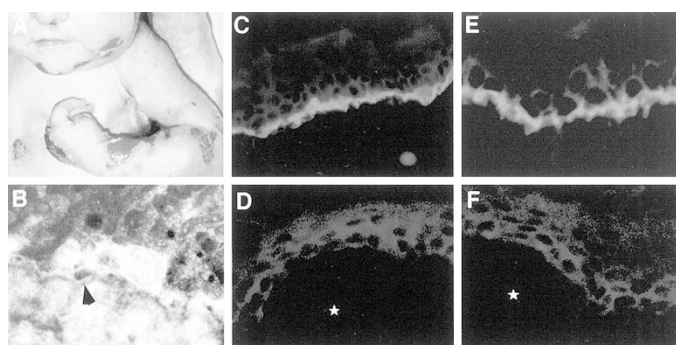
In each family, the verification of the mutation was performed by digesting the PCR products representing the proband and his or her immediate family members by restriction endonucleases (*Pst* I and *Nar* I for family A; *Ava* II and *Hga* I for family B; *Aci* I for family C; *Apa* I and *Hph* I for family D; *Hind*III, *Bfa* I and *Bsa*H I for family E; *Apa* I for family F; and *Bsm*A I for family G). For detection of the mutation 4790delTC in family G, specific primers were designed so that the C nucleotide at position 4787 was replaced by a G (underlined) in the forward primer: L, 5'-TTCTCTGCCCTGGGGC CCACATGTC-3', R, 5'-TTAGCGCTCAAGGGACTTGG-3'.

## RESULTS AND DISCUSSION

**Clinical and genetic features of patients with EB-PA.** A total of seven families, each with an affected child with EB-PA,

were subjected to study (Table 1). In each case, except family F (Table 1), there was no family history of a blistering disease, and the parents in each case were clinically unaffected. Family F lived in an isolated aboriginal community where similar cases were reported, one of which was in a distant family member (26). Typically, the affected newborn was diagnosed as having pyloric atresia that required surgical intervention, and, in most cases, skin blistering was noted at birth or shortly thereafter (Fig. 1A). In one case, family B (Table 1), the diagnosis of aplasia cutis congenita was also made at the time of birth. The absence of skin was particularly extensive, covering approximately 30% of the entire skin surface on all extremities. Among the seven probands, four of them (families A, B, D, and F) died within the first year of life from complications of EB-PA, and these individuals were classified as having the lethal variant of EB-PA. In contrast, in three of the patients (families C, E, and G) the blistering tendency improved with time. For example, a 13-y-old patient (family E), a ballet dancer, who had extensive blistering involving skin and the mucous membranes at birth, showed relatively mild skin fragility at the time of this study, and blisters develop on her toes only when she is dancing *en pointe*. She continues, however, to note increased skin fragility on her upper arms and trunk, to the extent that adhesive tape and bandages will induce blisters and erosions. Thus, the patients surviving beyond 1 y of age have been classified as nonlethal variants of EB-PA.

Several of the individuals also exhibited extracutaneous manifestations in addition to pyloric atresia (27, 28; Kambham *et al.*, unpublished data). For example, vesico-urinary tract involvement was noted in patient E at approximately 3 y of age

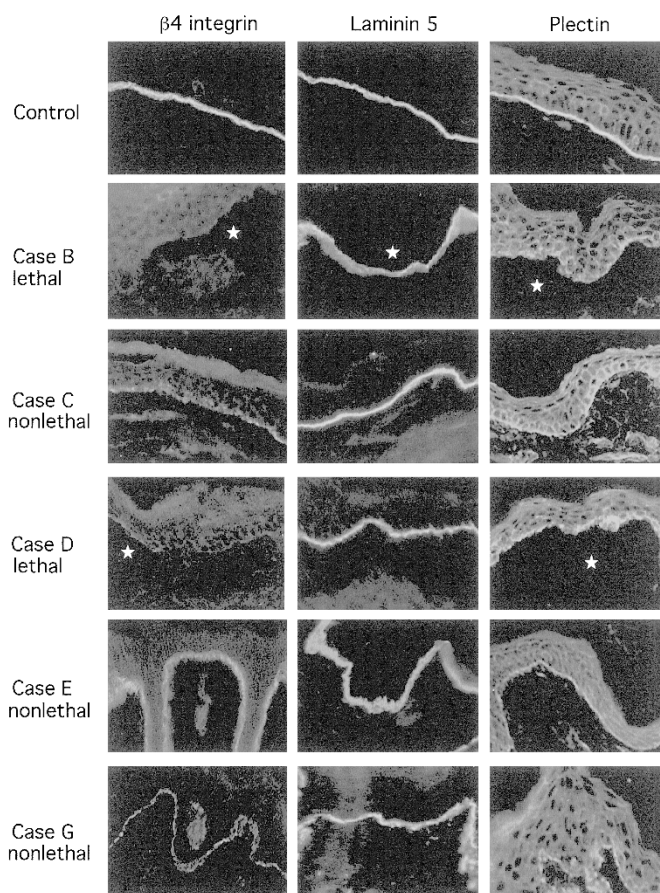


**Figure 1.** Clinical features, electron microscopy, and immunofluorescence staining of the skin of proband in family A. (A) The affected infant at 10 d of age shows multiple erosions. (B) Electron micrograph of the proband's skin shows separation at the level of lamina lucida and hypoplastic hemidesmosomes, sometimes on the floor of the blister (arrow). (C–F) Immunofluorescence mapping of a skin biopsy taken from freshly traumatized skin from the proband using normal human skin as a control. Both of the 3E1 and the GoH3 staining recognizing  $\beta 4$  and  $\alpha 6$  integrin polypeptides, respectively, are negative in the proband's skin (D, F) compared with normal skin (C, E). Blister cavity of patient's skin is indicated by asterisks.

as a result of increased blood pressure and included ureteral obstruction that resulted in hydronephrosis and chronic renal failure. The patient is now a candidate for renal transplant and the progress of renal failure may require dialysis in the near future. This patient also had evidence of laryngeal obstruction and ocular abnormalities. Patient B also had kidney involvement including multicystic dysplasia and hydronephrosis as well as involvement of tracheal, esophageal, and small intestinal tissues. Patient G had relatively mild skin involvement but had severe corneal erosions with considerable morbidity (Table 1).

**Confirmation of the diagnosis by immunofluorescence and electron microscopy.** In all cases, skin biopsy specimens were subjected to histopathology as well as diagnostic immunofluorescence staining and/or TEM. Hematoxylin-eosin stain showed dermal-epidermal blistering. In five cases (families A–D and G), immunofluorescence with antibodies recognizing the  $\alpha 6$  and  $\beta 4$  integrins was entirely negative or weakly positive (Fig. 1, C–F, and Fig. 2). In family E, staining for  $\beta 4$  integrin (Fig. 2) and  $\alpha 6$  integrin (not shown) was normal. Basement membrane-specific staining with HD121 for plectin was clearly reduced in patients B, C, D, and G, whereas in patient E it was close to normal (Fig. 2). No immunofluorescence data were available from family F.

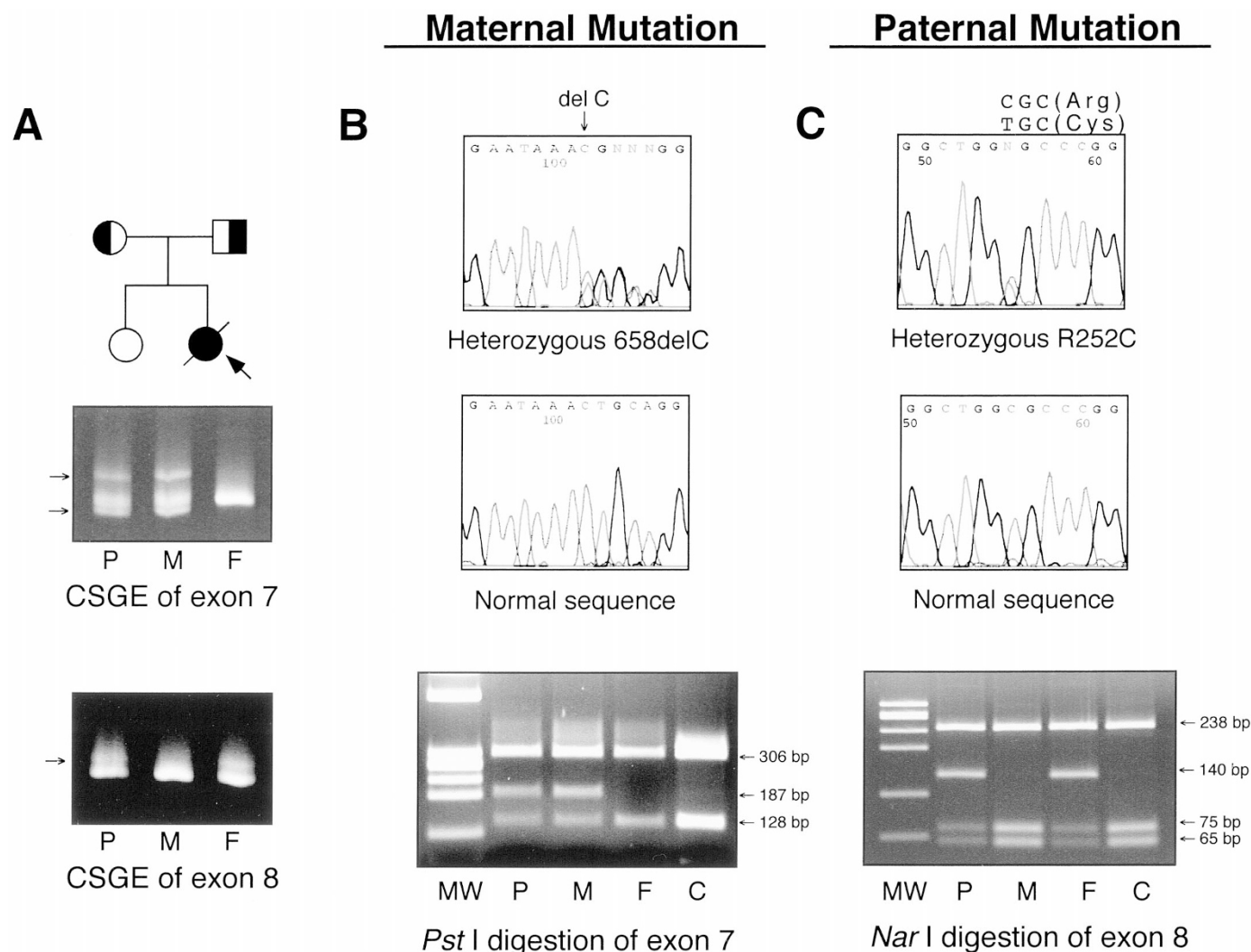
TEM revealed tissue separation at the dermal-epidermal junction in four cases (families A, C, D, and G), the cleavage being above the lamina densa at the level of the lamina lucida or the hemidesmosomes (Fig. 1B). In family F, the separation was apparently intracellular, cytoplasmic material being noted in association with the basal lamina on the floor of the blister. Thus, all seven patients examined in this study had characteristic clinical features of EB-PA, and this diagnosis was confirmed by negative or attenuated immunofluorescence of the  $\alpha 6\beta 4$  integrin or by ultrastructural examination in six cases, the exception being family E, in which the immunofluorescence was clearly positive for  $\alpha 6\beta 4$  integrin.



**Figure 2.** Immunofluorescence staining of skin sections obtained from EB-PA patients and a healthy control subject using MAb 3E1 (Chemicon International, Temecula, CA, U.S.A.), GB3 (Harlan Bioproducts for Science, Indianapolis, IN, U.S.A.), and HD121 (a gift from Dr. K. Owaribe) recognizing  $\beta 4$  integrin, laminin 5, and plectin, respectively. The linear staining was observed with all antibodies on the basement membrane of the control skin (upper panel). In addition, HD121 for plectin staining was also noted throughout the epidermis.

**Identification of novel mutations in the  $\beta 4$  integrin gene.** To identify potential mutations in the  $\alpha 6\beta 4$  integrin genes (ITGA6 and ITGB4), a mutation detection strategy consisting of heteroduplex scanning of these genes, followed by automated nucleotide sequencing, has been established (11). First, DNA from the probands and their immediate family members, including their parents and unaffected siblings, if available, was subjected to PCR amplification of exonic sequences in ITGB4. These PCR products were then examined by CSGE; an example of the methodology is presented in Figure 3. In case of a heteroduplex, the corresponding PCR product was subjected to automated nucleotide sequencing. All sequence variants were confirmed by restriction enzyme digestion if a restriction site was altered, or by direct sequencing in other members of the family (Fig. 3).

Among the seven probands studied, 12 distinct ITGB4 mutations were found in 13 alleles of these individuals, 11 of the mutations being previously unpublished (Table 1). Among these mutations, six were missense mutations, whereas four were nonsense mutations or small out-of-frame insertions or deletions resulting in PTC downstream from the site of the



**Figure 3.** Mutation analysis in family A. (A) Heteroduplex analysis of the PCR-amplified DNA spanning exon 7 and flanking intronic sequences of ITGB4 demonstrated heteroduplex bands in the mother's (lane M) and proband's (lane P) DNA (arrows), whereas the father's (lane F) DNA showed a homoduplex band only. Conversely, the father's and proband's DNA spanning exon 8 and flanking intronic sequences demonstrated heteroduplex bands (arrow), whereas the mother's DNA showed a homoduplex band only. (B) Direct nucleotide sequencing of the proband's PCR product spanning exon 7 revealed the heterozygous deletion of C at nucleotide position 658 (658delC), as shown in the upper panel, whereas the normal sequence is shown in the middle panel. Verification of the maternal mutation by digestion with *Pst* I is shown in the lower panel. The mutation abolishes the restriction enzyme site for *Pst* I, which cuts the 494-bp PCR product in case of normal allele to 306-bp, 128-bp, and 60-bp bands, and in case of the mutant allele to 306-bp and 187-bp bands (the size difference reflects the 1-bp deletion). (C) Direct nucleotide sequencing of the proband's PCR product spanning exon 8 revealed the heterozygous C→T transition at nucleotide position 754, which resulted in substitution of an arginine codon (CGC) by a cysteine codon (TGC) at amino acid position 252 (R252C), as shown in the upper panel, whereas the normal sequence is shown in the middle panel. Verification of the paternal mutation by digestion with *Nar* I is shown in the lower panel. The mutation abolishes the restriction enzyme site for *Nar* I, which cuts the 378-bp PCR product in case of normal allele to 238-bp, 75-bp, and 65-bp bands, and in case of the mutant allele to 238-bp and 140-bp bands. MW, molecular weight markers  $\phi$  X174/*Hae*III.

genetic lesion. In addition, one allele containing a missense mutation (L336P) (family E) also revealed the presence of a putative splice site mutation (3112-3C→T) in combination with a missense mutation R1225H *in trans*. Among the probands, two were homozygous for their mutation (families F and G), whereas the remainder were compound heterozygotes (Table 1). In each case, the mutations disclosed in the patients' DNA were confirmed in their parents in the heterozygous state. Thus, EB-PA in each case was inherited in an autosomal recessive manner. The mutations discovered in this study bring the total number of mutations in ITGB4 to 33 (Table 2). In addition to pathogenetic mutations, a number of neutral sequence variations in ITGB4 were discovered in this study

(Table 3). Most of these polymorphisms were relatively rare, found in only a small number (less than five) of the normal controls screened.

Examination of DNA from 100 unrelated control individuals did not reveal either the presence of the missense mutations or a putative splicing mutation, indicating that they were not common polymorphisms. Furthermore, all missense mutations affected amino acid residues that were conserved between human, rat, and mouse ITGB4, although not all of them were conserved between different human  $\beta$  integrin chains or the  $\beta\nu$  integrin chain ( $\beta$  neu) in *Drosophila* (Fig. 4). It should be noted that these missense mutations, with the exception of R1225H, which resides in the fibronectin III-like domain in the intracel-

**Table 2.** *ITGB4* mutations reported in the literature and in this study

Case no.	Mutations*	Consequences†	Affected exons	References
<i>Lethal</i>				
1	1141delC/3793+2insT	PTC/Spl	10/30	17
2	3434delT/4050del8	PTC/PTC	28/32	18
3	3793+1G→A/3793+1G→A	Spl/Spl	30/30	10
4	4501delTC/4501delTC	PTC/PTC	34/34	11
5	120delTG/C245G	PTC/missense	3/7	11
6	Q73X/ND	PTC/ND	4/ND	11
7	C738X/4791delCA	PTC/PTC	18/36	12
8	C61Y/C61Y	Missense/missense	4/4	12
9	658delC/R252C	PTC/missense	7/8	This study (family A)
10	D131Y/G273D	Missense/missense	5/8	This study (family B)
11	1874delTCTinsC/V325D	PTC/missense	16/8	This study (family D)
12	Q1767X/Q1767X	PTC/PTC	41/41	This study (family F)
<i>Nonlethal</i>				
13	C562R/C562R	Missense/missense	14/14	12
14	R1281W/R252C	Missense/missense	31/8	12
15	R1281W/R1281W	Missense/missense	31/31	12
16	L156P/R554X	Missense/PTC	5/14	13
17	3793+1G→A/W1478X	Spl/PTC	30/34	19
18	C38R/4776delG	Missense/PTC	3/36	19
19	3976-19T→A/3793+1G→A	Spl/Spl	32/30	20
20	ND/R283C	ND/missense	ND/8	This study (family C)
21	3112-3C→T,L336P/R1225H	Spl, missense/missense	27,9/30	This study (family E)
22	4790delTC/4790delTC	PTC/PTC	36/36	This study (family G)

\* Nucleotide numbers refer to the translation-initiation codon (ATG) as being +1/+3 in  $\beta 4$  integrin cDNA sequence (GenBank accession nos. X53587 and NM000213).

† ND, not determined; Spl, putative splicing mutation.

lular segment, are found within the extracellular domain of the  $\beta 4$  integrin subunit, thus possibly interfering with ligand binding and/or  $\alpha 6/\beta 4$  subunit interactions.

The mutations resulting in PTC as a result of nonsense mutations or small out-of-frame insertions or deletions predict synthesis of a truncated polypeptide and/or down-regulation of the *ITGB4* mRNA levels as a result of nonsense-mediated mRNA decay (29, 30). Thus, no functional  $\beta 4$  integrin polypeptides are synthesized, apparently resulting in EB-PA phenotype in affected individuals.

**Examination of the *ITGB4* mutation database reveals genotype/phenotype correlations.** Identification of the 11 novel mutations in this study brings the total number of distinct  $\beta 4$  integrin mutations disclosed thus far in EB-PA to 33 (Table 2 and Fig. 5). These mutations have been found in a total of 22 individuals with EB-PA, 12 of them being classified as lethal and 10 as nonlethal cases (Table 2). Examination of the mutation database in *ITGB4* reveals a predominance of PTC-causing mutations in the lethal variant of EB-PA, whereas missense mutations are more prevalent in the nonlethal variant. In fact, at least 13 out of 23 alleles in 12 lethal cases harbor PTC mutations (57%) and 7 alleles are missense mutations (30%), whereas, in cases of nonlethal variants, at least 5 out of 19 alleles in 10 individuals are PTC mutations (26%) and 11 alleles are missense mutations (58%).

**Missense/missense mutations associated with a lethal phenotype.** Two cases with a "lethal" variant of EB-PA carried missense/missense combinations, suggesting that the position or the type of these mutations influences the phenotype. However, one of these cases (case 8 in Table 2) with homozygous C61Y/C61Y mutations died during the first week of life before

surgery for pyloric atresia was performed. Thus, the consequence of this mutation to clinical outcome remains unclear (12). The second case (case 10 in Table 2) is a compound heterozygote for D131Y/G273D missense mutations with entirely negative immunostaining for  $\alpha 6$  and  $\beta 4$  integrin epitopes with antibodies recognizing the extracellular domains of the protein. The corresponding aspartic acid-131 and glycine-273 are located in a highly conserved region (Fig. 4). Furthermore, it has been shown that several missense mutations in the corresponding region in  $\beta 3$  integrin abolish ligand-binding activity (31, 32). Thus, this region in  $\beta 4$  integrin may have ligand-binding properties similar to those of  $\beta 3$  integrin. Based on these observations, the mutations D131Y and G273D are predicted to abolish ligand binding of  $\beta 4$  integrin, and could at least partially explain the severe phenotype of the patient.

**Missense/PTC mutations associated with a lethal phenotype.** Three other lethal cases studied previously and in this work (cases 5, 9, and 11 in Table 2) are compound heterozygotes for PTC and missense mutations. In all of them, the PTC mutations may cause mRNA decay or, alternatively, result in the synthesis of truncated nonfunctional  $\beta 4$  integrin polypeptides. In the absence of functional  $\beta 4$  integrin polypeptide from the PTC allele, the missense mutation will determine the phenotype of each patient. One of them (case 9) harbors the mutation R252C, which has been previously characterized in a nonlethal patient (case 14) in combination with another missense mutation R1281W (12). When mutation R252C is combined with a PTC mutation, the phenotype is lethal, suggesting more severe consequences of this mutation on the function of  $\beta 4$  integrin than mutation R1281W. Case 5 harbors the cysteine-to-glycine substitution at position 245 (C245G), which,

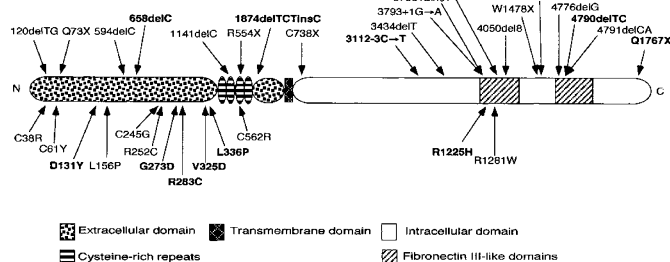
**Table 3.** Nonpathogenic nucleotide variations in the *ITGB4* gene

Location	Nucleotide position	Nucleotide		Restriction enzyme site*	Allele frequencies
		Major	Minor		
Exon 6	513	T(Phe)	C(Phe)	<i>Hinf</i> I	0.991/0.009
Exon 7	702	C(Gly)	T(Gly)	<i>Aci</i> I	0.959/0.041
Exon 15	1821	G(Ser)	A(Ser)	<i>Tsp45</i> I	0.932/0.068
Intron 19	2255–41	G	A	<i>Hinf</i> I	0.958/0.042
Exon 27	3240	C(His)	T(His)	<i>Nla</i> III	0.983/0.017
Exon 29	3648	C(His)	A(Gln)	( <i>Bfa</i> I)	0.983/0.017
Intron 36	4867+59	T	A	<i>BsmA</i> I	0.508/0.492
Exon 41	5567	G(Arg)	A(Gln)	<i>Msp</i> I	0.988/0.012

\* These enzymes are used for confirmation of the nucleotide changes; *Bfa* I in parenthesis requires the use of specific primers.

	D131	R252	G273	R283	V325	L336
Dros $\beta$ nu	SYRTARNNPLDLYVLMDLIW	CTKEIGWKEQARKVVLLVTDGFMHLAGDGL--LAGIIQRNDKQCHLN			RKINVIFAVTEEVVSSYWELSSALMK	
rat $\beta 4$	KVFEPLESFVDLYILMDFSN	CTRDIGWRADSTHLLVFSSTESAFHYEADGANVLAGIMNRNDEKCHLD			HNIIPIFAVTNYSYSYKELHKYFP	
mouse $\beta 4$	QVFEPLESFVDLYILMDFSN	CTRDIGWRADSTHLLVFSSTESAFHYEADGANVLAGIMNRNDEKCHLD			HNIIPIFAVTNYSYSYKELHKYFP	
human $\beta 4$	EVFEPLESFVDLYILMDFSN	CTRDIGWRPDSHLLVFSSTESAFHYEADGANVLAGIMSRNDRCHLD			HNIIPIFAVTNYSYSYKELHTYFP	
human $\beta 1$	KFKRAEDYPIIDLYVLMDSL	CGSLIGWRN-VTRLLVFSSTADAGFHAFAGDGK--LGGTVLPNDGQCHLE			NNIQTIFAFTVEEFQPVYKELKNLIP	
human $\beta 2$	TFRRAKGYPIIDLYVLMDSL	CPEEIGWRN-VTRLLVFAATDDGFHFAGDGK--LGALLTPNDGRCHLE			NNIQPIFAVTSRMVTKYEKLTBIIP	
human $\beta 3$	QVRQVEDYPIIDLYVLMDSL	CDEKIGWRNDASHLLVFTDAKTHIALDGR--LAGIVQPNDDGQCHVG			KNINLIFAVTENVVNLQNYSELIP	
human $\beta 5$	QVRQVEDYPIIDLYVLMDSL	CKEKIGWRKDALHLLVFIIDVPHIALDGGK--LGGILVQPHDQCHLN			NNINLIFAVTKNHYMLYKNTALIP	
human $\beta 6$	HVRQVEDYPIIDLYVLMDSL	CKEKIGWRNDSLHLLVFSVSDAFSDFGMDSK--LAGIVIPNDGCHLD			NNVLLIFAVTQEQVHLYENYAKLIP	
human $\beta 7$	RFLRAEGYPIIDLYVLMDSL	CQEQIGWRN-VSRLLVFSSTDTFTACDGK--LGGIFMPSDGHCHLD			ANNIQPIFAVTSALPVYQELSKLIP	
human $\beta 8$	KVHPLKYPVVDLYVLDVSA	CESHIGWRKEAKRLLVMTDQTSHLALDSK--LAGIVVFPNDGNCHLK			NNINVIFAVQKGFHWYKDLLPLIP	

**Figure 4.** Comparison of human integrin  $\beta 4$  polypeptide chain, spanning the regions containing the aspartic acid, arginine, glycine, arginine, valine, and leucine residues affected by mutations at positions 131, 252, 273, 283, 325, and 336, respectively, with rat and mouse  $\beta 4$  integrins and *Drosophila*  $\beta v$  ( $\beta$  neu) chain, as well as with human  $\beta 1$ – $\beta 8$  integrins. The shaded regions indicate conserved amino acids. GenBank accession numbers for  $\beta$  integrin genes are as follows: rat  $\beta 4$ , NM013180; mouse  $\beta 4$ , L04678; human  $\beta 4$ , X53587, NM000213; *Drosophila*  $\beta v$ , L13305; human  $\beta 1$ , X07979; human  $\beta 2$ , NM000211; human  $\beta 3$ , NM002210; human  $\beta 5$ , X53002; human  $\beta 6$ , NM000888; human  $\beta 7$ , NM000889, M62880, M68892; and human  $\beta 8$ , NM002214.



**Figure 5.** Schematic illustration of the  $\beta 4$  integrin polypeptide with domain organization and positions of all *ITGB4* mutations disclosed thus far in patients with EB-PA. The PTC and putative splice site mutations are shown above the molecule, whereas missense mutations are shown below the molecule. Novel mutations disclosed in this study are shown in bold.

along with arginine-252, is a conserved amino acid located in a putative ligand-binding region (32). When in the hemizygous state, these mutations may be especially deleterious inasmuch as creating or abolishing cysteine residues may change disulfide bonding and alter the secondary structure of the polypeptide. Such conformational changes may result in the loss of the putative ligand-binding function of the  $\beta 4$  integrin. The missense mutation V325D (case 11 in Table 2) substitutes a nonpolar for an acidic residue and also occurs in a position relatively conserved between different  $\beta$  integrin chains as well as between rat and human  $\beta 4$  integrin (Fig. 4).

**Mutations associated with a nonlethal phenotype.** Among the nonlethal EB-PA cases, three of them were either compound heterozygous (cases 17 and 19) or homozygous (case 22, family G) for putative PTC-causing mutations and three were either compound heterozygous or homozygous for mis-

sense mutations (cases 13, 14, and 15) (Table 2). In two of the nonlethal cases, severe nephrosis was reported (cases 15 and 21) (see Ref. 12 and this study). One of these patients is homozygous for R1281W, whereas another patient harbors two putative mutations, a splice site (3112–3C→T) and a missense (L336P) on one allele, and a missense mutation (R1225H) on the other allele. The mutations R1281W and R1225H are located specifically within the first pair of fibronectin III-like domains in the intracellular domain. This region is involved in the  $\beta 4$  integrin interaction with plectin as well as in directing its hemidesmosomal localization (33–35). It should be noted that the BMZ-specific staining of plectin was reduced in all patients, indicating that absent or mutated  $\beta 4$  integrin polypeptides apparently disturb the interaction between plectin and  $\beta 4$  integrin. In addition, in case 21 (family E), the putative splice site mutation affects the nucleotide at position –3 at the intron 26/exon 27 border, indicating that this mutation may cause alternatively spliced products potentially affecting exon 27. On the other hand, leucine at position 336 is conserved between rat, mouse, and human  $\beta 4$  integrin, whereas the human  $\beta 3$ ,  $\beta 5$ , and  $\beta 6$  integrin chains have different amino acids in the corresponding position (Fig. 4). Therefore, leucine may play a less critical role at this position and only minor effects of mutations at this site are predicted. These observations could explain the mild phenotype in the case 21 (family E).

Case 17 was a compound heterozygote for 3793+1G→A and W1478X. Although the mutation 3793+1G→A has been previously shown to result in at least two different frameshift splice variants affecting exon 30, recent studies have suggested that functional splicing can be restored, at least *in vitro*, by changing the culture conditions of keratinocytes (20). Thus, it

is conceivable that the mutation 3793+1G→A may result, under certain circumstances *in vivo*, in some functional  $\beta 4$  integrin polypeptides, perhaps explaining the milder, nonlethal phenotype with a nonsense mutation, W1478X, *in trans*. Case 22 (family G), with a nonlethal clinical presentation, is homozygous for a PTC/PTC mutation, 4790delTC/4790delTC. This mutation predicts truncation of the  $\beta 4$  integrin polypeptide close to the carboxyl-terminal end, potentially eliminating approximately 14% of the polypeptide (Fig. 4). The 4790delTC mutation seems not to cause total absence of mRNA through nonsense-mediated decay, inasmuch as the skin of the patient demonstrated the presence, although in reduced amounts, of  $\alpha 6$  and  $\beta 4$  integrin polypeptides (Fig. 2). In contrast, in case 12 (family F), the nonsense mutation Q1767X results in a stop codon on both alleles, from which we can predict deletion of 39 amino acids from the C-terminus of the  $\beta 4$  integrin polypeptide. This patient, who is from a remote Aboriginal community, died at about 2.5 mo of age with a relatively mild skin condition compared with other lethal cases. This emphasizes the importance of continuing care and the possible role of other clinical manifestations on the outcome. TEM findings in case 12 (family F) revealed intracellular cleavage in the skin, a finding that is reinforced by recent studies that have shown that this region of the  $\beta 4$  integrin polypeptide is critical for the interaction with BP180 (34, 36). Because tissue for immunofluorescence was not available from this patient, the  $\alpha 6$  and  $\beta 4$  integrin staining patterns could not be studied.

In two patients listed in Table 2, the second mutation was not identified despite screening and complete sequencing of the ITGB4 gene. The possible promoter region mutations resulting in absent expression of ITGB4 from one allele was investigated by sequencing the ITGB4 promoter region from genomic DNA in these patients without evidence of mutations. Unfortunately, RNA was not available for reverse transcription-PCR studies in either case. Large genomic deletions were ruled out by Southern blots of the ITGB4 region, and the identification of heterozygosity for several intragenic polymorphisms further supported this finding. The second mutations in these two cases, therefore, remain undetected.

In summary, with a few exceptions, an overall theme explaining the severity of the EB-PA phenotype, lethal *versus* nonlethal, seems to be emerging from these studies. In general, a combination of missense mutations on both alleles may be associated with a milder skin phenotype depending upon the functional significance of the affected amino acids within the protein, whereas PTC in both alleles cause a severe phenotype. However, the phenotype of the patient is clearly influenced by the position of the missense mutation in the functional domains of the  $\beta 4$  integrin protein. The missense mutations in the putative ligand-binding domain cause a severe phenotype, especially when the mutations affect conserved amino acids, whereas mutations affecting less conserved amino acids cause milder phenotypes. It should be noted that the immunofluorescence studies in this and previous works correlate relatively well with the phenotype, *i.e.* in nonlethal cases  $\alpha 6\beta 4$  integrin expression is positive whereas in lethal cases this protein is absent. This emphasizes the importance of immunofluorescence examination, in addition to mutation analysis, as a

diagnostic procedure with prognostic implications for EB-PA patients. Precise prediction of the phenotype will require further knowledge of the interactions of ITGB4 with other structural proteins in the skin and other affected tissues. This will be accomplished, in part, by further examination of the expanding ITGB4 mutation database in patients with EB-PA and their associated phenotypes, as well as by further characterization of the functional domains of the  $\alpha 6\beta 4$  integrin molecule through experimental studies.

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