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Novel ITGB4 Mutations in Lethal and Nonlethal Variants of Epidermolysis Bullosa with Pyloric Atresia: Missense versus Nonsense

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Summary

Epidermolysis bullosa with pyloric atresia (EB-PA), an autosomal recessive genodermatosis, manifests with neonatal cutaneous blistering associated with congenital pyloric atresia. The disease is frequently lethal, but nonlethal cases have also been reported. Expression of the $\alpha 6\beta 4$ integrin is altered at the dermal-epidermal basement-membrane zone; recently, mutations in the corresponding genes (ITGA6 and ITGB4) have been disclosed in a limited number of patients, premature termination codons in both alleles being characteristic of lethal variants. In this study, we have examined the molecular basis of EB-PA in five families, two of them with lethal and three of them with nonlethal variants of the disease. Mutation analysis disclosed novel lesions in both ITGB4 alleles of each proband. One of the patients with lethal EB-PA was a compound heterozygote for premature termination–codon mutations (C738X/4791delCA), whereas the other patient with a lethal variant was homozygous for a missense mutation involving a cysteine residue (C61Y). The three nonlethal cases had missense mutations in both alleles (C562R/C562R, R1281W/R252C, and R1281W/R1281W). Immunofluorescence staining of skin in two of the nonlethal patients and in one of the lethal cases was positive, yet attenuated, for $\alpha 6$ and $\beta 4$ integrins. These results confirm that ITGB4 mutations underlie EB-PA and show that missense mutations may lead to nonlethal phenotypes.

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Introduction

Epidermolysis bullosa with pyloric atresia (EB-PA; MIM 226730) is a recessively inherited genodermatosis manifesting at birth or shortly thereafter, with blistering of the skin and mucous membranes and with associated congenital gastrointestinal abnormalities, including esophageal, pyloric, or duodenal atresia (Nazarro et al. 1990; Fine et al. 1991; Lestringant et al. 1992). Clinically, both lethal and nonlethal variants have been recognized, the lethal forms resulting in the demise of the affected individuals usually within a few weeks or months of the postnatal period; in nonlethal variants the clinical severity of the skin involvement often tends to improve with age. The patients often demonstrate nail dystrophy and dental anomalies, including enamel hypoplasia. Ultrastructurally, the skin lesions are characterized by blister formation at the level of the lamina lucida or at the lamina lucida/basal keratinocyte interface, associated with hypoplastic hemidesmosomes (Niessen et al. 1996; Pulkkinen et al. 1998*b*). Consequently, this disease has been traditionally classified as a form of junctional epidermolysis bullosa (EB), but recent reclassification of various forms of EB, based on molecular evaluation of these patients, has suggested that EB-PA is a hemidesmosomal variant (Uitto et al. 1997; Pulkkinen and Uitto 1998).

Initial clues to the underlying molecular defects in EB-PA were suggested by the demonstration of absent or reduced expression of the $\alpha 6$ and/or $\beta 4$ integrin in the skin of these patients, by immunofluorescence microscopy using antibodies specific to these integrin epitopes (Gil et al. 1994; Brown et al. 1996; Shimizu et al. 1996). As a result, the corresponding genes, ITGA6 and ITGB4, encoding the $\alpha 6$ and $\beta 4$ integrin polypeptides, respectively, were considered as candidate genes for mutations in EB-PA. In accordance, we and others have recently disclosed distinct mutations in both of these genes in EB-PA patients representing nine families. In seven families,

Table 1

Diagnostic Features and Mutation Analysis in Patients with EB-PA

Family	Proband Age (Sex)	Clinical Features	Disease Variant	Ultrastructural Features/ Immunofluorescence	ITGB4 Mutations	Consequences of Mutations
1	7 years (F)	PA at birth; skin involvement noted at age ~2 years	Nonlethal	Hypoplastic hemidesmosomes without distinct inner and outer plaques, reduced expression of $\alpha 6$ and $\beta 4$ integrin epitopes	C562R/C562R	Missense/missense
2	Newborn (M)	PA at birth, generalized blistering and erosions of the skin at birth	Lethal	Hypoplastic hemidesmosomes, tissue separation at the level of plasma membrane, reduced staining with antibodies against $\alpha 6$ and $\beta 4$ integrins	C738X/4791delCA	PTC/PTC
3	10 mo (M)	Antral atresia at birth, blisters on fingers and toes at end of 1st wk of life	Nonlethal		R1281W/R252C	Missense/missense
4	Newborn (F)	PA at birth, extensive blistering within 1st wk of life	Lethal	Absence of hemidesmosomes	C61Y/C61Y	Missense/missense
5	4 mo (M)	PA at birth, blisters on the sacral area noted at age ~3 mo	Nonlethal	$\beta 4$ integrin expression reduced, $\alpha 6$ integrin normal	R1281W/R1281W	Missense/missense

the mutations were present in the ITGB4 gene, whereas in two of them mutations in the ITGA6 gene were found (Vidal et al. 1995; Pulkkinen et al. 1997*a,* 1997*b,* 1998*a,* 1998*b;* Ruzzi et al. 1997; Takizawa et al. 1997). Eight of the nine probands in whom the mutations have been disclosed thus far have a lethal phenotype, and the majority of mutations resulted in a premature termination codon (PTC) for translation (see Pulkkinen and Uitto 1998). In one case with lethal EB-PA, a combination of a PTC-causing mutation (120delTG) and a missense mutation (C245G) was noted (Pulkkinen et al. 1998*b*). On the other hand, compound heterozygosity for a missense (L156P) and a nonsense (R554X) mutation in the $\beta 4$ integrin gene was shown to underlie the relatively mild, nonlethal phenotype (Pulkkinen et al. 1998*a*). These observations suggested, therefore, that, in general, PTC mutations in both alleles predict a lethal phenotype, whereas a missense mutation in the second allele may signify a milder phenotype with a nonlethal outcome. In this study, we have examined the molecular basis of EB-PA in five patients, two presenting a lethal phenotype and three presenting a nonlethal phenotype.

Subjects and Methods

Clinical and Genetic Features

Family 1.—The proband of this family was a 7-year-old female who was the third child from a consanguineous union, the parents being first cousins of Turkish extraction. The proband manifested at birth with PA, which was surgically corrected at age 4 d. Fragility of the skin was noted during the 2d year of life, and at age 7 years the child had very localized blistering, affecting primarily the extremities and perioral areas of the skin, associated with nail dystrophy and enamel pitting of the teeth (table 1 and fig. 1).

Immunoepitope mapping of the skin, using a battery of monospecific antibodies recognizing cutaneous basement-membrane zone (BMZ) epitopes revealed reduced expression of both $\alpha 6$ and $\beta 4$ integrin subunits at the roof of the blister cavity, as well as on sweat glands, in comparison with normal control skin (fig. 2). In contrast, laminin 5 staining using the monoclonal antibody GB3 showed essentially normal results for the blister floor, in comparison with control skin (fig. 2*G* and *H*).

Transmission electron microscopy revealed the presence of hypoplastic hemidesmosomes without distinct inner and outer plaques. Most hemidesmosomes appeared to be connected to the intermediate-filament network.

Family 2.—The proband of this family, a newborn male, was the second child of nonconsanguineous parents. He had a 3-year-old sister who was clinically unaffected. At birth, the proband exhibited blistering that was confined to the fingers, ankles, and feet, at the sites of mechanical trauma, as well as in the oral mucosa. Despite successful gastroduodenostomy at age 13 d to repair the pyloric atresia, he was unable subsequently to be weaned from nasogastric tube feeding, because of a secondary swallowing dysfunction that resulted in as-

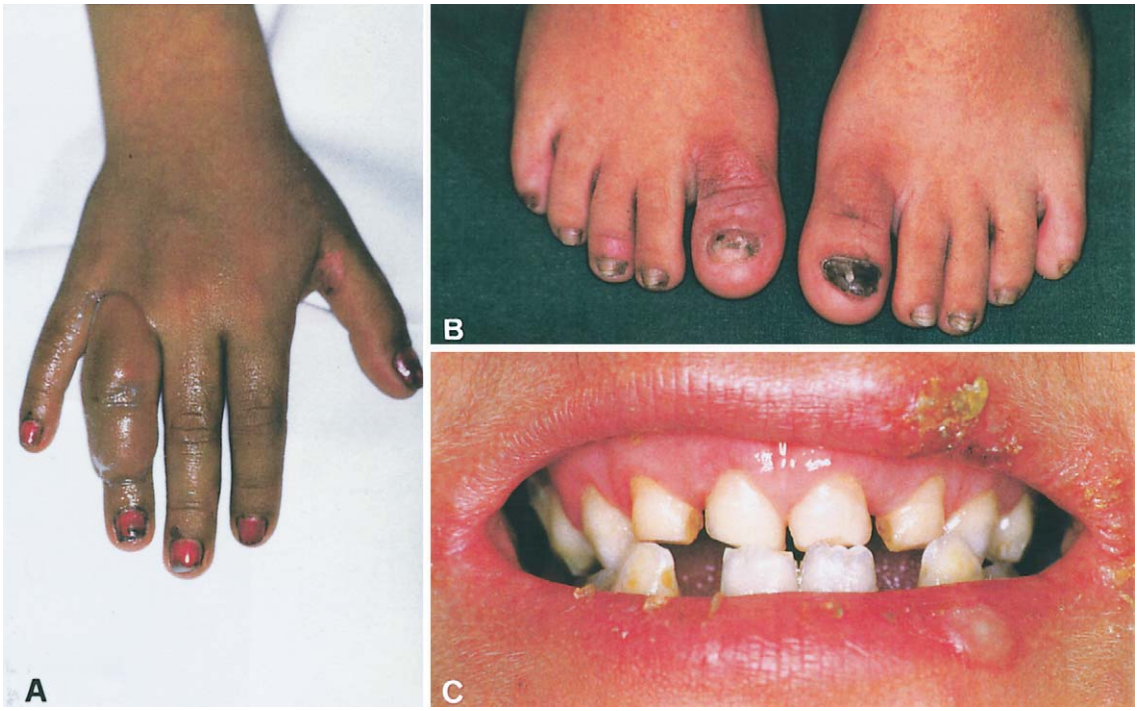

Figure 1 Clinical features of proband of family 1. *A,* Trauma-induced localized blistering of right hand. *B,* Dystrophy of toenails. Note that the left big toenail had additional onychomycosis. *C,* Blistering of lips, as well as dental anomalies with enamel hypoplasia and caries.

piration. He failed to thrive and died during the 3d mo of the postnatal period.

Transmission electron microscopy revealed hypoplastic hemidesmosomes, and tissue separation was noted at the level of the plasma membrane (fig. 3). Immunohistochemical staining of unaffected skin, with a battery of monoclonal antibodies to BMZ antigens, revealed normal staining intensity and distribution for laminin 5; collagen types IV, VII, and XVII; BP230; and plectin/HD1. However, staining for the $\alpha 6$ and $\beta 4$ integrin epitopes was positive but reduced (fig. 4).

Family 3.—The proband of this family was a 10-mo-old male who, shortly after birth, was diagnosed as having antral atresia, which was immediately corrected by surgery. The parents were of Bangladeshi ethnic origin and were not known to be related.

At the end of the 1st wk of life, the patient developed blisters on his fingers, toes, and scalp. He has continued to develop blisters, but their frequency has lessened during the 1st year of life. The mother had three earlier pregnancies; the first and the third resulted in healthy males. The second pregnancy resulted in a male, delivered at home, with congenital aplasia cutis on the arm. This patient also had clinical signs of gastrointestinal atresia, but no surgical intervention was attempted, and the patient died at age 6 d.

Family 4.—The proband in this family was a newborn female who had congenital PA, which was not corrected, and she developed extensive blistering shortly after birth. Transmission electron microscopy of the skin revealed hypoplastic hemidesmosomes, but immunofluorescence analysis was not done. The newborn died at age 2 wk, from complications of the disease.

Family 5.—The proband in this family is a male infant of first-cousin Orthodox Jewish parents. He had PA at birth and developed nail dystrophy at age 2 mo. At age 3 mo, he developed blistering of the skin, as well as severe nephrotic syndrome. Indirect immunofluorescence staining of cryosections containing dermis (epidermis was lost) from the proband revealed positive but reduced staining of sweat glands for $\beta 4$ integrin, whereas $\alpha 6$ integrin, laminin 5, and the 230-kD bullous pemphigoid antigens were normally expressed.

Collectively, the clinical material consisted of five patients with diagnostic features of EB-PA. Three of them (patients 1, 3, and 5) had a nonlethal phenotype, whereas two of them (patients 2 and 4) died during the early postnatal period, from complications of the disease (table 1).

Immunofluorescence and Electron Microscopy

The distribution of antigens in skin samples was analyzed by microscopic examination of cryostat sections

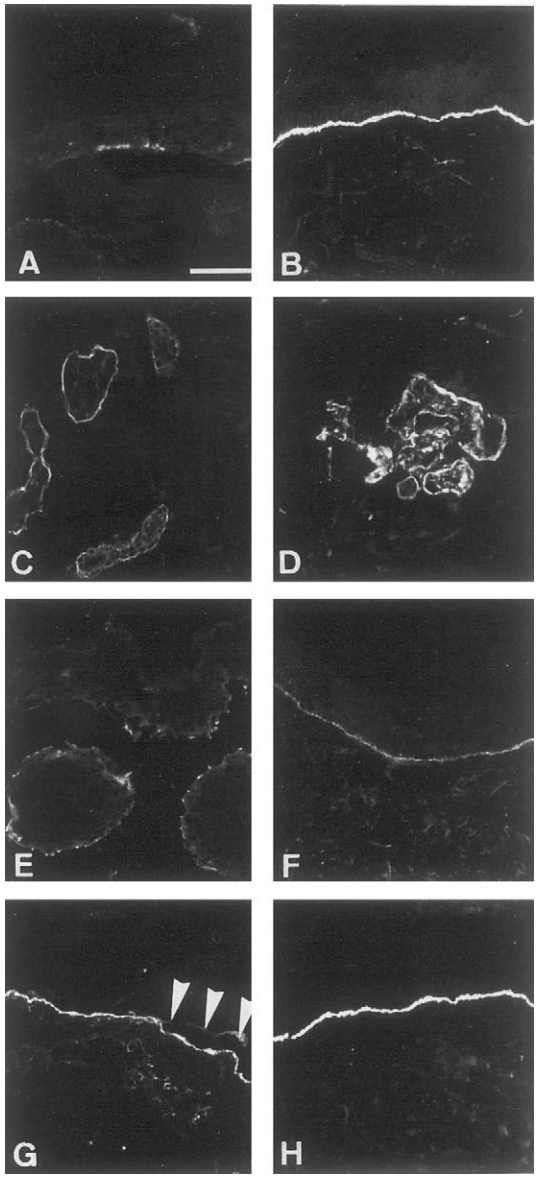

Figure 2 Indirect immunofluorescence staining of skin of proband of family 1 and of normal control skin. Antibodies to β4 integrin (*A–D*) revealed attenuated staining at the roof of a blister (*A*) and of the sweat glands (*C*) of the patient, compared with staining of the dermo-epidermal junction (*B*) and sweat glands (*D*) in normal control skin. Staining with antibodies to α6 integrin showed a slightly attenuated reaction in the patient's skin (*E*), compared with that of the control (*F*). Laminin 5 staining was found in the blister base of the patient's skin (*G*); the arrowheads indicate the blister roof. Normal control skin stained with antibodies to laminin 5 is shown for comparison (*H*).

stained either by the indirect immunofluorescence method using FITC-conjugated secondary antibodies or by the indirect immunoperoxidase method described elsewhere (Brown et al. 1996).

Skin biopsies were prepared for electron microscopy, according to standard protocols. In brief, samples were placed in half-strength Karnovsky's fixative, postfixed in osmium tetraoxide, en bloc–stained in uranyl acetate, dehydrated by graded ethanol and propylene oxide, and embedded into epoxy resin. Ultrathin sections were stained sequentially with phosphotungstic acid, uranyl acetate, and lead citrate and were viewed by means of a Philips 420 STEM operated at 60 kV.

Characterization and Verification of Mutations

Mutation-detection strategy 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, as described elsewhere (Pulkkinen et al. 1998*b*). Specifically, the following primers were used to amplify ITGB4 exons 4, 8, 14, 18, 31, and 36, by use of total genomic DNA isolated from peripheral blood as the template: E4-L, 5′-TCGGGAATAGCTGGTGGAAA-3′; and E4-R, 5′-CCCATAAATAGCCAGGCTGA-3′; E8-L, 5′-GCCGTGATGCGTGTCAGCAG-3′; and E8-R, 5′-CCATGGACCCCCTACCTTGA-3′; E14-L, 5′-AGGTGGGCAGCCTGGACTAC-3′; and E14-R, 5′-TAGGAGGGGGCACCAGTGAG -3′; E18-L, 5′-AGCTCGGTGGGGAGGACAGG-3′; and E18-R, 5′-AGAACTCAGGCGGGGCTGG-3′; E31-L, 5′-CTGTGTCAGGGGTGGTGTTG-3′; and E31-R, 5′-CCACGATAGGGATGTCAGGG-3′; E36-L, 5′-GGGGGCAGCACTGTGACTCC-3′; and E36-R, 5′-AGGGACTTGGGTGGGTTCCT-3′.

The PCR reactions were performed in a total volume of 50 μl containing 1 × PCR buffer, either 1.25 U of *Taq* polymerase (Qiagen) (for exons 4, 8, 14, 31, and 36) or Expand™ High Fidelity PCR System (Boehringer Mannheim) (for exon 18), 4% dimethylsulfoxide, 12.5 pmol of each primer, and 200 ng of genomic DNA. The amplification conditions were as follows: 5 min at 95°C for one cycle, followed by 38 cycles of 45 s at 95°C, 45 s at the annealing temperature (60°C for exons 4, 8, and 31; 62°C for exon 18; and 64°C for exons 14 and 36), and 45 s at either 72°C (*Taq* polymerase; Qiagen) or 68°C (Expand™ High Fidelity Enzyme; Boehringer Mannheim). The PCR products were analyzed by conformation-sensitive gel electrophoresis (CSGE), as described elsewhere (Ganguly et al. 1993). All PCR products demonstrating heteroduplexes were directly sequenced by an automated DNA sequencer (ABI).

In each family, the verification of the mutation was performed on the basis of the PCR products representing the proband and his or her immediate family members, by use of either restriction-endonuclease digestions (*Bsr*I for family 1, *Sfa*NI for family 2, *Nar*I and *Bsa*WI for family 3, and *Bsa*WI for family 5) or allele-specific oligonucleotide hybridization (ASO) (for families 2 and 4).

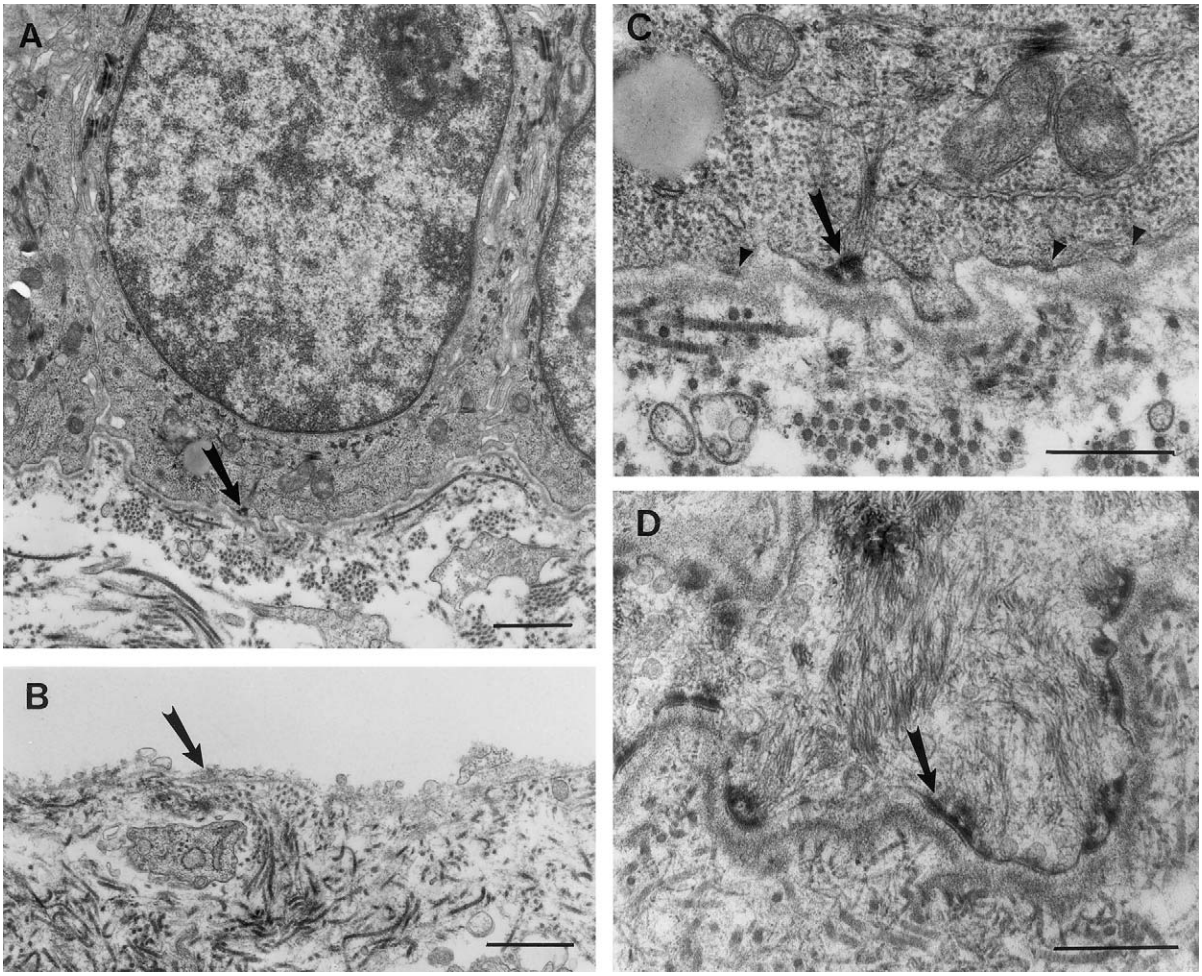

Figure 3 Transmission electron microscopy of skin. *A–C,* Proband of family 2. *D,* Unrelated normal control. Basal keratinocytes have a reduced number of hypoplastic hemidesmosomes (*A* and *C* [*arrows*]). There are perinuclear arrays of keratin filaments, but the abundance of keratin filaments is diminished in the basal compartment of the cell. Electron-dense material along the basal plasma membrane suggests the presence of incomplete hemidesmosomes (*C* [*arrowheads*]). Normal hemidesmosomes are shown in control skin (*D* [*arrow*]). Separation of the epidermis from the basement membrane left fragments of the basal keratinocyte associated with the basal lamina (*B* [*arrow*]). (Scale bars are 1 μm [*A* and *B*] and 500 nm [*C* and *D*].)

The allele-specific oligomer primers for verification of mutation C61Y in family 4 were as follows: wild type, 5′-CCGGCGCTGCAACACCC-3′; mutant, 5′-CCGGCGCTACAACACCCA-3′.

For verification of mutation 4791delCA in family 2, the ASO primers were as follows: wild type, 5′-ACATCTCTCAGAGTGAGCT-3′; mutant, 5′-CCACATCTCTGAGTGAGCT-3′.

The oligomers were 5′ end-labeled with $\gamma[^{32}P]$-dATP. The PCR products were dotted onto nylon filters (Zeta probe; Bio-Rad), immobilized by UV cross-linking, and hybridized with radioactive oligomers at 37°C, according to the manufacturer's recommendations. The filters were washed to the final stringency of 0.2 × SSPE, 0.1% SDS, at the melting temperatures of the oligomers. The filters were exposed to x-ray film.

Results

Identification and Verification of Mutations

The mutation screening in each proband and his or her immediate family members was performed as described in Subjects and Methods, starting with PCR amplification of each exon of ITGB4, followed by heteroduplex analysis on CSGE gels and direct sequencing of the PCR products showing heteroduplexes (Pulkkinen et al. 1998*b*). This strategy is illustrated in figure 5.

Family 1.—Screening of ITGB4 for mutations in this family revealed heteroduplex bands in the case of exon 14, in DNA from both parents. Direct nucleotide sequencing of the corresponding PCR products revealed that both parents were heterozygous for a C→T substi-

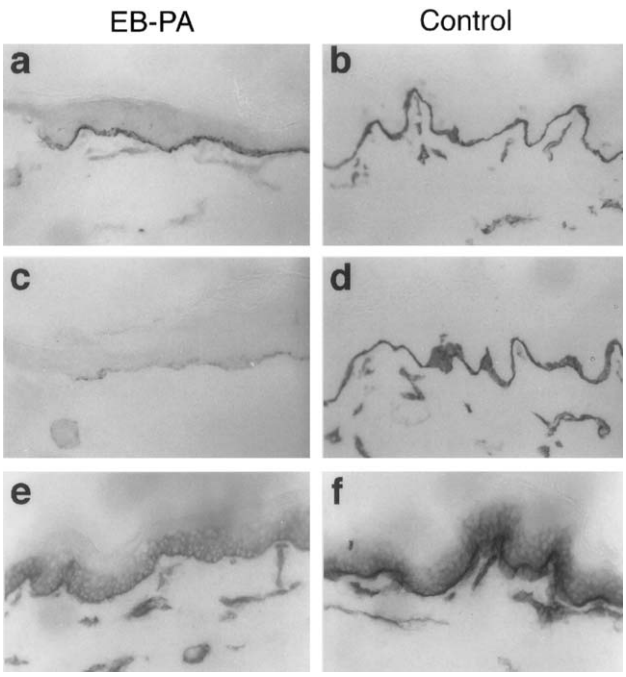

Figure 4 Altered $\alpha 6\beta 4$ integrin expression in EB-PA skin from the proband of family 2. Immunoperoxidase-labeled cryostat sections of EB-PA patient's skin (*a, c,* and *e*) and of normal control skin (*b, d,* and *f*) were stained for integrin subunits, with either anti-$\beta 4$ (clone 3E1; *a–d*) or anti-$\alpha 6$ (clone GoH3; *e* and *f*) monoclonal antibodies. Tissue sections in panels *c* and *d* were extracted with 0.5% Triton X-100 prior to formaldehyde fixation. Staining intensity for both integrin subunits was reduced in the EB-PA patient's skin (*a* and *e*) vs. that in the normal control skin (*b* and *f*) but remained polarized predominantly to the basal-cell surface. A major fraction of the integrin $\beta 4$ subunit could be removed in the EB-PA patient's skin (*c*), but not in the normal control skin (*d*), by Triton X-100 extraction, suggesting an altered structural organization for this integrin in EB-PA. Similar results were obtained with staining with several other anti-$\alpha 6$ and $\beta 4$ integrin subunit-specific monoclonal antibodies.

tution at nucleotide position 1684, whereas the proband was homozygous for this transition (table 1). This nucleotide substitution resulted in a change from cysteine (TGT) to arginine (CGT), at amino acid position 562 (C562R) within the third cysteine-rich repeat of the extracellular domain of $\beta 4$ integrin polypeptide. This nucleotide substitution also abolished a restriction site for *Bsr*I, which was used to verify the presence of this mutation in the parents' (heterozygous) and proband's (homozygous) DNA. Screening of 100 chromosomes from normal, healthy controls by *Bsr*I digestion demonstrated the absence of C562R, suggesting that it was a pathogenetic mutation rather than a common polymorphism.

Family 2.—In this family, scanning of the ITGB4 gene by CSGE revealed a heteroduplex band when exon 18 was amplified from the proband's and the father's DNA, whereas the mother's DNA revealed the presence of a homoduplex band only (fig. 5*A, upper panel*). Direct sequencing of the corresponding PCR products revealed the presence of a heterozygous C→A nucleotide substitution at nucleotide position 2214, which changed a codon for cysteine (TGC) to a stop codon (TGA), at amino acid position 738 (fig. 5*B*). This nucleotide substitution created a new restriction site for *Sfa*NI; digestion of the PCR products confirmed that the proband and the father were heterozygous for this mutation, whereas the mother's DNA showed the presence of the wild-type allele only (fig. 5*C*).

Further scanning of the ITGB4 revealed heteroduplex bands in exon 36 in the proband's and the mother's DNA, whereas the father's DNA revealed the presence of a homoduplex band only (fig. 5*A, lower panel*). Direct sequencing of the PCR products revealed the presence of a 2-bp deletion at nucleotide position 4791 (4791delCA), which created a PTC for translation, 77 nucleotides downstream from the site of deletion (fig. 5*D*). The presence of this genetic lesion in the proband's and the mother's DNA was verified by ASO, which also revealed that the father did not have this mutation (fig. 5*E*). Thus, the proband in this family was a compound heterozygote for ITGB4 mutations, C738X/4791delCA, both mutations causing PTCs.

Family 3.—In this family, heteroduplexes were noted with PCR products spanning exons 31 and 39 of ITGB4 when DNA from the proband and the mother was examined, whereas the father's DNA revealed homoduplex bands only. Sequencing of the PCR products corresponding to exon 39 revealed a G→C nucleotide change at nucleotide position −25 in intron 38 (5213–25G → C), which was a polymorphism recognized by restriction enzyme *Bgl*I (see table 2). Sequencing of the PCR product spanning exon 31 and flanking intronic sequences revealed a C→T transition at nucleotide position 3841, which resulted in an amino acid change from an arginine (CGG) to a tryptophan (TGG), at amino acid position 1281 (R1281W; table 1). This nucleotide change abolished a restriction-enzyme site for *Bsa*WI, which was used for verification of the inheritance of the mutation from the mother to the proband. This nucleotide change was not noted either in the father's DNA or in 120 chromosomes from unrelated healthy control individuals, suggesting that it is a pathogenetic mutation rather than a polymorphism.

No other heteroduplexes were found in ITGB4, and results of scanning of the ITGA6 gene by CSGE (Pulkkinen et al. 1997*a*) was negative. Subsequently, all other PCR products corresponding to the entire coding sequence of ITGB4 from the proband were sequenced directly. This revealed two other nucleotide changes. Exon 34 had a silent G→C transversion at nucleotide position 4521, which was recognized by restriction enzymes *Bst*NI and *Eco*RII (see table 2). A C→T transition was noted in exon 8, at nucleotide position 754, which re-

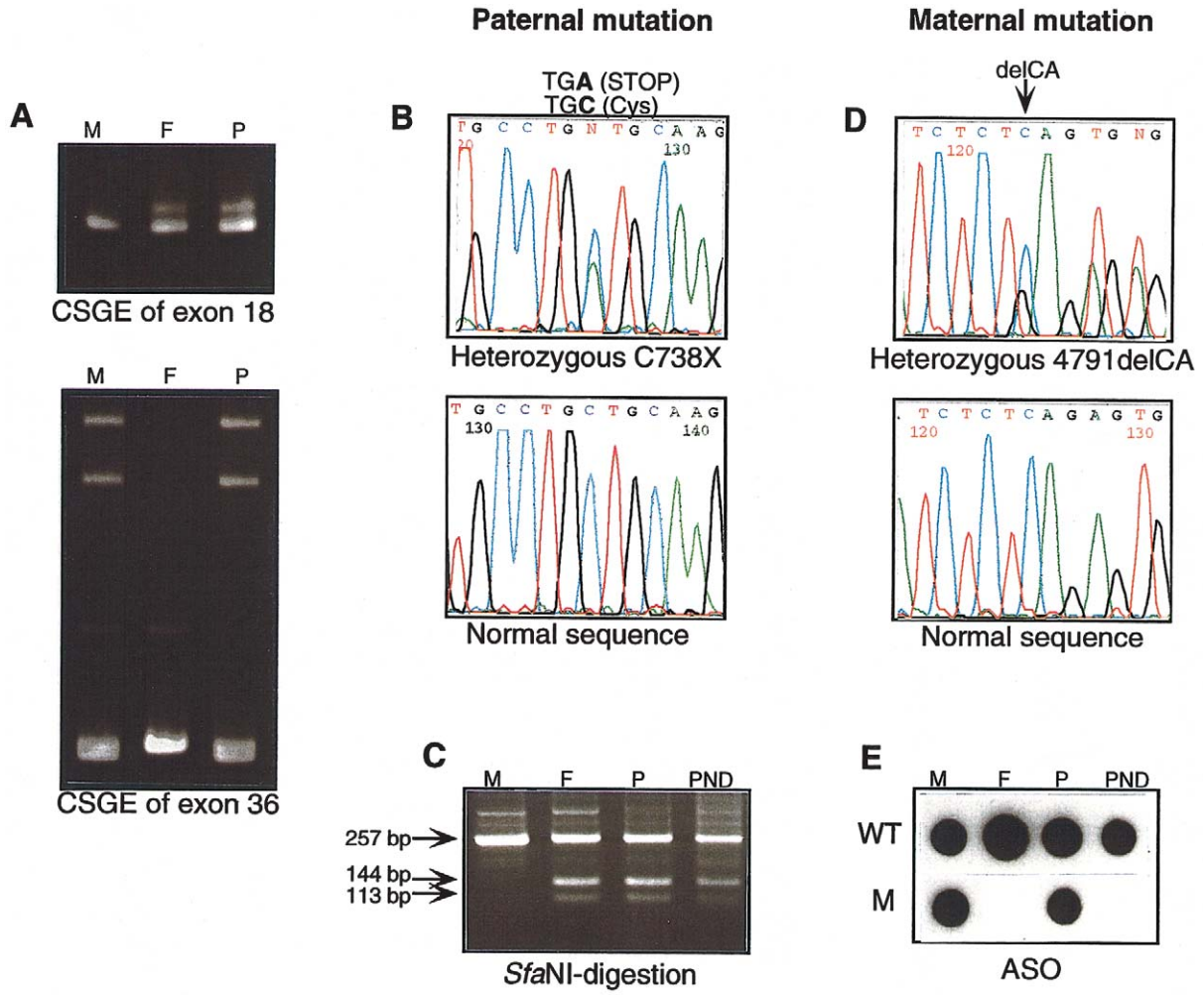

Figure 5 Mutation analysis and prenatal testing in family 2. *A,* CSGE analysis of ITGB4, which reveals heteroduplex bands in father's (lane F) and proband's (lane P) PCR products spanning exon 18 (*upper panel*) and in mother's (lane M) and proband's PCR products spanning exon 36 (*lower panel*). *B,* Direct nucleotide sequencing of the PCR product from the proband, spanning exon 18, revealed a heterozygous C→A transversion, which resulted in substitution of a codon for cysteine (TGC) by a stop codon (TGA); the mutation was designated "C738X" (*upper panel*). *C,* Verification of inheritance of mutation, by use of restriction enzyme *Sfa*NI, which recognized the site of the mutation. Specifically, the mutant allele was digested to 144- and 113-bp bands, whereas the normal allele, corresponding to the 257-bp band, resisted digestion. The results showed that the father and the proband were heterozygous carriers of the mutation whereas the mother harbored the normal alleles in both chromosomes. *D,* Direct nucleotide sequencing of PCR product from proband, spanning exon 36, revealing a heterozygous 2-bp deletion (CA) at nucleotide position 4791 (*upper panel*), compared with the normal sequence (*lower panel*). *E,* Verification of inheritance of mutation 4791delCA , by allele-specific oligomer hybridization as described in Subjects and Methods. Specifically, the PCR products from the mother and the proband hybridized both to wild-type (WT) and to mutant (M) oligomers, indicating that these individuals are heterozygous for this mutation, whereas the father's PCR product hybridized to the normal oligomer only, indicating that he is homozygous for the normal sequence. In a subsequent pregnancy, the family requested prenatal testing of the fetus at risk for EB-PA. DNA from amniotic cells was amplified for exons 18 and 36, and the PCR products were studied for the mutations C738X and 4791delCA, by use of *Sfa*NI digestion and ASO, respectively. The *Sfa*NI digestion revealed that the fetus had inherited the paternal mutation (*C*; PND) and a normal maternal allele (*E*; PND). Thus, the fetus was predicted to be an unaffected carrier of the paternal mutation.

sulted in the change of a codon for arginine (CGC) to a codon for cysteine (TGC), at amino acid position 252 (R252C). This nucleotide change abolished a restriction-enzyme site for *Nar*I, which was used to verify the inheritance of the transition from the father to the proband. Furthermore, the *Nar*I screening demonstrated the absence of this nucleotide change not only in the maternal DNA but also in 110 chromosomes from unrelated healthy control individuals, suggesting that R252C is the paternally inherited pathogenetic mutation.

Family 4.—Screening of the ITGB4 gene for mutations in this family revealed similar heteroduplexes when the PCR products spanning exon 4 and flanking intronic sequences in both parents were analyzed by CSGE,

Table 2

Nucleotide Variations in the ITGB4 Gene

Location	Nucleotide Position	Nucleotide Variant		Restriction-Enzyme Site(s)	Allelic Frequencies[a]
		Major	Minor		
Exon 7	579	T (Pro)	C (Pro)	*Sty*I, *Ban*II	Not determined
Exon 13	1544	G (Arg)	A (His)	*Eag*I, *Nco*I	.932/.068
Intron 25	2962+61	T	G	*Msp*I	Not determined
Intron 27	3317−46	C	T	*Hph*I	.900/.100
Intron 28	3474+21	C	T	*Pal*I/*Hae*III/*Bsu*RI	.635/.365
Intron 28	3474+78	G	A	*Sty*I	.875/.125
Exon 30	3730	G (Pro)	A (Pro)	…	.990/.010
Exon 34	4521	G (Pro)	C (Pro)	*Bst*NI/*Eco*RII	.550/.450
Intron 34	4558+146	ACAT deletion		…	.850/.150
Exon 38	5097	G (Pro)	A (Pro)	*Cac*8 I	.987/.013
Intron 38	5213−25	G	C	*Bgl*I	.600/.400
Intron 39	5378−15	A	G	*Drd*I	.989/.011
Exon 41	5495	C (Pro)	T (Leu)	…	.690/.310
Exon 41	5653	T	C	*Mae*II	Not determined

[a] In 50 unrelated healthy controls.

whereas the proband showed a homoduplex band only. Sequencing of the PCR products demonstrated that both parents were heterozygous for a G→A transition at nucleotide position 182, which resulted in the change of a codon for cysteine (TGC) to a codon for tyrosine (TAC), at amino acid position 61 (C61Y). The proband was shown to be homozygous for this nucleotide change. Since this nucleotide substitution could not be recognized by any restriction endonuclease, verification of this transition in this family, as well as verification of its absence in 100 chromosomes of unrelated healthy individuals, was performed by ASO hybridization, which suggested that it was a pathogenetic mutation rather than a polymorphism.

Family 5.—In this family, heteroduplex scanning of the parents' DNA revealed identical heteroduplex bands when the PCR amplification products corresponding to exon 34 of ITGB4 were examined by CSGE. Direct sequencing of the PCR products revealed a C→T transition at nucleotide position 3841 within exon 31, which changed a codon for arginine (CGG) to a codon for tryptophan (TGG), a mutation designated as "R1281W." The proband was shown by sequence analysis to be homozygous for this mutation (R1281W/R1281W), and these results were confirmed by *Bsa*WI restriction-enzyme digestion (see case 3).

DNA-Based Prenatal Testing in Family 2

As indicated above, the proband of family 2, who had died prior to being tested, was found to be a compound heterozygote for ITGB4 mutations, C738X/4791delCA, present in exons 18 and 36, respectively. At the time of mutation analysis, the parents requested prenatal testing of a subsequent pregnancy, which was considered to be at risk for recurrence of EB-PA. Sequencing as well as *Sfa*NI digestion and ASO-analysis of PCR products spanning exons 18 and 36 from DNA isolated from amniotic cells at the 15th wk of gestation revealed that the fetus was a carrier of the paternal mutation, C738X (see fig. 5). However, the fetus did not have the maternal mutation, predicting a phenotypically normal child, and a healthy baby was born at term.

Identification of Novel Polymorphisms in ITGB4

During the search for pathogenetic mutations in the ITGB4 gene in these five families, as well as in those that we have reported elsewhere (Pulkkinen et al. 1997*a*, 1998*a*, 1998*b*), a number of polymorphisms were detected (table 2). Many of these polymorphisms change a restriction-enzyme site, thus allowing expedient screening of the general population. Of these polymorphisms, two changed the corresponding amino acid (R515H in exon 13 and P1832L in exon 41). These amino acid substitutions were found in the general population (see table 2), indicating that they are normal polymorphisms. The remainder of the polymorphisms were either silent exonic or intronic. Also, a biallelic ACAT-deletion polymorphism was disclosed in intron 34. These polymorphisms will assist in further genetic linkage analysis when the ITGB4 gene, which has been mapped to chromosomal locus 17q25 (Bhalerao et al. 1997), is examined in the context of families with EB-PA.

Discussion

In this study, we have identified novel mutations in the ITGB4 gene in five families with EB-PA. Of the five probands, three were homozygous (C562R/C562R, C61Y/C61Y, and R1281W/R1281W) and one was

compound heterozygous (R1281W/R252C) for novel missense mutations. The fifth proband was a compound heterozygote for two PTC mutations (C738X/4791delCA). Previously, either PTC mutations in both alleles or a PTC mutation in one allele and a missense mutation in the other allele have been detected in the ITGB4 gene in seven families with EB-PA (fig. 6; Pulkkinen and Uitto 1998). In addition, homozygous nonsense mutations in the ITGA6 gene encoding the $\alpha 6$ subunit of the $\alpha 6\beta 4$ integrin have been reported in two families with EB-PA (Pulkkinen et al. 1997*b;* Ruzzi et al. 1997). In both cases, the phenotype was lethal, indistinguishable from those with nonsense mutations in both alleles of the ITGB4 gene.

The repertoire of mutations disclosed thus far suggests that the presence of PTCs in both alleles, either in a homozygous or in a compound-heterozygous state, will result in a lethal phenotype. However, the presence of a missense mutation, either in one allele in combination with a PTC mutation or in both alleles, could predict a more variable and occasionally milder nonlethal phenotype. For example, in a previous study a leucine-to-proline substitution in the amino acid position 156 of $\beta 4$ integrin polypeptide, in combination with a PTC mutation (R554X), resulted in a mild clinical phenotype, whereas a PTC mutation combined with C245G missense mutation resulted in a lethal condition (Pulkkinen et al. 1998*a,* 1998*b*). In the present study, the homozygous cysteine substitution mutation C562R, as well as the homozygous and compound-heterozygous missense mutations R1281W/R1281W and R1281W/R252C, were found in nonlethal variants of EB-PA. In fact, the skin involvement in some of these patients was so mild that blistering was not noted until the age of ~2 years, as in case 1. This raises the intriguing possibility that some patients with PA but without evidence of skin blistering may harbor mutations in the $\alpha 6\beta 4$ integrin genes. The patient with the homozygous missense mutation C61Y died at age <2 wk, because of extensive skin involvement as well as complications of PA that was not operated on. Thus, it remains unclear whether this homozygous mutation would have ultimately resulted in a lethal or nonlethal condition if the pyloric abnormalities would have been corrected.

The expression of $\alpha 6\beta 4$ integrin was studied by immunohistochemistry in the skin of three patients (cases 1, 2, and 5) with lethal and nonlethal phenotypes, respectively. Interestingly, this work reports, for the first time, clearly positive, although markedly reduced, expression of the $\alpha 6$ and the $\beta 4$ integrin epitopes in a patient (case 2) with a lethal phenotype and PTC mutations in both alleles. Triton X-100 extraction removed most of the immunoreactive material, suggesting an altered structure of the $\beta 4$ polypeptide containing the epitope. In this patient, the mutation R738X resides within the intracellular domain just adjacent to the transmembrane segment and is predicted, therefore, to result in deletion of the entire intracellular domain of the $\beta 4$ integrin polypeptide. In vitro transfection studies have shown that tailless $\beta 4$ integrin affects hemidesmosome assembly but that ligand binding is preserved (Spinardi et al. 1995). The construct used in the latter studies was 137 amino acids longer than the putative polypeptide corresponding to the allele containing the R738X mutation. The other mutation, 4791delCA, is predicted to delete the region spanning the last 278 amino acids in the intracellular domain, which has been shown to interact with the 180-kD bullous pemphigoid antigen (Aho and Uitto 1998). Thus, the extracellular domain is retained in both alleles, suggesting the possibility of $\alpha 6\beta 4$

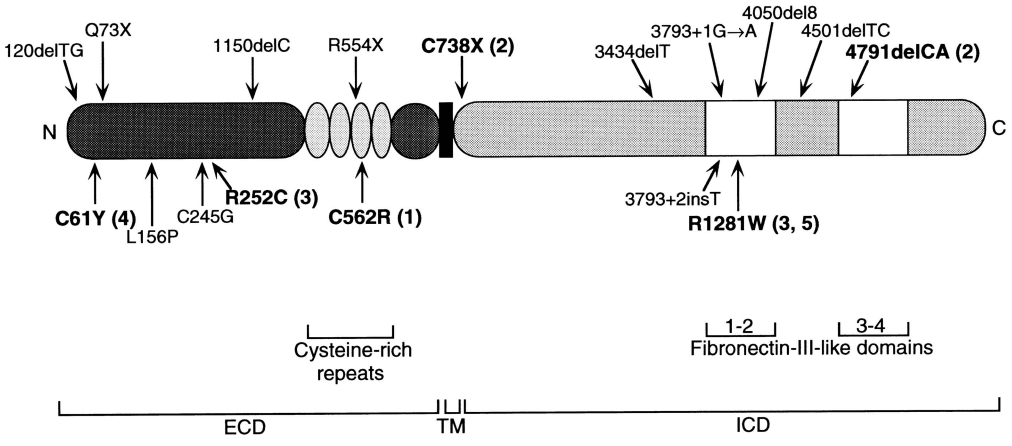

Figure 6 Schematic illustration of $\beta 4$ integrin polypeptide and locations of all ITGB4 mutations disclosed thus far. The PTC mutations are drawn above the molecule, and the missense mutations or a splicing mutation resulting in in-frame deletion are shown below the molecule. ECD = extracellular domain; TM = transmembrane domain; ICD = intracellular domain.

dimer formation and a positive signal on indirect immunofluorescence. It should be noted, however, that the expression of the truncated polypeptide may be significantly reduced because of nonsense-mediated decay of the corresponding mRNA transcripts (Cui et al. 1995). In nonlethal cases with homozygous C562R and R1281W mutations, the $\alpha 6\beta 4$ expression was clearly detectable yet attenuated. A similar situation has been described elsewhere, in a mild EB-PA case who was a compound heterozygote for a PTC mutation and a missense mutation (R554X/L156P). In contrast, the expression of $\alpha 6\beta 4$ was absent in the skin of another patient with compound heterozygosity for missense and PTC mutations (120delTG/C245G) resulting in a lethal phenotype. Thus, it appears that the type and location of the missense mutations influence the expression levels of the $\alpha 6\beta 4$ integrin as well as the clinical phenotype. It should be noted that two homozygous missense mutations (C562R and C61Y) disclosed in this study both affect cysteine residues within the extracellular domain of the $\beta 4$ integrin polypeptide (see fig. 6). It is expected that loss of these cysteine residues could potentially interfere with the formation of intra- or interchain disulfide bonds, with subsequent changes in the conformation and/or ligand-binding affinity of this protein. At the same time, one of the missense mutations, R252C, created a new cysteine residue, which may result in mispairing of the cysteines in the extracellular domain.

The intracellular domain of the $\beta 4$ integrin has been shown to interact with other hemidesmosomal components of basal keratinocytes, including plectin and the 180-kD bullous pemphigoid antigen (Borradori et al. 1997; Niessen et al. 1997; Sanchez-Aparicio et al. 1997; Aho and Uitto 1998). It is conceivable, therefore, that missense mutations such as R1281W, shown to be present in the probands of families 3 and 5, could alter such binding. In case of the proband of family 3, both mutations were missense mutations, one affecting the intracellular and the other one affecting the extracellular domain of the $\beta 4$ integrin (see fig. 6). Interestingly, in this case the skin phenotype was particularly severe at the early stages of postnatal life, but significant improvement during the subsequent months was noted. In comparison, in the proband of family 5, who was homozygous for the R1281W mutation, the skin problems started at age 3 mo, together with nephrotic syndrome. Since renal involvement is not a consistent clinical feature in EB-PA, the relationship between the nephrosis and the $\beta 4$ integrin mutation is not clear, especially since $\beta 4$ integrin has been reported not to be expressed in the kidney (van Leusden et al. 1997).

In case of family 2, DNA-based prenatal testing was performed by amniocentesis at the 15th wk of gestation. The results indicated that the fetus was a heterozygous carrier of the paternal mutation, and a phenotypically unaffected child was predicted. This prediction was fulfilled by the birth of a healthy child. This case attests to the feasibility of DNA-based prenatal testing in severe forms of EB, including the EB-PA variant. It should be noted that such prenatal testing can be performed as early as the 10th wk of gestation, with a chorionic villus sample (Christiano et al. 1996, 1997).

In summary, we have identified a total of six novel mutations in all 10 ITGB4 alleles of five patients affected with EB-PA. The results indicate that missense mutations in either the homozygous or the heterozygous state, in addition to PTC mutations that previously have been demonstrated in both alleles of the ITGA6 and ITGB4 genes, can lead to EB-PA. The involvement of two organ systems—namely, the skin and the gastrointestinal tract—can be explained by the fact that the $\alpha 6\beta 4$ integrin is developmentally expressed in both of these tissues and apparently plays a critical role in providing stability to the association of the epithelial layers of the underlying mesenchyme (Stepp et al. 1990; Sonnenberg et al. 1991; Thorsteinsdóttir et al. 1995). This conclusion is supported by the development of transgenic mice with targeted ablation of either the $\alpha 6$ or the $\beta 4$ integrin gene, which results in both marked fragility of the skin and congenital intestinal abnormalities reminiscent of those noted in patients with EB-PA (Dowling et al. 1996; Georges-Labouesse et al. 1996; van der Neut et al. 1996).

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Electronic-Database Information

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim (for EB-PA [MIM 226730])

References

Aho S, Uitto J (1998) Direct interaction between the intracellular domains of bullous pemphigoid antigen 2 (BP180) and $\beta 4$ integrin, hemidesmosomal components of basal keratinocytes. Biochem Biophys Res Commun 243:694–699

Bhalerao J, Barnes R, Massa H, Trask B, Leach R, Bowcock A (1997) A radiation hybrid and physical map of human chromosome 17q25. Am J Hum Genet Suppl 61:A232

Borradori L, Koch PJ, Niessen CM, Erkeland S, van Leusden MR, Sonnenberg A (1997) The localization of bullous pem-

phigoid antigen 180 (BP180) in hemidesmosomes is mediated by its cytoplasmic domain and seems to be regulated by the β4 integrin subunit. J Cell Biol 136:1333–1347

Brown TA, Gil SG, Sybert VP, Lestringant GG, Tadini G, Caputo R, Carter WG (1996) Defective integrin α6β4 expression in the skin of patients with junctional epidermolysis bullosa and pyloric atresia. J Invest Dermatol 107:384–391

Christiano AM, LaForgia S, Paller AS, McGuire J, Shimizu H, Uitto J (1996) Prenatal diagnosis for recessive dystrophic epidermolysis bullosa in ten families by mutation and haplotype analysis in the type VII collagen gene (COL7A1). Mol Med 2:59–76

Christiano AM, Pulkkinen L, McGrath JA, Uitto J (1997) Mutation-based prenatal diagnosis of Herlitz junctional epidermolysis bullosa. Prenat Diagn 17:343–354

Cui Y, Hagan KW, Zhang S, Peltz SW (1995) Identification and characterization of genes that are required for the accelerated degradation of mRNAs containing a premature translational termination codon. Genes Dev 9:423–436

Dowling J, Yu Q-C, Fuchs E (1996) β4 integrin is required for hemidesmosome formation, cell adhesion, and cell survival. J Cell Biol 134:559–572

Fine J-D, Bauer EA, Briggaman RA, Carter DM, Eady RAJ, Esterly NB, Holbrook KA, et al (1991) Revised clinical and laboratory criteria for subtypes of inherited epidermolysis bullosa: a consensus report by the Subcommittee on Diagnosis and Classification of the National Epidermolysis Bullosa Registry. J Am Acad Dermatol 24:119–135

Ganguly A, Rock MJ, Prockop DJ (1993) Conformation-sensitive gel electrophoresis for rapid detection of single-base differences in double-stranded PCR products and DNA fragments: evidence for solvent-induced bends in DNA heteroduplexes. Proc Natl Acad Sci USA 90:10325–10329

Georges-Labouesse E, Messaddeq N, Yehia G, Cadabert L, Dierich A, Le Meur M (1996) Absence of integrin α6 leads to epidermolysis bullosa and neonatal death in mice. Nat Genet 13:370–373

Gil SG, Brown TA, Ryan MC, Carter WG (1994) Junctional epidermolysis bullosa: defects in the expression of epiligrin/nicein/kalinin and integrin beta 4 that inhibit hemidesmosome formation. J Invest Dermatol Suppl 103:31S–38S

Lestringant GG, Akel SR, Qayed KI (1992) The pyloric atresia-junctional epidermolysis bullosa syndrome: report of a case and review of the literature. Arch Dermatol 128:1083–1086

Nazzaro V, Nicolini U, De LL, Berti E, Caputo R (1990) Prenatal diagnosis of junctional epidermolysis bullosa associated with pyloric atresia. J Med Genet 27:244–248

Niessen CM, Hulsman EHM, Oomen LCJM, Kuikman I, Sonnenberg A (1997) A minimal region on the integrin β4 subunit that is critical to its localization in hemidesmosomes regulates the distribution of HD1/plectin in COS-7 cells. J Cell Sci 110:1705–1716

Niessen CM, van der Raaij-Helmer LMH, Hulsman EHM, van der Neut R, Jonkman MF, Sonnenberg A (1996) Deficiency of the integrin β4 subunit in junctional epidermolysis bullosa with pyloric atresia: consequences for hemidesmosome formation and adhesion properties. J Cell Sci 109:1695–1706

Pulkkinen L, Bruckner-Tuderman L, August C, Uitto J (1998*a*) Compound heterozygosity for missense (L156P) and nonsense (R554X) mutations in the β4 integrin gene (ITGB4) underlies mild, nonlethal phenotype of epidermolysis bullosa with pyloric atresia. Am J Pathol 152:935–941

Pulkkinen L, Kim D-U, Uitto J (1998*b*) Epidermolysis bullosa with pyloric atresia: novel mutations in the β4 integrin gene (ITGB4). Am J Pathol 152:157–166

Pulkkinen L, Kimonis VE, Xu Y, Spanou EN, McLean WHI, Uitto J (1997*a*) Homozygous α6 integrin mutation in junctional epidermolysis bullosa with congenital duodenal atresia. Hum Mol Genet 6:669–674

Pulkkinen L, Kurtz K, Xu Y, Bruckner-Tuderman L, Uitto J (1997*b*) Genomic organization of the β4 integrin gene (ITGB4): a homozygous splice-site mutation in a patient with junctional epidermolysis bullosa associated with pyloric atresia. Lab Invest 76:823–833

Pulkkinen L, Uitto J (1998) Hemidesmosomal variants of epidermolysis bullosa: mutations in the α6β4 integrin and the 180-kD bullous pemphigoid antigen/type XVII collagen genes. Exp Dermatol 7:46–64

Ruzzi L, Gagnoux-Palacios L, Pinola M, Belli S, Meneguzzi G, D'Alessio M, Zambruno G (1997) A homozygous mutation in the integrin α6 gene in junctional epidermolysis bullosa with pyloric atresia. J Clin Invest 99:2826–2831

Sánchez-Aparicio P, Martínez de Velasco AM, Niessen CM, Borradori L, Kuikman I, Hulsman EHM, Fässler R, et al (1997) The subcellular distribution of the high molecular mass protein, HD1, is determined by the cytoplasmic domain of the integrin β4 subunit. J Cell Sci 110:169–178

Shimizu H, Suzumori K, Hatta N, Nishikawa T (1996) Absence of detectable α6 integrin in pyloric atresia-junctional epidermolysis bullosa syndrome. Arch Dermatol 132:919–925

Sonnenberg A, Calafat J, Janssen H, Daams H, van der Raaij-Helmer LM, Falcioni R, Kennel SJ, et al (1991) Integrin alpha 6/beta 4 complex is located in hemidesmosomes, suggesting a major role in epidermal cell-basement membrane adhesion. J Cell Biol 113:907–917

Spinardi L, Einheber S, Cullen T, Milner TA, Giancotti FG (1995) A recombinant tail-less integrin β4 subunit disrupts hemidesmosomes, but does not suppress α6β4-mediated cell adhesion to laminins. J Cell Biol 129:473–487

Stepp MA, Spurr-Michaud S, Tisdale A, Elwell J, Gipson IK (1990) α6β4 integrin heterodimer is a component of hemidesmosomes. Proc Natl Acad Sci USA 87:8970–8974

Takizawa Y, Shimizu H, Nishikawa T, Hatta N, Pulkkinen L, Uitto J (1997) Novel ITGB4 mutations in a patient with junctional epidermolysis bullosa-pyloric atresia syndrome and altered basement membrane zone immunofluorescence for the α6β4 integrin. J Invest Dermatol 108:943–946

Thorsteinsdóttir S, Roelen BAF, Freund E, Gaspar AC, Sonnenberg A, Mummery CL (1995) Expression patterns of laminin receptor splice variants α6Aβ1 and α6Bβ1 suggest different roles in mouse development. Dev Dyn 204:240–258

Uitto J, Pulkkinen L, McLean WHI (1997) Epidermolysis bullosa: a spectrum of clinical phenotypes explained by molecular heterogeneity. Mol Med Today 3:457–465

van der Neut R, Krimpenfort P, Calafat J, Niessen CM, Sonnenberg A (1996) Epithelial detachment due to absence of hemidesmosomes in integrin β4 null allele. Nat Genet 13:366–369

van Leusden MR, Kuikman I, Sonnenberg A (1997) The

unique cytoplasmic domain of the human integrin variant β4E is produced by partial retention of intronic sequences. Biochem Biophys Res Commun 235:826–830

Vidal F, Aberdam D, Miquel C, Christiano AM, Pulkkinen L, Uitto J, Ortonne J-P, et al (1995) Mutations in the gene for the integrin β4 subunit are associated with junctional epidermolysis bullosa with pyloric atresia. Nat Genet 10: 229–234