## CLINICAL REPORT

# Life-long Course and Molecular Characterization of the Original Dutch Family with Epidermolysis Bullosa Simplex with Muscular Dystrophy due to a Homozygous Novel Plectin Point Mutation

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**Plectin is one of the largest and most versatile cytolinker proteins known. Cloned and sequenced in 1991, it was later shown to have nonsense mutations in recessive epidermolysis bullosa with muscular dystrophy. A dominant mutation in the gene was found to cause epidermolysis bullosa simplex Ogna without muscular dystrophy. Here we report the DNA sequencing of the plectin gene *(PLEC1)* in a Dutch family originally described in 1972 as having epidermolysis bullosa with muscular dystrophy. The results revealed homozygosity for a new plectin nonsense mutation at position 13187 and its specific 8q24 marker haplotype profile. Western blotting of cultured fibroblasts and immunofluorescence microscopy of skin biopsy confirm that the plectin protein expression is grossly reduced or absent. A summary of the life-long clinical course of the two affected brothers homozygous for the new E1914X mutation is given. *Key words: EBS-MD; genotype-phenotype; nonsense mutation; plakins; plectin; PLEC1.***

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Epidermolysis bullosa simplex with muscular dystrophy (EBS-MD) is a rare recessive disease associated with plectin deficiency (1) and caused by nonsense mutations, small deletions or insertions in the *PLEC1* gene (2 – 14) coding for the protein plectin. Targeted inactivation of the plectin gene in mice caused a phenotype similar to that of human EBS-MD patients (15). Plectin, a member of the plakin family, acts as a versatile cytoskeletal linker protein (16). It is expressed in epidermal cells, muscle cells and various other tissues

(19). Plectin was first cloned and sequenced from rat (18) and later from man (19) and mouse (20). The only dominant mutation described in *PLEC1* has been shown to cause the rare blistering skin condition epidermolysis bullosa simplex Ogna (EBS-O) (21).

The first detailed clinical report on epidermolysis bullosa with muscular dystrophy was published in 1972 in Dutch by the neurologist C. J. de Weerdt and dermatologist S. Castelein, who named it EB dystrophica because of skin sequelae and nail dystrophies (22). Electron microscopy of the skin of the two affected brothers revealed low intraepidermal basal cell blistering and hemidesmosomes with hypoplastic attachment plates and impairment of keratin filament insertion into the inner hemidesmosomal plaque (23). This blister pathogenesis differed from both dystrophic and junctional EB (23 – 26). The group was initially denoted "pseudojunctional" (P-JEB), but has later been renamed EBS-MD (27).

Altogether 15 patients with individual mutations suffering from EBS-MD have been described (2 – 14), all suffering from a generalized blistering skin condition or fragile skin with blistering due to trauma at birth or in infancy, nail dystrophy and a severe muscular dystrophy resembling the limb-girdle type with a variable onset from infancy (delayed walking) to their early 30s.

We report Western blotting of fibroblast cultures, immunofluorescence microscopy of the skin, and molecular findings in the genomic plectin *(PLEC1)* locus and its flanking 8q24-haplotype in the original Dutch EBS-MD family. The life-long clinical course of the two adult patients is also summarized.

## PATIENTS AND METHODS

### Patients

The Dutch family (NEB1) first published in 1972 (22) comprises both lethal and non-lethal cases. The parents did not report consanguinity, but both knew of ancestral lines to rural Western Germany. Later other relatives reported that

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the parents were third cousins, hence the inbreeding coefficient is 0.00391 for the patient generation.

In a sibship of 14, 3 girls and 3 boys had generalized blisters from birth, and 4 of those died in infancy (see Fig. 4 for a partial pedigree). The 3 girls (nos. 3, 6, 8) died within a few weeks because of their skin condition; the boy (no. 12) died at 5 months of age due to pneumonia. He was recalled to have fewer blisters than his affected siblings. Another boy (no. 2) looked normal at birth, but was never able to move out of bed, never learned to walk or talk, had epilepsy and was severely psychomotorically retarded. He died at 10 years of age.

Two boys with EB (nos. 9, 11) grew up and both developed progressive muscular dystrophy from age 15 (no. 11) and age 17 (no. 9), respectively, but none of their 7 healthy living siblings did so. Based on the suspicion that this family did not have dystrophic EB, but rather a non-Herlitz EB junctional type of the disease, their neurologist, Dr. de Weerdt, kindly arranged for two of us (CPdG, TGD) to see the whole family in their home town in North Holland, the Netherlands, in 1982. In addition to a detailed clinical history from the parents and the two patients, blood samples for classical genetic marker testing and skin shave biopsies for fibroblast cultures were taken of all 11 living family members. The parents and the 2 affected boys had normal karyograms (analyses by Anton Brøgger, Oslo, Norway).

The patients were followed up by one of us (C. J. de Weerdt) in their home town and had frequent admissions until their deaths in 1990 (no. 9, 44 years) and 1995 (no. 11, 47 years). A skin biopsy for immunofluorescence study was taken several hours post-mortem from patient no. 11. Autopsies were not done.

*Immunofluorescence study*

Skin samples were processed for immunofluorescence as previously described (28). The primary antibodies raised against human and rat antigens are listed in Table I. In combination with primary mouse IgG we used biotinylated horse anti-mouse IgG (Vector Laboratories, Inc., Burlingame, CA, USA) and FITC (fluorescein isothiocyanate) conjugated neutravidin (Southern Biotechnology Associates (SBA), Inc., Birmingham, AL, USA). For the primary mouse IgM antibody we used FITC conjugated goat anti-mouse IgM (SBA). In combination with primary rat IgG we used FITC-conjugated goat anti-rat IgG (SBA). Digital fluorescence images of tissue sections were obtained with a Leitz Orthoplan microscope equipped with a video imaging

Table I. *Rodent antibodies used for immunofluorescence microscopy of skin (35, 36)*

| Antigen (source) | Antibody | Isotype |
| --- | --- | --- |
| keratin 5 (Hu) | AE14 | IgG1 |
| keratin 14 (Hu) | RCK107 | IgG1 |
| 500-kD HD-1 (Hu) | HD121 | IgG1 |
| plectin (I-2) (Rt) | 6C6 | IgG2 |
| plectin (II) (Rt) | 10F6 | IgG 1 |
| plectin (III-1) (Rt) | 5B3 | IgG 1 |
| plectin (III-1) (Rt) | 5C10 | IgM |
| BP230 (Hu) | R815 | IgG 1 |
| BP180 ic (Hu) | 1A8c | IgG 1 |
| integrin a6 ec (Hu) | GoH3 | IgG 2a |
| integrin b4 ec (Hu) | 439-9B | IgG2 |
| laminin-5 g2 (Hu) | GB3 | IgG1 |

system with exposure time control board designed for the detection of low levels of fluorescence (29).

*Fibroblast cultures*

Fibroblasts stored in liquid nitrogen after establishment in 1982 were recovered and used for DNA extraction by standard protocols from all 11 family members and for plectin protein expression from 2 patients (nos. 9 and 11) and one unaffected sibling (no. 10). Primary fibroblasts derived from a non-EB-MD patient (EB107-1) were used in the preparation of control lysates for Western blotting.

*Western blotting*

Cultured patient and control primary fibroblasts were lysed directly in hot $2 \times$ PAGE sample buffer and run on SDS 3.5% polyacrylamide mini gels. Separated proteins were transferred to PVDF membranes (Millipore, Bedford, MA, USA) and membranes blocked with 3%BSA over night. Immunodetection of plectin on the blots was carried out with mAbs 10F6 and 5B3, followed by goat anti-mouse antibodies conjugated to alkaline phosphatase (Promega, San Luis Obispo, CA, USA). Plectin purified from cultured rat glioma C6 cells was used as size marker.

*Genetic markers and linkage studies*

After plectin was reported in situ assigned to 8q24 (19) we typed the then most 8q telomeric DNA marker, the tetranucleotide repeat D8S373 (23). Recently, we added the following 8q-assigned dinucleotide Genethon STR-markers: D8S274, D8S1717, D8S1836, AFMa128xh5, D8S1925 and D8S2334. The primers were as specified from Genethon and/or the Genome Data Bank (gdbwww.gdb.org) and labelled with FAM or HEX. Fragment sizes were determined on an ABI 377 sequencer. The results were entered in Cyrillic format. Lod scores for linkage analysis were calculated where needed by standard methods.

*DNA sequencing*

Plectin sequences deposited in GenBank (acc. nos. U63610; Z54367; X59601; U53204; and NM_000445) were used to design PCR primers that would yield overlapping 400–600 bp-long DNA fragments covering the sequence from the start of exon 2 to the end of exon 32 (19) (exon 33 in the McLean et al. terminology (3)), and skipping only the longest introns.

All PCR primers were designed as described earlier (21). The PCR products were purified either using the QIA quick PCR Purification Kit (Qiagen) or the PCR product pretreatment kit (USB) and subsequently sequenced using PRISM AmpliTaq FS Dye Primer Cycle Seq. Kit (Perkin Elmer) and run on the ABI 377 automatic sequencer. Sequences were aligned and compared using Sequencher 4.1 from Gene Codes Corporation.

*Mutation detection by restriction enzyme*

The restriction patterns after 4 h (at 37°C) *Hinf*I digestion of exon 31 DNA amplified with the PLE47F/PLE48R primers (PLE47F: AGGACGAGAGCCAGCGTAAG; PLE48R:CCT CTGCTTGGACTTCTC) followed by nested PCR with the PLE47F/R primers (PLE47F: AGGACGAGAGCCAGCGTAAG;

PLE47R:TTCCAGCTCCTGCCGTTTC), were read after a 2 h run at 70V on a 10% PAGE gel.

## RESULTS

### Clinical features and course of the disease

The course of the disease shared the following skin manifestations: Blisters at birth and in 2 of them specifically on the fingers, soon widespread occurrence of new blisters, buttocks invariably affected with sores. Lifting up the baby in the usual way induced blisters on lateral thorax in a pattern showing the finger grips. No blister sequelae, including no miliae, remained upon healing. In childhood, both of them had had attacks of eye irritation. No. 9 had a hoarse voice throughout life. By age 33, no. 11 had lost all his permanent teeth, while his 38-year-old brother, no. 9, had decayed teeth similar to those often seen in severe-dystrophic EB. Both patients had dystrophic nails (Fig. 1), no oral blistering but new blisters at 2–4 week intervals on the peripheral extremities, leaving many spots of pale atrophic skin often surrounded by hyperpigmentation.

No. 11 was deaf in his right ear and had reduced hearing in the left. From age 15 he had experienced slowly increasing muscular weakness. At first he had problems getting out of bed without the help of his arms, and the knees tended to sink together when he tried to get up from a lying position. Muscular biopsy of the m. quadriceps at that time revealed extensive variation of the diameter of muscle fibres – with big rounded fibres with several central nuclei and signs of regeneration.

At 33 years, the muscular atrophy was still most striking at the proximal extremities (limb-girdle-like; Fig. 1), but included facial muscles, ptosis, Bell's phenomenon and bulbar dysarthria. At age 46 he could still walk, but had a typical waddle and drop foot. He died the same year as a result of respiratory insufficiency probably caused by his muscular disease.

The older brother (no. 9) experienced the same muscular dystrophy from age 17. He had recurrent pulmary infections during childhood and died at 44 years of age because of respiratory insufficiency and kidney failure.
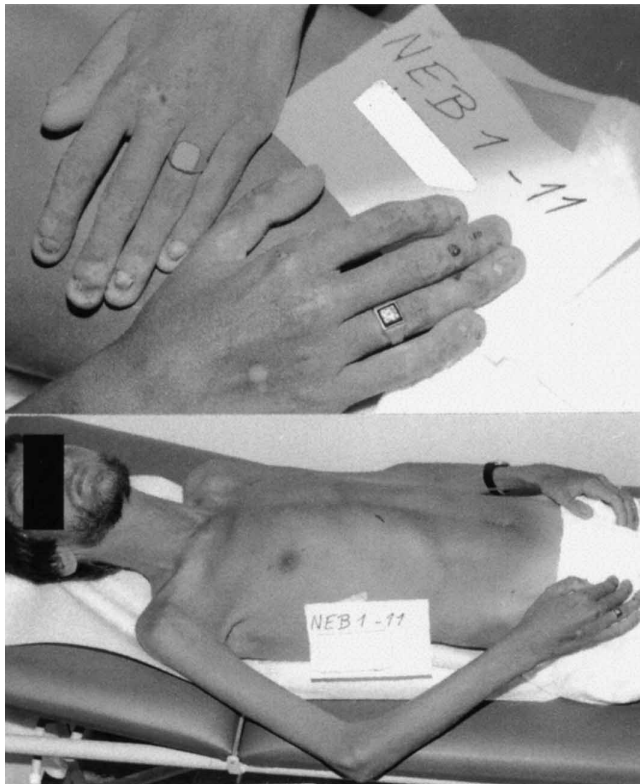


*Fig. 1.* The younger brother (no. 11) with EBS-DM at age 33 years. Note the generalized muscle atrophy with predominantly limb-girdle distribution, faint atrophic skin areas and superficial blood blisters dorsally on the fingers and hands, with variable nail dystrophies.
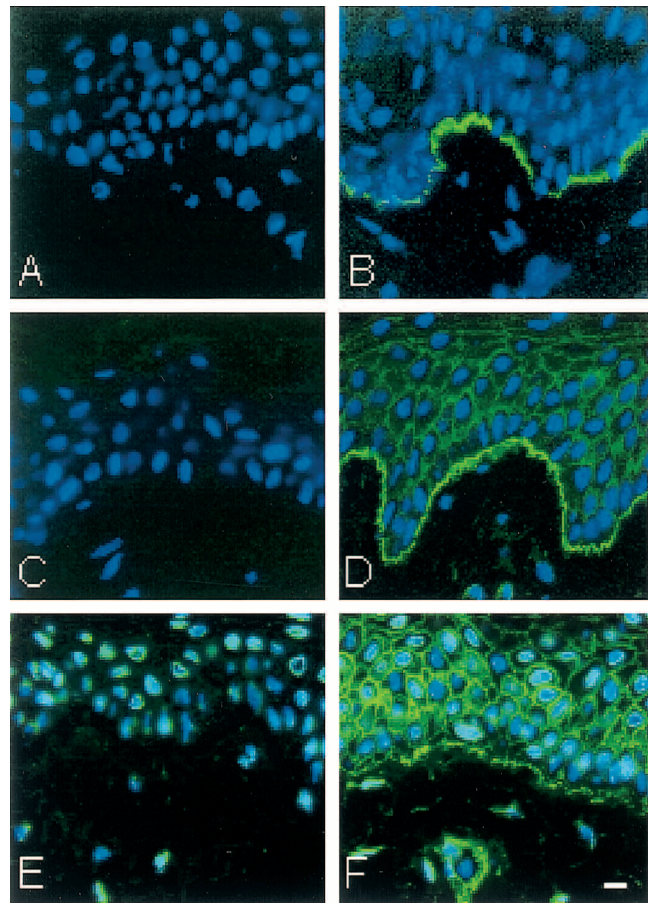


*Fig. 2.* Immunofluorescence microscopy of the skin of no. 11 with recessive epidermolysis bullosa simplex with muscular dystrophy (A, C, E) and of a normal human individual (B, D, F). The nuclei are counterstained in blue with bisbenzimid. Anti-HD1/plectin antibody does not bind to the epidermal basement membrane zone in the patient (A). Anti-plectin antibodies (5B3 and 5C10) also do not bind to this region (C, E). The staining with anti-plectin 5C10 antibody at the cell periphery in the suprabasal layers is also lost, whereas the perinuclear staining in epidermal cells, fibroblasts and endothelial cells remains conserved (E). Bar is 10 μm.

*Absence of plectin in skin and fibroblast cultures*

Immunofluorescence microscopy of the skin: mono-clonal antibody (mAb) HD-121 to 500-kD HD-1/plectin (30) did not bind to the epidermal basement membrane zone (EBMZ) in patient no. 11 (Fig. 2A, B). Also mAbs 10F6, 5B3 (Fig. 2C, D), 6C6 and 5C10 (Fig. 2E, F) against plectin did not bind to the EBMZ, although some epitopes appear to remain in the suprabasal layers (6C6) or perinuclear region (5C10) of the epidermis.

Knowing that the patient's skin lacks plectin, the tissue distribution of other hemidesmosome components becomes important. Basal keratins 5 and 14 were nor-mally present in the basal epidermal cells. The 230-kD and 180-kD bullous pemphigoid antigens, as well as the integrin subunits α6 and β4 and laminin-5, were all unreduced and normally polarized in the EBMZ (data not shown).

Western blotting: When lysates from cultured fibro-blasts from the patients (nos. 9 and 11) were subjected to Western blotting, no protein band at the position of plectin was seen, contrary to lysates from an unaffected sibling (no. 10) and control lysate (Fig. 3).

*Linkage and haplotype studies*

All family members were homozygous 1-1 in glutamate pyruvate transaminase (chromosome 8q24) (Fig. 4). When short tandem repeat markers became available, the two common limb-girdle muscular dystrophy loci (on chromosome 2 and chromosome 15), the two laminin-5 loci tested (on chromosome 1q) and the

collagenase locus (on chromosome 11q) were also excluded as candidate loci.

When plectin became assigned to chromosome 8q24, we first tested D8S373, most telomeric on the tetra-nucleotide marker CHLC (Collaborative Human Link-age Center) map, and confirmed full co-segregation with the EBS-MD disease (23). We have elaborated the extended 8q24 haplotypes, as shown in Fig. 4. During our current plectin DNA sequencing studies we have observed several polymorphisms, including an inser-tion/deletion polymorphism with alleles S and F (to be published). In Fig. 4 one intragenic plectin marker on each side of the disease mutation is flanked by short tandem repeat markers located according to our ongoing 8q24 fine mapping. This reveals an identical long haplotype from both parents strongly suggesting identity in descent and therefore predicting homozyg-osity for an 8q24 disease-causing mutation.

*Mutation detection by gene sequencing and restriction enzyme*

DNAs from two unaffected siblings (nos. 7 and 13) and one patient (no. 11), representing one normal and one disease-carrying chromosome (as shown in the haplotype analysis), together with DNAs from 12 other haplotypes (21), were subjected to systematic sequencing from exon 2 to exon 32 (see Methods). A new plectin nonsense mutation was found using the Ple48 primer (PLE48F AAACGGCAGGAGCTG-GAAG: PLE48R) (Fig. 5). The substitution which was verified homozygous in both patients is a G>T transversion at genomic position 13187 (GenBank
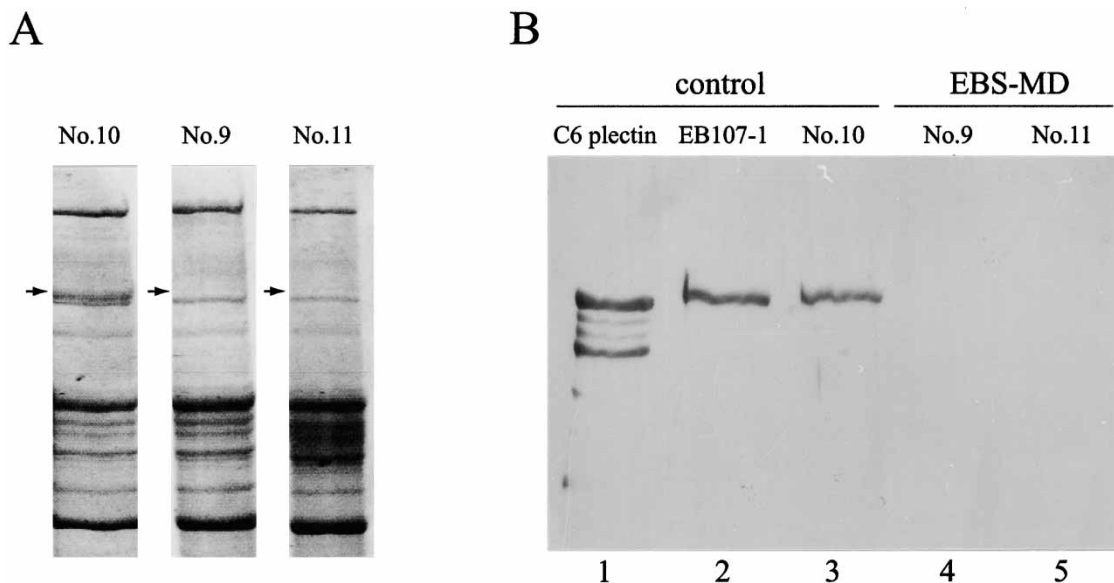


*Fig. 3.* Immunoblotting of fibroblast cell lysates from EBS-MD patients. (A) Coomassie staining of lysates from control (unaffected sibling no. 10) and affected patients nos. 9 and 11. Arrows point at position of plectin band. (B) Anti-plectin immunostaining of Western blots using mAbs 5B3. Lane 1: purified rat glioma C6 plectin; lane 2: EB 107-1 patient (positive control); lane 3: unaffected sibling no. 10; lanes 4 and 5, EBS-MD patient nos. 9 and 11.
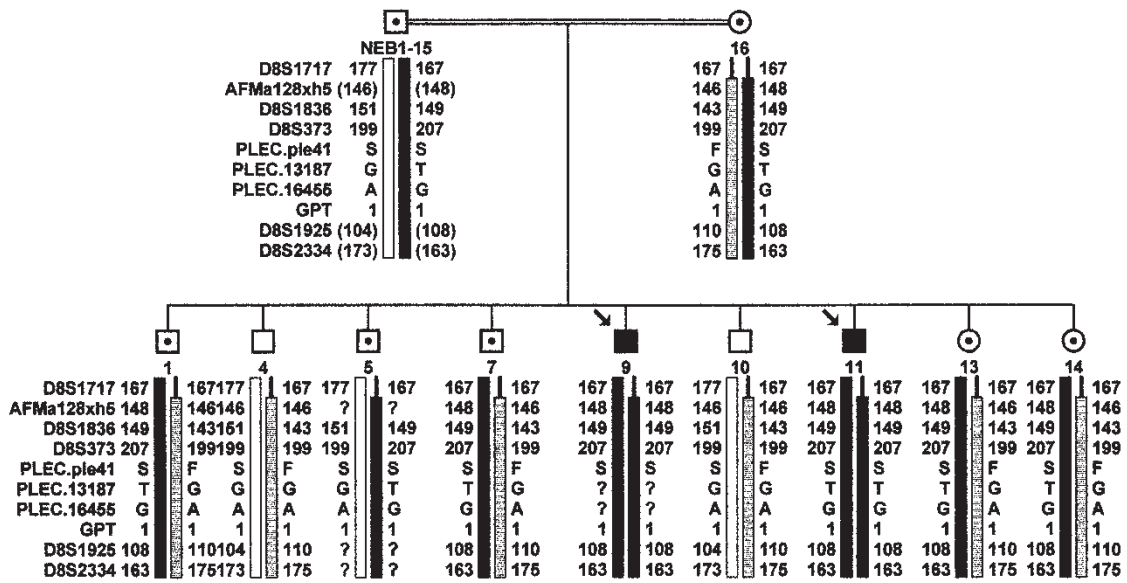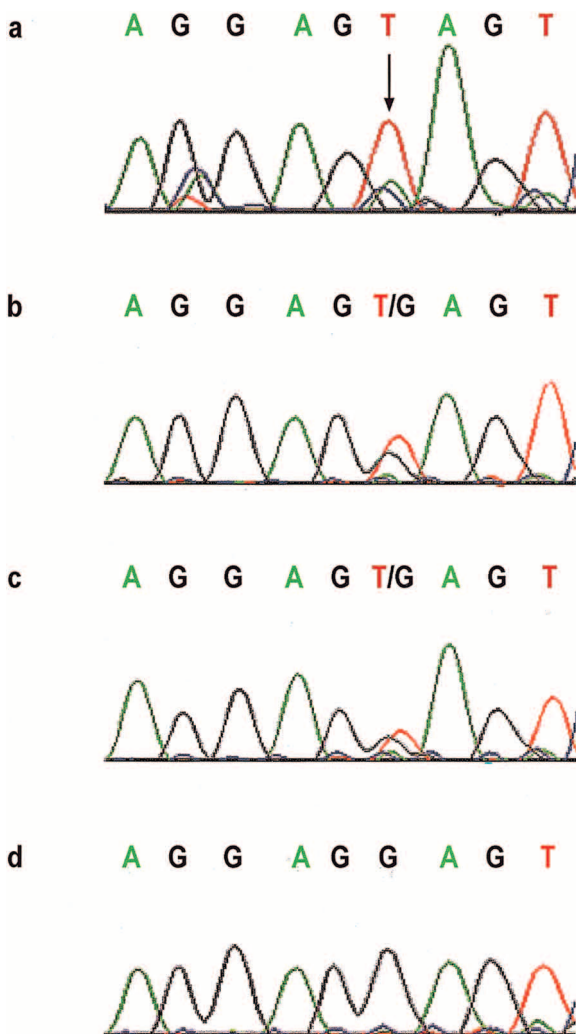
*Fig. 4.* The 8q24 haplotypes in the NEB1 family. The given order is based on our ongoing family and radiation hybrid studies. Note the long identical haplotype shared by both parents and carrying both the new disease mutation 13187 G->T (E1914X) and the 16455 A->G substitution.



accession no. U63610), corresponding to cDNA position 5740 (codon 1914) in GenBank accession no. Z54367 (position 5461 in cDNA U53204) located in exon 31 (exon 32 in (3)). This mutation leads to a shift from GAG > TAG resulting in the substitution of glutamic acid codon with a stop codon (E1914X) in the plectin polypeptide. This base substitution abolishes a normal *Hinf*I restriction site and allows a restriction-based identification of the mutation in all carriers of the NEB1 family (Fig. 6). Normal DNA has a restriction site splitting the 156 bp nested PCR product into 106 bp and 50 bp fragments (including m13 tail), whereas the EBS-MD mutation abolishes this restriction site leaving the pattern of the homozygote (no. 11) with unsplit fragments similar to undigested fragments. One hundred Norwegian random chromosomes (50 individuals) lacked this mutation. Homozygosity for the stop mutation in both affected individuals and heterozygosity in unaffected family members are in full accordance with the distribution of 8q24 haplotypes, supporting that this nonsense point mutation is in fact the cause of EBS-MD in this family.

Further downstream another mutation was found using the PLE57 primer (PLE57F: TGTGAAGGA-GGGTGTGGTG; PLE57R: CTGCCGTGAAGTAT-TCCG). This is an A > G substitution in position 16455 in genomic DNA (U63610) creating a restriction site for *Ngom*IV. It corresponds to cDNA position 8900

*Fig. 5.* DNA sequencing revealing a G > T mutation in the affected proband NEB1-9 (a), G/T heterozygosity in non-affected family members NEB1-7 (b) and NEB1-13 (c), whereas the healthy control shows homozygous G (d).
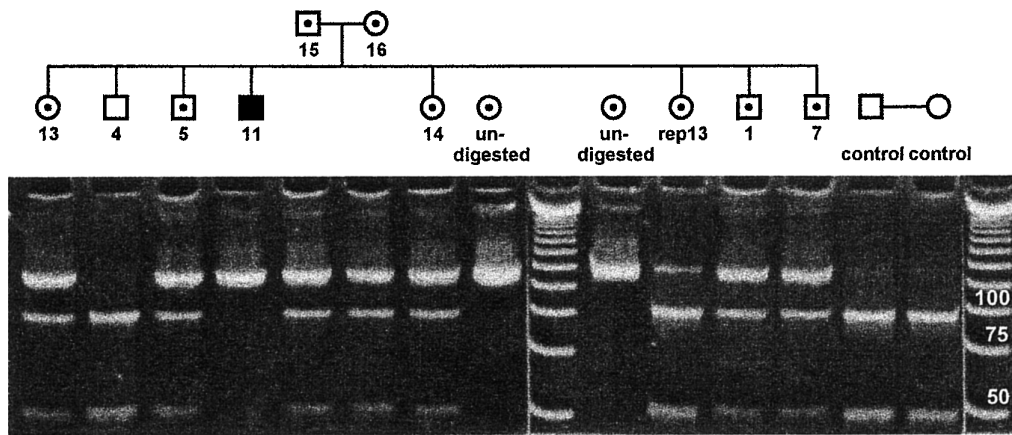
*Fig. 6.* The pathogenic EBS-MD 13187 G->T plectin mutation identified in a *Hinf*I restriction enzyme test, verifying the mutation in relatives carrying the same haplotype. In controls the 156 bp nested PCR product splits into 106 and 50 bp fragments (including m13 tail), whereas the EBS-MD mutation abolishes this restriction site (see no. 11).

(codon 2967) in Z54367 and position 8621 (codon 2857) in U53204. This change results in alteration of the amino acid tyrosine (TAC) to cysteine (TGC). Of 140 chromosomes, 7 carried G, shown by *Ngom*IV typing, which reflects a polymorphism (A = 0.95, G = 0.05).

## DISCUSSION

All EBS-MD mutations known to us up to December 2002 are detailed in Table II. Our novel genomic

mutation 13187G->T or cDNA 5740G->T, changing a GAG glutamic acid codon to a UAG termination codon (E1914X), is located in exon 31 (19) between the Italian 5728C->T (Q1910X) (5) and the Japanese 5806C->T (Q1936X) (9,10) EBS-MD mutations. The skin of patient no. 11 with the homozygous *PLEC1* late stop mutation makes mapping of monoclonal antibodies in the absence of plectin possible. Note-worthy is the remaining expression of plectin epitopes in the suprabasal layers (6C6) of the epidermis and

Table II. *Reported disease mutations in the plectin gene (PLEC1) as in December 2002. The total of 34 alleles depict 23 different recessive EBS-MD mutations and one dominant EBS-O mutation. Updated after Table 145.3 in ref. 34*

| McLean et al. (3) exon 2- exon 33 | PLEC1 cDNA (U53204) | 4574aa codon (U53204) | Ethnic origin | Disease alleles (no.) | Original reports (ref.) |
|---|---|---|---|---|---|
| exon 10 | 1008uns3 | | Austria | 1 | 3 |
| exon 23 | 2719del9 | | Japan | 2 | 4,1 |
| exon 23 | 2745-9del21 | | France | 1 | 11 |
| exon 25 | 3156C->T | Q1053X | Japan | 1 | 9 |
| exon 32 | | Q1408X | Austria | 1 | 13 |
| exon 32 | 4359ins13 | | Japan | 1 | 8,1 |
| exon 32 | 4416delC | | Japan | 1 | 8 |
| exon 32 | 5069del19 | | England | 2 | 7 |
| exon 32 | 5083delG | | France | 1 | 11 |
| exon 32 | 5148del8 | | Spain | 2 | 3 |
| exon 32 | 5188C->T | Q1713X | Germany | 1 | 10 |
| exon 32 | 5461G->T | E1804X | Netherlands | 2[a] | This study |
| exon 32 | 5728C->T | Q1910X | Italy | 2 | 5 |
| exon 32 | 5806C->T | Q1936X | Japan | 1 | 9,1 |
| exon 32 | 5866delC | | Japan | 2 | 4,6 |
| exon 32 | 5905del2 | | Italy | 2 | 4 |
| exon 32 | 5907ins8 | | Italy | 2 | 2 |
| exon 32 | 6049C->T | R2000W | Norway, Germany | 2[b] | 21 |
| exon 32 | 6064G->T | E2005X | Spain | 1 | 11 |
| exon 32 | 7102C->T | R2351X | Germany | 1 | 13 |
| exon 32 | 7261C->T | Q2421X | Japan | 1 | 9,1 |
| exon 33 | 12633ins4 | | Japan | 1 | 9,1 |
| exon 33 | 13429A->T | K4460X | | 1 | 11 |
| exon 33 | 13524 | | | 2[c] | 14 |

[a]In U63610 (gDNA): 13187; in Z543067 (cDNA): 5740G->T and E1914X.
[b]In U63610: 13775; in Z543067: 6328C->T and R2110W.
[c]In U63610: 21358; in Z543067: 13805.

perinuclear (5C10) region in all cells of the skin. These antigens may represent alternative splice variants of plectin, which is distributed in most tissues. Western blotting of the huge plectin molecule often results in a ladder of bands that may not always be degradation products, but instead alternative splice variants. Similar to other cases of EBS-MD, it should be noted that plectin mRNA was significantly reduced (to ~5% of its normal level), as determined by RNase protection assays using fibroblasts derived from patient nos. 9 and 11 (data not shown).

The absence of plectin did not result in altered distribution of other hemidesmosome components such as BP230, BP180, and integrin α6β4, nor in its associated intermediate filaments keratins 5 and 14. Thus plectin is not essential for the recruitment of these hemidesmosome components (31).

Compared to extensive tissue sampling and examinations recently performed on young adult patients (14), we only had fibroblasts and 2 postmortem skin samples available for study. However, the size of the extensively sampled family 2 decades ago made it ideally suited for gene mapping by linkage. Unfortunately both parents happened to be homozygous in the GPT isozyme system, so no association was made with the GPT-linked dominant epidermolysis bullosa simplex Ogna (32) back in 1982. EBS-Ogna is now explained by an exon 31-specific base substitution and plectin amino acid exchange (R2110W) (21).

In the history of clinical delineation of Mendelian disease the coexistence of different organ signs and symptoms has led to prolonged discussions of (a) the chance co-occurrence of independent diseases, (b) the pleiotropic effects of a single disease mutation, or (c) the close genetic linkage of different Mendelian diseases. This is also the case with EBS-MD.

When the Dutch report (22) of EB dystrophica with muscular dystrophy was later complemented by a "similar" association in a Finnish family with EBS and muscular dystrophy (25), both chance coincidence and linkage of two separate loci became less likely, but classification of the EB involved was at stake. Like Anton-Lamprecht's ultrastructural results in the Dutch family, the Finnish workers saw the low basal cell intraepidermal origin of the blistering. The intraepidermal pathogenesis led to their term EB simplex, although the clinical signs were similar to junctional EB, hence the original term pseudojunctional EB (23, 24). The term EBS-DM was recommended by Fine et al. (27). The low intrabasal cell split level categorizes this form as EBS (27). The same very low split level within the basal cell is also seen in EBS due to a *ITGB4* mutation deleting part of the cytoplasmic tail of integrin beta4 (33). When plectin first came into focus, our initial D8S373-typing revealed full linkage to this at that time the most

telomeric marker on the genetic chromosome 8q map (23).

The extended haplotypes (Fig. 4) define a region of 8q24 for which the EBS-MD patients of the family are homozygous and haplotype identical from both parents. The considerable length of the haplotype common to both parents is explained by the inbreeding (parents 3rd cousins), but we do not know if this inbreeding line includes the initially reported West German ancestors. When other detected EBS-MD mutations are likewise haplotyped, this will give a rapid indirect way of pinpointing previously known mutations in new patients.

In the review by Anton-Lamprecht & Gedde-Dahl (34) no specific Dutch origin mutations, but 2 previously reported exon 31 German mutations (5188C->T, Q1713X: and 7102C->T, R2351X), are described (12). To this can be added the exon 32 North-East German 16 basepair insertion mutation 13803ins16 which leads to loss of the normal 35 last amino acids in the plectin polypeptide and thereby the keratin binding motif (14).

## ACKNOWLEDGEMENTS

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