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# Homozygous α6 integrin mutation in junctional epidermolysis bullosa with congenital duodenal atresia

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**Junctional epidermolysis bullosa with congenital pyloric or duodenal atresia is a distinct variant within this group of autosomal recessive blistering skin diseases. In this study we demonstrate, for the first time, a homozygous mutation in the α6 integrin gene (ITGA6) in a family with three affected individuals. For this purpose, we first determined the genomic organization of ITGA6, and placed the gene on chromosome 2q by high resolution radiation hybrid mapping. Heteroduplex analysis of PCR products containing the individual exons of ITGA6, followed by direct nucleotide sequencing, revealed that the proband was homozygous for a G-to-T transversion in the +1 position of intron 12. This mutation, 1856+1G→T, affects an invariant base of the 5′ donor splice site predicting aberrant splicing involving exon 12. The mutation was verified in the proband's DNA by restriction enzyme digestion which also confirmed that the parents were heterozygous carriers of this mutation. Altered expression of α6 integrin, which forms a heterodimer with the β4 subunit at the dermal–epidermal junction, would explain fragility and blistering as a result of minor trauma to the skin.**

## INTRODUCTION

Junctional epidermolysis bullosa (JEB) is a heterogeneous group of autosomal recessive skin diseases characterized by fragility of the dermal–epidermal junction (1,2). The ultrastructural hallmark of JEB is abnormality of the hemidesmosome-anchoring filament attachment complex at the cutaneous basement membrane zone (BMZ), and tissue separation occurs within the lamina lucida at the level of anchoring filaments or at the lamina lucida/basal keratinocyte interphase at the level of hemidesmosomes (3). The hemidesmosomes consist of at least four protein components, the α6β4 integrin, the 230 kDa and the 180 kDa bullous pemphigoid antigens, as well as plectin/HD1, while the anchoring filaments consist predominantly, if not exclusively, of laminin 5 (4,5). The polypeptide subunits of the hemidesmosomes and anchoring filaments are encoded by eight distinct genes, each of which could potentially serve as a candidate gene for the junctional forms of EB.

Previously, a number of mutations in the classic lethal (Herlitz) type of JEB have been demonstrated in the three genes, LAMA3, LAMB3 and LAMC2, encoding the subunit polypeptides of laminin 5 (6). In non-lethal variants of JEB, specific mutations have been found, in addition to laminin 5 genes, also in the genes encoding the hemidesmosomal proteins. Specifically, in a variant known as generalized atrophic benign EB (GABEB), mutations reside in the gene encoding the 180 kDa bullous pemphigoid antigen/type XVII collagen (7,8), while in a form associated with late-onset muscular dystrophy mutations reside in the plectin/HD1 gene (9–12). Furthermore, in one case with JEB associated with pyloric atresia, compound heterozygosity for a paternal 1 bp deletion leading to premature termination codon and a maternal in-frame exon skipping mutation in the β4 integrin gene has been reported (13). In this study, we describe the first mutation in the gene encoding the α6 integrin subunit, which forms a heterodimer with the β4 subunit, in a family with a variant of JEB associated with congenital duodenal atresia.

## RESULTS

### Intron–exon organization and fine mapping of ITGA6

The complete genomic organization of the human ITGA6 gene was determined by PCR, using primers derived from the cDNA sequence (GenBank no. X53586; 14). The gene consists of 27 exons, one of which (exon 5B) is an additional exon present only in certain tissue-specific alternate transcripts (Fig. 1a).

A human-specific PCR marker for the ITGA6 gene was developed for radiation hybrid mapping using the Genebridge4 panel. Using the RHMapper program, the resultant data vector placed the gene 12.33 cR from framework marker WI-3728 on

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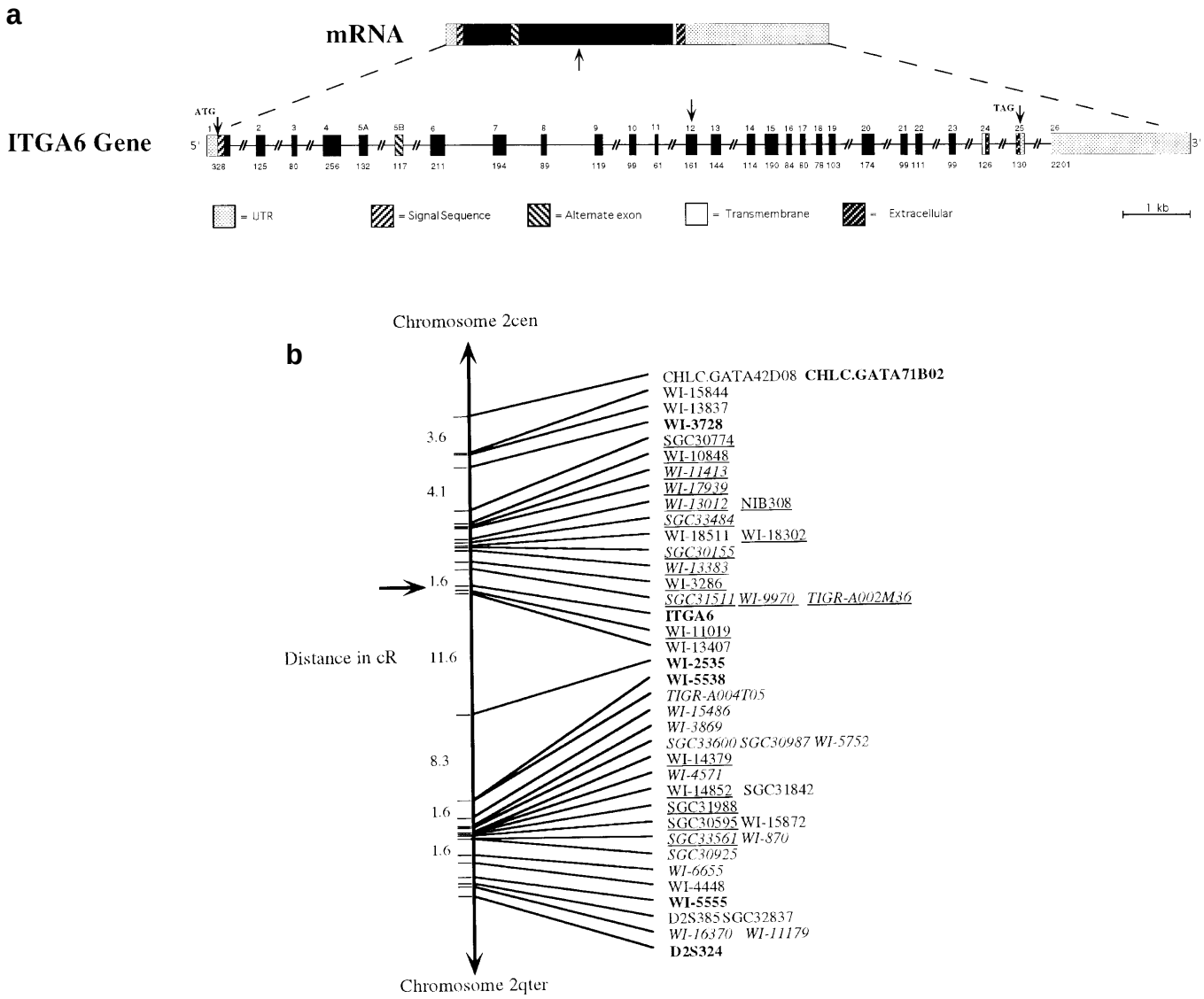


**Figure 1.** (**a**) Genomic organization of the human ITGA6 gene and structure of the corresponding mRNA. The exons (vertical blocks), numbered beginning at the 5′ end of the gene, and the introns without interruption (—) are drawn to scale. The sizes of the exons (bp) are indicated below each exon. The introns with interruptions (/ /) have not been sequenced in their entirety or are >1 kb in size. Exon 12 involving the splice site mutation and the putative site of the protein truncation within the ITGA6 mRNA are marked by arrows. The mRNA encodes a signal sequence, an extracellular domain, a transmembrane segment, and an intracellular portion, as indicated. Exon 5a, which is alternatively spliced (28), is also indicated. (**b**) High resolution radiation hybrid map of the region flanking the ITGA6 locus on 2q. Distances are in centiRads (cR), and framework markers are indicated in bold. The gene is flanked by polymorphic markers CHLC.GATA71B02 and *D2S324*.

chromosome 2, in the interval bounded by polymorphic markers CHLC.GATA71B02 and *D2S324*, as shown in Figure 1b.

## Detection and verification of the mutation

Cloning of the integrin α6 and β4 genes, ITGA6 (see above) and ITGB4 (Pulkkinen *et al.*, manuscript in preparation), respectively has allowed us to develop a mutation detection strategy using genomic DNA as template for PCR amplification of exons, followed by heteroduplex analysis and direct nucleotide sequencing (7,8,15). In this study, DNA isolated from the peripheral blood lymphocytes of the parents, or from a paraffin tissue block from the proband representing a family with three affected individuals (Fig. 2 and Fig. 3). DNA was first used to amplify all 39 exons of ITGB4 encoding the entire β4 integrin polypeptide (GenBank no. X52186; 16), followed by amplification of the ITGA6 gene corresponding to the entire coding sequence of cDNA (GenBank no. X53586; 14,17). PCR amplification of individual exons of both genes was performed, and the DNA products were subjected to heteroduplex analysis by conformation-sensitive gel electrophoresis (CSGE) (18). No evidence for sequence variation within the ITGB4 gene was found (results not shown). However, examination of exon 12 and flanking intronic sequences of the ITGA6 gene (see Fig. 1a), corresponding to the cDNA bases 1696–1856, revealed a heteroduplex band in both parents, while the proband's DNA showed a homoduplex band only (Fig. 4A). Direct sequencing of the parents' DNA revealed that both were heterozygous for a G-to-T transversion in the first nucleotide of intron 12 downstream from exon 12, and sequencing of the proband's DNA revealed that he was homozygous for

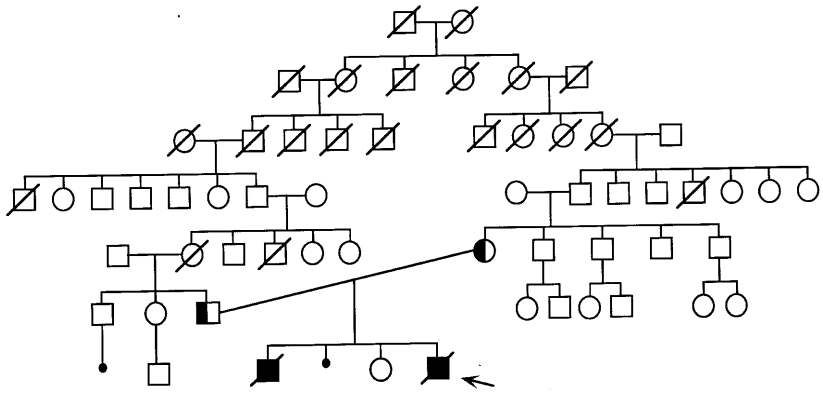

**Figure 2.** Extended pedigree of the proband (arrow) in a family with junctional epidermolysis bullosa and duodenal atresia.

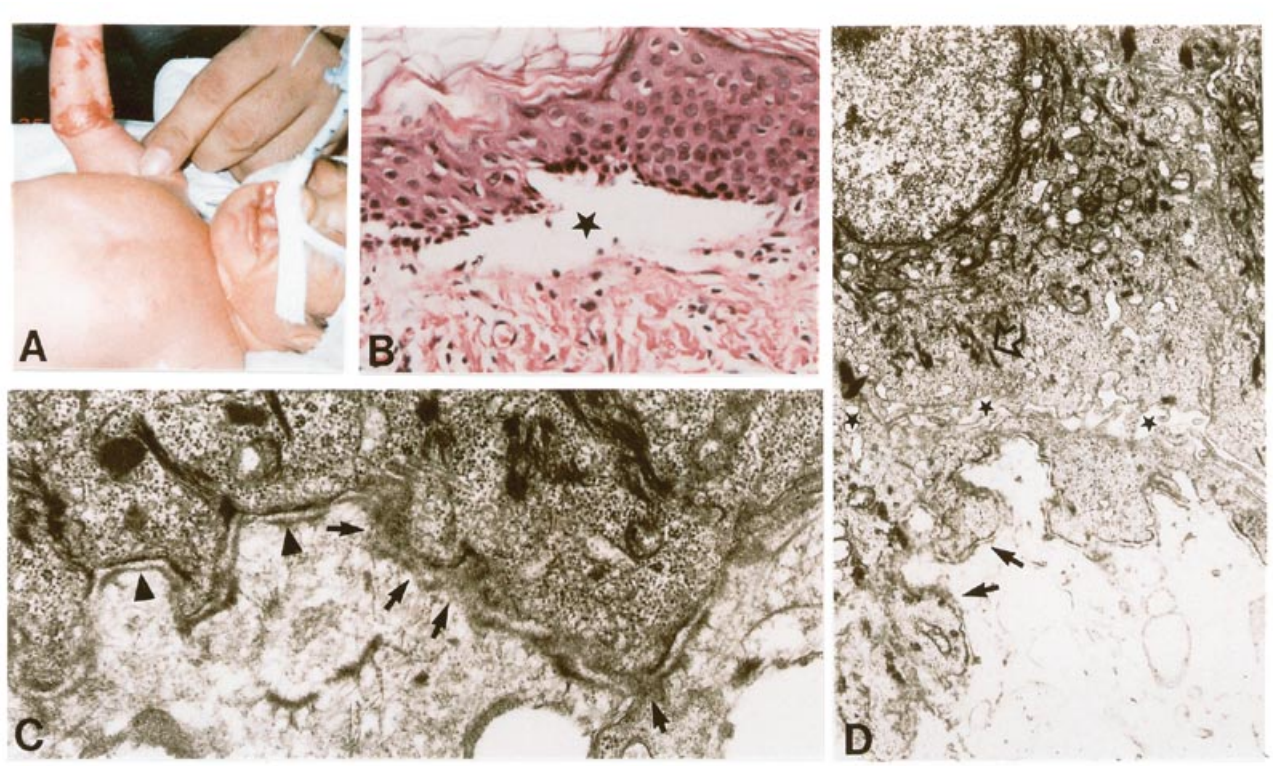


**Figure 3.** Clinical features, histopathology and transmission electron microscopy of the proband. (**A**) Note extensive blistering on the right arm of the proband who also had bilateral cleft lip and cleft palate. (**B**) Light microscopy revealed non-inflammatory tissue separation at the dermal–epidermal junction (H & E, ×40). (**C**) Transmission electron microscopy of the dermal–epidermal junction revealed the presence of hemidesmosomes lacking the inner plaque (arrowheads), and segments of the basement membrane zone which were morphologically perturbed (arrows). (**D**) Intermediate filaments were severed from the lower portion of the basal keratinocytes and condensed perinuclearly (open arrow). There was vacuolization within the lower portion of the keratinocytes (*) and local herniation of the basal cells into the upper papillary dermis (arrows).

this nucleotide substitution (Fig. 4B). The mutation abolished a recognition site for *Hph*I restriction endonuclease which was used for verification of the mutation in the family members (Fig. 4C). The mutation, 1856+1G→T, affects an invariant base of the 5′ donor splice site (EXON-gt) predicting aberrant splicing involving exon 12 (19). If the aberrant splicing results in the skipping of the entire exon, which consists of 161 bp, this would be out-of-frame potentially resulting in a frameshift and premature termination codon 3 bp downstream from the site of the mutation and predicting a protein truncated within the extracellular domain (Fig. 3A). No RNA was available from the proband or his parents to test this possibility, however, screening of 124 unrelated control individuals using *Hph*I restriction enzyme failed to reveal the presence of this nucleotide substitution, suggesting that it is pathogenetic in this family.

## DISCUSSION

In this study, we have disclosed a novel mutation in the ITGA6 gene encoding the α6 subunit of integrins in a family with JEB and duodenal atresia. This finding further attests to the molecular heterogeneity of JEB (6). Previously, mutations in the gene

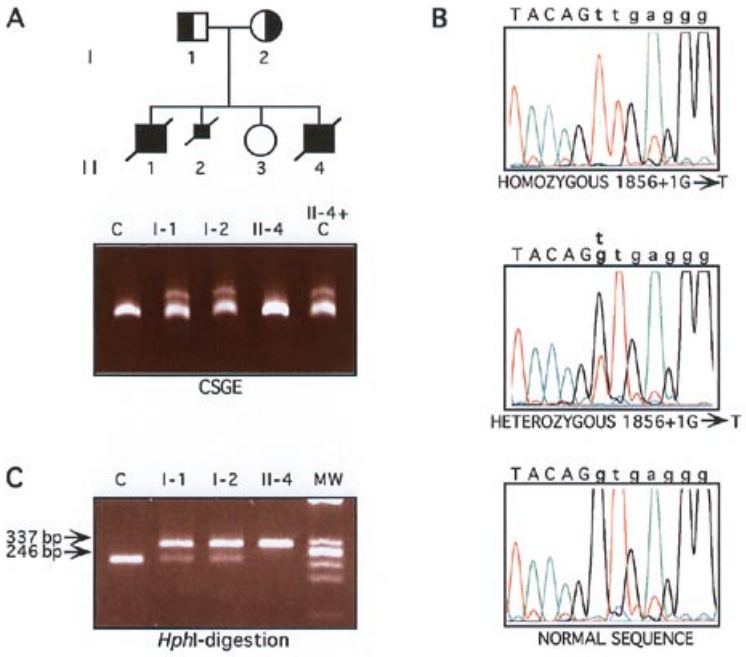


**Figure 4.** Identification and verification of mutations in ITGA6. (**A**) CSGE revealed a heteroduplex band in case of the proband's parents (I-1 and I-2), while the proband (II-4) showed a homoduplex band only, similar to an unrelated healthy control (C). However, when the probands DNA was mixed with control DNA (II-4+C), a heteroduplex band, similar to that seen in the parents, was noted. (**B**) Direct sequencing of the proband's PCR product indicated that he was homozygous for G→T substitution (upper panel) when compared to the normal sequence (lower panel); the parents were heterozygous carriers of this mutation (middle panel). (**C**) The mutation, 1856+1G→T, affects the first nucleotide of the intron 12 downstream from the 161 bp exon 12. This mutation abolished a restriction enzyme site for*Hph*I endonuclease. Digestion of the 337 bp PCR product spanning this exon in control DNA resulted in 246 bp (arrow) and 91 bp (not shown) fragments. In the proband's parents (I-1 and I-2) one allele was not digested, indicating that they were heterozygous carriers of this mutation, while both alleles of the proband (II-4) remained undigested, indicating homozygosity. MW: molecular weight markers 174-*Hae*III.

encoding the β4 integrin subunit, which dimerizes with α6 in the dermal–epidermal junction, have been reported in one patient with JEB with pyloric atresia (13). In the present family, duodenal atresia was found in two previous pregnancies, and the proband also had bilateral cleft palate and cleft lip. The correlation of the latter clinical finding to the α6 gene mutation is unclear. It should be noted, however, that α6 integrin serves as a subunit not only in the α6β4 integrin, but can also combine with the β1 integrin subunit (14,17). Furthermore, the α6 integrin splice variants are expressed in a number of tissues, including various epithelia, as well as the cardiovascular, nervous and nephrogenic organ systems (20,21). It is conceivable that the relatively broad distribution of the α6 integrin and its role during fetal development (22) may explain the widespread clinical manifestations, including duodenal atresia and cleft palate. In support of this interpretation are the phenotypic findings in a genetically engineered mouse line with absence of the α6 integrin gene (23). Specifically, these mice developed extensive blistering of the skin, as well as detachment of epithelia of the tongue, the oral and nasal cavities, and the larynx and esophagus. In this context, it should be noted that ablation of ITGB4 in transgenic mice results in a remarkably similar phenotype (24,25), indicating that both subunits of the α6β4 integrin are required for the function of this hemidesmosomal protein to contribute to stable association of the epidermis to the underlying basement membrane.

In summary, determination of the intron–exon organization of the ITGA6 gene has allowed us to develop a genomic DNA mutation detection strategy covering the entire coding region. We have also mapped ITGA6 on chromosome 2q, allowing identification of flanking microsatellites to enable rapid initial assessment of further JEB families by linkage analysis. Finally, we have disclosed a homozygous ITGA6 mutation in a family with a variant of JEB with duodenal atresia and the proband having cleft palate and cleft lip. Altered expression of the α6 integrin in the skin would explain the extreme fragility and blistering as a result of minor trauma, which caused early demise of the affected infants in this family.

## MATERIALS AND METHODS

### Clinical features of the family

The proband was the product of the fourth pregnancy of clinically unaffected parents who were distant relatives of Greek ethnic origin residing in Cyprus (Fig. 2). The first pregnancy had resulted in a male child who had duodenal atresia which was surgically corrected. On the first post-operative day (the third day of life) the neonate developed blistering which progressed to cover the entire skin, and the patient died at the age of 40 days. The fetus in the second pregnancy was also found to have JEB by fetal skin biopsy, and duodenal atresia was noted on ultrasound; this pregnancy was subsequently terminated. The third pregnancy resulted in a clinically healthy girl. The proband was found by prenatal ultrasound examination to have bilateral cleft lip but no evidence of duodenal atresia was noted. The pregnancy was allowed to continue to term, and a baby boy was delivered with bilateral cleft lip and cleft palate (Fig. 2A). On the third day of life, the child developed extensive blistering (Fig. 3A) and died at the age of 29 days. Histology of the proband's skin revealed blister

formation at the dermal-epidermal junction (Fig. 3B). Transmission electron microscopy of the unaffected perilesional skin revealed abnormalities in hemidesmosomes (Fig. 3C and D). Specifically, although numerous hemidesmosomes were present many of them lacked both inner plaques and sub-basal dense plates (Fig. 3C). In several areas, the BMZ was seen to be highly disorganized and there was apparent herniation of the basal cells into the papillary dermis. Finally, the association of keratin intermediate filaments with hemidesmosomes was severed leading to perinuclear condensation of these filaments within the basal cells with apparent vacuolization of the lower portion of the cells (Fig. 3C and D). These findings, together with the clinical association of JEB with duodenal atresia, were consistent with an α6β4 integrin defect (13,26,27), but unfortunately immunohistochemistry was not performed before the demise of the proband.

### Elucidation of ITGA6 intron–exon borders

To allow amplification of the α6 integrin exonic sequences from genomic DNA, intron–exon organization of the corresponding gene, ITGA6, was first determined. For this purpose, oligonucleotide primers corresponding to the α6 integrin cDNA sequences (GenBank no. X53586) were used as primers for PCR amplification of total genomic DNA. The PCR amplification products were then subjected to direct automated sequencing (ABI PRISM Method). Intron–exon borders at the 5′- and 3′ ends were sequenced directly from the P1 clone (Genome Systems, Inc.). The intron–exon borders were determined by comparison of the genomic sequences with published cDNA (14).

### Radiation hybrid mapping

A 450 bp human sequence-specific fragment of the the ITGA6 gene was amplified using intronic primers α6sts.L (5′ CAA GAG GCT TGT ATG GTA ATG A 3′) and α6sts.R (5′ ATT AGG GAA GTG AAA AGT GTC C 3′) using standard PCR buffer with 4% DMSO. The following PCR program was used: denaturation at 94°C for 5 min; followed by 38 cycles at 94°C 30 s, 56°C 45 s, 72°C 45 s; extension at 72°C for 5 min. This PCR product was used to analyze the Genebridge4 radiation hybrid panel (Genome Systems, Huntsville AL). The resultant data vector (0200000 00000212 11011100 11100000 02100000 01101001 00001000 10000100 20101000 00000100 10100101 02001) was mapped using a lod score of >15 by the RHMapper program, accessed on-line at the Whitehead Institute for Genome Research, Massachusetts Institute of Technology.

### Mutation detection strategy

For identification of mutations in the genes encoding the α6 and β4 integrin subunits, a mutation detection strategy was developed, consisting of direct amplification of the genomic sequences using primers placed on flanking introns. The PCR products were subjected to heteroduplex analysis by CSGE (18), and in case of evidence for a sequence variant, the PCR products were subjected to direct nucleotide sequencing.

### Verification of the mutation

The mutation, 1856+1G→T, abolished the recognition site for *Hph*I restriction endonuclease. To verify the mutation the following primers were used to amplify exon 12 containing the mutation, and flanking intronic sequences, from genomic DNA of the proband, his parents, and 124 unrelated healthy controls:

Sense primer: 5′-AGG AGT TAC AAC ATG CTT GTG G-3′
Antisense primer: 5′-TTG ATT AAG AGG TTG CCA GAA T-3′

The PCR amplification conditions were the same as above, except that an annealing temperature of 55°C was used. The 337 bp PCR product was subjected to *Hph*I digestion according to the manufacturer's recommendation (New England Biolabs) and analyzed on 3% agarose gels.

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