A Novel Homozygous Mutation Affecting Integrin α6 in a Case of Junctional Epidermolysis Bullosa with Pyloric Atresia Detected *In Utero* by Ultrasound Examination

To the Editor:

We report a new case of junctional epidermolysis bullosa with pyloric atresia (PA-JEB) in the product of a consanguineous union of healthy parents that had previously generated a clinically normal child. At 24 wk of gestation, an amniocentesis was requested because of polyhydramnios. The karyotype revealed a normal 46XX haplotype, and analysis of the amniotic fluid detected high levels of acetylcholinesterase. A week later, ultrasound examination of the fetus using an Aloka 2000–5 MHz transducer apparatus confirmed the presence of polyhydramnios with dilated stomach. Careful examination detected abnormal limbs with fisted hands, overlapping fingers, and malposition of the first toe. Ears were hypoplastic, with the helix merged with the temporal muscle, absent lobule and the concha opening obstruded by collapsed epidermis. Facial deformities and extensive desquamation of the integument were observed.

Five skin biopsies were obtained by fetoscopy at 28 wk gestation under local anesthesia and ultrasound control. Electromicroscopic analysis of the skin samples showed detachment of the epithelium, displaying a cleavage plane within the lamina lucida of the basement membrane zone. No hemidesmosomal structure could be detected, which correlated with the extreme fragility of the fetal skin. The pregnancy was terminated. At the delivery the fetus presented with aplasia cutis congenita and almost total detachment of the integument, involving the complete separation of the pilosebaceous units, nails, and nailbeds from the dermis. The oral and nasal mucosa were detached from the mesenchyme, and the vaginal mucosa had prolapsed externally. Pathologic examination confirmed the presence of the pyloric obstruction.

Immunofluorescence analysis of samples of the proband's skin obtained at the delivery showed a complete absence of reactivity to antibodies directed against integrins α6 and β4, and also against the bullous pemphigoid antigen PB230 (**Fig 1*B***, ***D***, ***H***). Labeling of bullous pemphigoid antigen BP180 was strongly reduced and displayed a faint pericellular staining of basal keratinocytes (**Fig 1*F***). Staining of plectin was decreased and irregular (**Fig 1*J***). In view of these results, and considering that PA-JEB has been associated with mutations in the genes for integrin α6 and β4, expression of these integrin subunits was assessed by northern analysis of total RNA purified from secondary cultures of epidermal keratinocytes obtained from the proband. Hybridization with an integrin β4 cDNA probe resulted in a strong signal, whereas with an integrin α6 cDNA probe no hybridization band was detectable (**Fig 2*A***). Expression of the major hemidesmosome components was then examined by western analysis of protein extracts obtained from the proband's cultured keratinocytes. Absence of integrin α6 was confirmed, whereas integrin β4, BP230, plectin, and BP180 were synthesized *in vitro* by the PA-JEB keratinocytes (**Fig 2*B***).

To identify the causative mutation hampering the expression of integrin α6, a search for mutations was performed on the corresponding cDNA obtained by reverse transcriptase-polymerase chain reaction amplifications of total RNA purified from cultured keratinocytes isolated from the proband. Overlapping polymerase chain reaction amplimers spanning the entire open reading frame of integrin α6 cDNA were obtained using eight pairs of primers (Ruzzi *et al*, 1997). Direct sequence analysis of the cDNA segment spanning nucleotides 1064–1815 detected a homozygous C-to-T

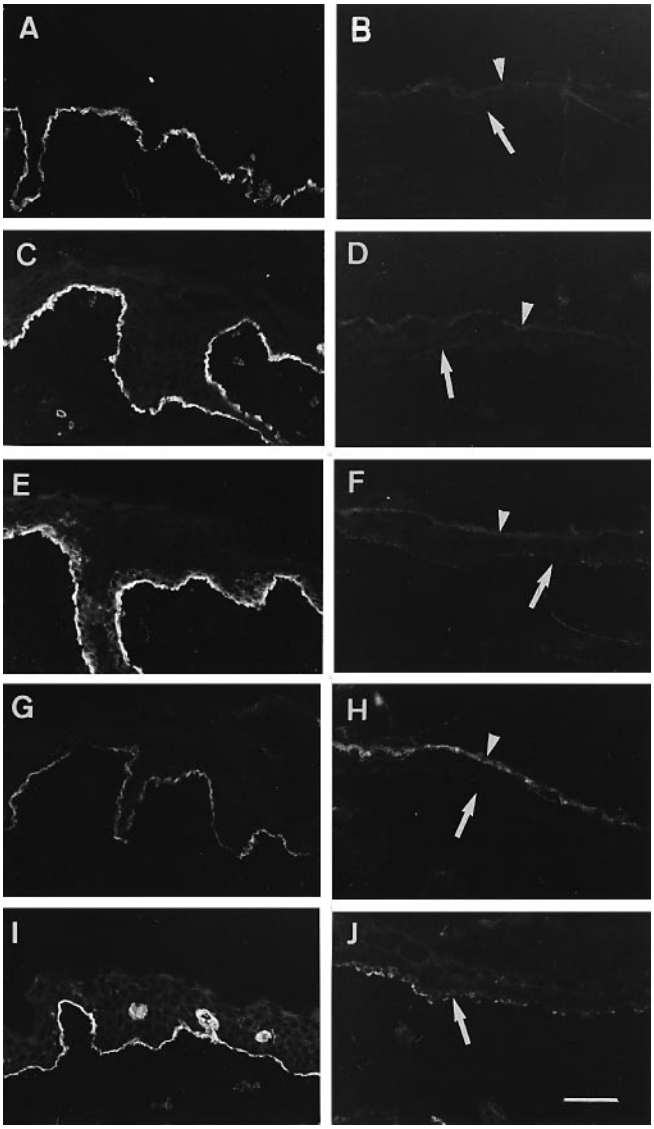

Figure 1. Immunofluorescence analysis of frozen skin biopsies. Cryostat sections of control (*A*, *C*, *E*, *G*, *I*) and proband (*B*, *D*, *F*, *H*, *J*) skin were stained with monoclonal antibodies GoH3 (Sonnenberg *et al*, 1987) (*A*, *B*) and 3E1 (Life Technologies, Cergy Pontoise, France) (*C*, *D*) specific to integrin α6 and β4, respectively, and with monoclonal antibodies 1A8C (Nishizawa *et al*, 1993) (*E*, *F*), FP1 (Tanaka *et al*, 1990) (*G*, *H*) and HD121 (Nishizawa *et al*, 1993) (*I*, *J*) directed against BP180, BP230, and plectin, respectively, as reported (Gagnoux-Palacios *et al*, 1997). The dermal–epidermal junction (*arrows*) and the stratum corneum (*arrowheads*) are indicated. *Scale bar*: 200 μm.

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transversion (CGA to TGA) at position 1764 (Genbank no X53586), resulting in a nonsense mutation (R540X) within the extracellular domain of the polypeptide. Direct nucleotide sequencing of genomic DNA obtained from the nuclear family, confirmed the presence of a homozygous C-to-T substitution in the patient, and disclosed the heterozygous state of the parents (**Fig 2*C***).

A prenatal testing of the fetus at risk for PA-JEB was requested for the third pregnancy of the mother. A search for mutation R540X was performed by polymerase chain reaction amplification of genomic DNA extracted from chorionic villous samples, as described in the legend to **Fig 2**. The mutation was not detected in the fetus, and the prenatal diagnosis was confirmed by the delivery of an unaffected child.

Integrin α6β4 plays a pivotal role in the nucleation of hemidesmosomes, therefore genetic mutations hampering the expression of this adhesion receptor interfere with the proper assembly of these adhesion structures and the stabilization of the dermal–epidermal junction. Two cases of PA-JEB caused by genomic mutations leading to premature termination codons in the gene for integrin α6 have thus far been reported (Pulkkinen *et al*, 1997; Ruzzi *et al*, 1997). In the two patients, the genetic defects predict an α6 polypeptide truncated in the extracellular domain. Immunohistologic examination of the skin of one of these patients detected a normal labeling pattern for BP180 and BP230, but ultrastructurally, the hemidesmosomes were rudimentary with a reduced or absent inner plaque and subbasal dense plate (Ruzzi *et al*, 1997). In contrast, in the PA-JEB patient presented in this study, the staining pattern of all hemidesmosomal components was altered. This may indicate an abnormal structuration of hemidesmosomes that would well correlate with the extreme fragility of the integument detected *in utero* by ultrasound examination of the fetus. Because ultrastructural studies of fetal skin have revealed that morphogenesis of the hemidesmosomes proceeds rapidly between 9 and 15 wk of gestation (McMillan and Eady, 1996), it remains unclear why, at the molecular level, the α6-defective fetus is distinct from the two α6-defective PA-JEB newborns described so far. Comparative analysis of fetal and newborn keratinocytes obtained from cases of lethal JEB is in progress and it may help to elucidate possible subtle mechanisms involved in the structuration of the hemidesmosomes during the different stages of development.

This study increases the repertory of mutations affecting integrin α6 in PA-JEB, and confirms that sonographic observations may provide clues to prenatal diagnosis of the disease.

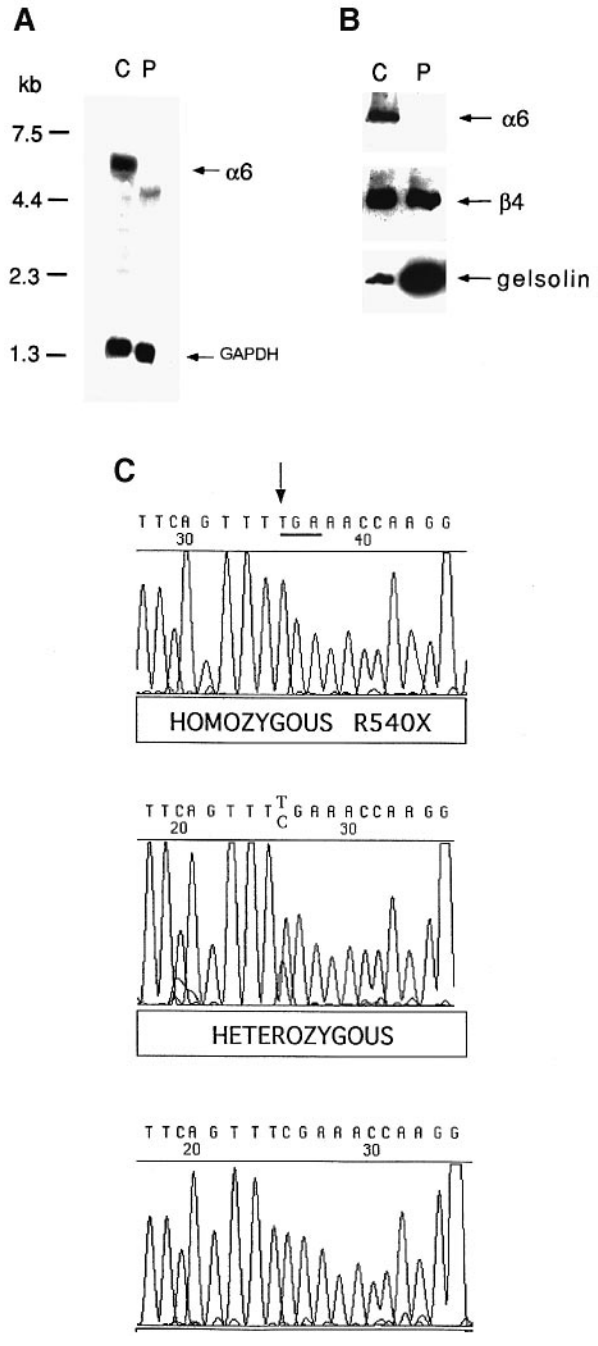

Figure 2. Identification of the genetic defect associated with this case of PA-JEB. (*A*) Northern analysis of total RNA. 30 μg of RNA was electrophoresed in a 1% agarose-formaldehyde denaturing gel, transferred onto a nitrocellulose membrane and hybridized with integrin α6 and GAPDH specific P^{32}-labeled cDNA probes. The α6 integrin mRNA signal is absent in PA-JEB keratinocytes. The faint 4.4 kb band visible in *lane P* is not specific and results from the overexposition of the northern blot. (*B*) Western analysis of cell extracts. Thirty micrograms of total proteins was fractionated on a 10% sodium dodecyl sulfate-polyacrylamide gel in nonreducing conditions, transferred onto a nitrocellulose filter, and reacted with monoclonal antibody 1A10, specific to integrin α6, or with a polyclonal antibody specific to integrin β4. An anti-gelsolin antibody was used as an internal control. Note the absent synthesis of the α6 polypeptide in proband cells. (*C*) Identification of tmutation R540X in the PA-JEB kindred: polymerase chain reaction amplification and direct sequencing of the proband's genomic DNA identified a homozygous C to T substitution in the patient (upper), and demonstrated heterozygosity of the parents for the mutation (middle) when compared with the normal sequence (lower). To detect the mutation at the genomic level, a DNA fragment was polymerase chain reaction-amplified using primers (L) 5′-CACTTGAAGC-TGAAAAAGAAAG-3′ and (R) 5′-GCCTCTTCAGAGTTAGTTC-3′ and, as a template, genomic DNA (100 ng) obtained from the proband and the proband's parents. The polymerase chain reaction conditions were: 94°C for 5 min, 94°C for 30 s; 54°C for 45 s; 72°C for 10 s (30 cycles). The amplified products were submitted to direct sequence analysis.

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