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Substantiating atypical phenotypes of epidermolysis bullosa

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Summary

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This thesis is a collection of studies centering around molecular diagnostics of epidermolysis bullosa (EB), produced by a collaboration between the Departments of Dermatology and Genetics in the Centre for Blistering Diseases of the University Medical Center Groningen in Groningen, the Netherlands.

The aim of the performed studies was twofold: I) to report and explain atypical phenotypes of EB with meticulous description of the clinical phenotype, supported by functional and molecular studies. In this way we aimed to further the understanding of specific adhesion molecules involved in EB; and II) to solve the cold cases of EB patients i.e. those patients lacking a molecular diagnosis with specific focus on the largest group in the Dutch registry, (basal) epidermolysis bullosa simplex (EBS).

In **Chapter 2**, we describe the novel, isoform specific mutation identified in the 1a isoform of *PLEC*. We studied two sisters who were born to consanguineous parents. They suffered from mild skin blistering which began at 1.5 years of age. From the age of 3 years, onychodystrophy of hand and toenails was present. Blistering became generalized in both girls during puberty, during which skin lesions healed with scarring and hyperpigmentation. Mucous membranes and tooth enamel were normal. Routine immunofluorescence antigen mapping (IF) of patient lesional skin revealed intraepidermal cleavage low in basal cells indicating a pseudojunctional split diagnosing EBS. Staining with antibodies targeting all isoforms of plectin (10F6) showed reduced intensity when compared to control, whereas a 1a isoform specific purified antibody (plectin-1a) showed negative staining. Transmission electron microscopy (TEM) revealed hypoplastic hemidesmosomes and intraepidermal pseudojunctional cleavage in the epidermis. In mutation analysis, a homozygous nonsense mutation in the 1a isoform (P1a), c.46C>T was disclosed, leading to a premature termination codon p.Arg16X. IF was performed with 10F6 and plectin-1a on control striated muscle and myocardium from normal human cadavers to study the expression of P1a in these tissues. P1a was not expressed in both tissues, whereas the shared plectin epitope was prominently stained at Z-disks of striated muscle and intercalated disks of myocardium. Examination by neurologist and cardiologist indeed showed no signs of myopathy or cardiomyopathy. By additional supporting functional studies, we were able to conclude that the 1a isoform is the dominant plectin isoform in the basal cell layer of the epidermis. This was shown by 1) the identical pattern of P1a and pan-plectin staining by IF of skin tissue, 2) the 84% reduction of pan-plectin polypeptide by Western blot in patient keratinocyte extracts, and 3) a higher level of plectin 1a mRNA transcript in cultured human keratinocytes. This novel mutation found in two sisters resulted in skin-only EBS, a new plectinopathy caused by P1a deficiency.

In **Chapter 3** we continued the investigation of unconventional *PLEC* genotypes by studying a cold case patient without known cause, who presented with clinical EBS with progressive muscular dystrophy in the subtype epidermolysis bullosa simplex with muscular dystrophy (EBS-MD). A 17-year old girl born to consanguineous parents suffered from acral blistering and nail dystrophy, which began shortly after birth. In addition, she had mild skeletal muscle dystrophy present since puberty. IF with pan-plectin antibodies showed strongly reduced expression of plectin. TEM indicated pseudojunctional cleavage. Neurological and cardiological consultations indicated myopathy and cardiomyopathy, respectively. Her father and brother had died from sudden cardiac death at very young ages, and two of her three sisters suffered from cardiomyopathy. Her older brother had also suffered from similar skin blister-

ing. Her clinical phenotype together with findings from skin biopsies pointed to *PLEC* as the candidate gene. Initial mutation screening with Sanger sequencing of the 32 coding exons of *PLEC* including intron/exon borders showed no mutations. Nonetheless, IF, TEM and the clinical picture was highly suspicious for EBS-MD. Therefore, the *PLEC* gene was carefully re-screened including complete intronic sequences. A homozygous deletion, c.906+19_40del in intron 8 was found in the index patient. Subsequent mRNA studies using RNA isolated from patient keratinocytes showed intron 8 retention leading to a frameshift and a premature termination codon, p.Val303_Pro313ins11*. Consequently, this mutation predicted complete loss of plectin expression affecting all isoforms. An unexpected finding during mRNA analysis was the identification of an additional transcript 12 nucleotides shorter than wild type. This product resulted from the use of an alternative splice site 12 base pairs upstream from the exon 8 splice donor site. The presence of the shorter transcript was also identified in healthy human control skin, striated muscle and myocardium. When the splice site was used, a resulting plectin protein short of 4 amino acids in the actin binding domain was produced. This slightly shortened protein explained the remaining, but reduced, plectin signal in patient skin. The location of the homozygous null mutation in the index patient predicted development of the lethal subtype of EBS occurring with pyloric atresia (EBS-PA), but due to the alternative splice site, EBS-PA was averted. Instead, the patient developed a forme fruste of EBS-MD due to the production of a functional physiological plectin isoform.

In **Chapter 4**, we studied the second EBS cold case in a four generation Dutch family presenting with many affected individuals. The presented subtype, EBS, generalized severe is caused in the majority of cases by autosomal dominant missense mutations, which affect the obligate coiled coil formation of the keratin heterodimers. All affected family members displayed cutis aplasia congenita at birth, herpetiform blistering of skin and mucous membranes all with marked improvement with age, and palmoplantar keratoderma. Skin biopsies were taken for diagnostic studies from the 50-year-old female index patient. IF indicated intraepidermal cleavage of the basal layer and unchanged expression of keratin 5 and 14. TEM showed lateral aggregation of keratin filaments, insufficient tonofilament insertion into the hemidesmosomes, and acantholysis. Mutation analysis performed in all affected individuals of *KRT5* showed a heterozygous intronic deletion c.1218+2_1218+31del. The deletion resulted on mRNA level in in-frame skipping of exon 6 of *KRT5*, explaining classic EBS, generalized severe phenotype. Interestingly, it was once again the intron that harboured the pathogenic mutation resulting in the first reported case of in-frame exon skipping in *KRT5* causing EBS, generalized severe. This case, stressed once more the importance of screening of introns in unsolved EBS cases.

In **Chapter 5** we investigated enamel pathology in junctional EB (JEB). Enamel abnormalities are a universal feature of JEB. Recently, our group identified that heterozygous carriers of null mutations in *LAMA3* displayed subtle enamel pitting in the absence of skin fragility. We identified two new *LAMA3* functional null mutations: nonsense c.2377C>T (p.Arg793Ter) and splice site c.4684+1G>A mutation in heterozygous carriers exhibiting such dental abnormalities. Both parents had offspring affected with JEB and displayed subtle enamel pitting of secondary dentition without any sign of skin blistering. We showed that the splice site mutation c.4684+1G>A behaved like a *LAMA3* null mutation, in that less than 2% expression of intact laminin α3 protein was observed in the performed immunoblot of

keratinocyte lysates obtained from the affected child. Enamel pitting seen in carriers of null *LAMA3* mutations is milder than reported enamel abnormalities seen in carriers of dominant-negative *LAMB3* mutations, who show generalized enamel hypoplasia termed amelogenesis imperfecta. We concluded that subtle enamel pitting can occur from haploinsufficiency for the α3 chain of laminin-332.

Comprehensive understanding of phenotypic traits in the immense clinical EB spectrum is revisited in **Chapter 6**, this time in a subtype of dystrophic EB (DEB) presenting with severe pruritus. We studied two young unrelated female patients affected with dominant DEB due to heterozygous glycine substitution mutations. Due to severe itch, both patients were further classified as EB pruriginosa (EBP), a subtype of DEB. However, both women had pruritus in the face and décolleté, a presentation that was not reported before in the literature of EBP. What was remarkable in our two patients was that the itch did not start in classic locations such as pretibial skin, nor did it lead to violaceus papules and plaques after traumatic scratching. It was not secondary to EB lesions, but localized to intact non-inflamed skin of the face and décolleté. The excoriations, which resulted from compulsive scratching, strongly resembled neurotic artefacts. Psychogenic causes were excluded and we classified this presentation as EBP-excoriée.
Finally, in **Chapter 7** our findings are discussed in the light of the newest genetic sequencing (NGS) techniques. We discuss the role of NGS is in the diagnostics and research of EB. With these techniques in mind, we suggest a comprehensive clinical approach to unsolved genetic cases of EB.