Summary, discussion and future perspectives

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The main focus of this thesis was the identification of the underlying genetic mutation and phenotype characterization of our ‘cold case’ epidermolysis bullosa (EB) patients from the Dutch National EB Registry. These patients were diagnosed and treated at the Department of Dermatology in Groningen, which is also the national referral centre for blistering diseases, both acquired and inherited, in The Netherlands. The investigation gave us an insight into several rare remarkable phenotypes resulting from mutations in genes, some of which not until recently were identified as involved in the pathophysiology of EB.

In chapter 2 we present a scientific review on blistering diseases related to the dermal-epidermal junction thus providing a deeper understanding of the proteins involved in EB. Since the publication of the review, two genes, KLHL24 and CD151 have been widely accepted for inclusion as EB candidate genes following a series of reports in the literature.\(^1\)\(^-\)\(^5\) Of note, CD151, was identified after the application of targeted next generation sequencing gene panel test following unfruitful Sanger sequencing of the candidate gene suggested by the clinical phenotype \(^5\). Interestingly, this has also been the approach that led to the identification of the gene mutations in the cases from our thesis and underscores the role of specifically designed next generation sequencing gene panel tests in the establishment of the genetic background of atypical and/or rare EB phenotypes. In the meanwhile, whole exome sequencing with the application with specific filters has taken the place of specifically designed next generation sequencing gene panel tests and will play an increased role in the molecular genetic analysis. The discovery of rare genetic mutations is accelerating. High-throughput DNA sequencing technologies provide unparalleled opportunities to discover new genes and variants underlying human disease. These findings must, however, rigorously be assed for evidence of implication. Clear guidelines for distinguishing disease-causing sequence variants from various potentially functional variants present in any human genome are certainly needed, otherwise there is a potential for false-positive publications of causality. This would impair the translation of genetic analysis results into the clinical findings and impede the understanding of disease pathophysiology. An excellent perspective on this issue was published by MacArthur et al. in Nature \(^6\). In this thesis, the challenge of reliably investigating the role of sequence variants was encountered in chapter 3 where we illustrated a family in which heterozygosity for a novel missense mutation in the \(\text{ITGB4}\) gene resulted in an autosomal dominant epidermolysis bullosa. This represents, to our knowledge, the first dominant phenotype associated with a \(\text{ITGB4}\) mutation. Bellow we outline in a point by point fashion the combined clinical, genetic and bioinformatical support for the pathogenicity of the reported mutation.
- Consistent segregation of the mutation in all affected individuals.

- Involvement of the external auditory canal, lacrimal duct, and urinary tract, as well as delayed wound healing, and granulation tissue formation in the larynx in some affected individuals, consistent with junctional EB.

- Novel mutation, not found in the Genome of the Netherlands, 7, 1000 genomes, or the ExAc Browser databases and, to our knowledge, was not earlier described in the literature.

- The pathogenicity prediction software tool Alamut (version 2.0, Interactive Biosoftware), classifies the missense mutation as probably pathogenic.

- Exclusion of other EB genes and genes implicated in pachyonychia congenita (KRT6A, KRT6B, KRT6C, KRT16, and KRT17) through our targeted next generation sequencing gene panel test.

- The c.433G>T, p.Asp145Tyr mutation is non-synonymous and changes the acidic side chained aspartate to hydroxyl side-chained tyrosine.

- Mutation is located in the von Willebrand factor type A domain (VWFA domain) of β4 integrin, a domain where most missense ITGB4 mutations reside and known to be implicated in a variety of important cellular functions: basement membrane formation, cell migration, ligand binding, and signaling. 8,9
**Figure 1.** All known ITGB4 missense mutations involved in epidermolysis bullosa (EB) are indicated above the schematic polypeptide with evident clustering within the Von Willebrand factor type A (VWFA) domain. The heterozygous p.Asp145Tyr substitution reported herein is shown in red below the schematic structure.

- Combination of normal and hypoplastic hemidesmosomes noted in EM studies; α6β4 integrin is essential for the assembly of hemidesmosomes. 10

- Affected protein region is highly conserved among species and also between different β integrin subunits. Analog protein region Asp-Asp-Leu (DDL), (where Asp145 is the middle residue) in β3 integrin represents the contact domain for the Arg-Gly-Asp (RGD)-containing integrin ligands.11

**Figure 2.** A, Conservation of β4-integrin residue Asp145 (D letter code) between species. B, Alignment of β-integrin subunits sequences shows evident conservation (except β8-integrin) of DDL peptide sequence (boxed area).

In eukaryotic cells, phosphorylation usually occurs on serine, threonine, and tyrosine residues. 12 The p. Asp145Tyr substitution may create a novel phosphorylation site in the extracellular domain of β4-integrin and modify its function.

Other examples of dominant phenotypes involving specific site mutations have been seen in EBS-Ogna (PLEC) and EBS with mottled pigmentation (KRT5 and KRT14). The association of the p. Asp145Tyr mutation and the respective clinical phenotype in a novel pedigree would provide further evidence for pathogenicity. Interestingly, considering the dominant manner of segregation in all affected family members, the easiest way to invalidate pathogenicity would be if there will be a newborn affected family member without the mutation or carrier mutation status in a non-affected individual. As Albert Einstein famously quoted, “No amount of experimentation can ever prove me right; a single experiment can prove me wrong.” As these data represent indirect proof, ultimately, the predicted damaging impact of the c.433G>T substitution in the ITGB4 gene resulting in p. Asp145Tyr should be validated experimentally using well-established models of gene function. Experimental data obtained from animal studies are sometimes used to recapitulate the phenomena underlying human pathology. Nevertheless, financial, technological and time limitations impede the development of an animal model for each genetic variation. It is, therefore, conceivable that a rare genetic variant associated with a mild phenotype would raise the question of costs and benefits. Also, the biological mechanisms observed in an animal model do not always accurately represent the mechanistic pathway(s) present in humans. Certainly, if abnormal function is observed, the association is strengthened. A negative result, however, does not disprove pathogenicity. In vitro studies could also be employed as experimental confirmations of pathogenicity in skin tissue. Just as in case of in vivo studies, it is not always possible to implement these investigations to characterize each identified genetic variation. Also, in vitro functional data does not always reflect in vivo biology. This phenomenon is due to the fact that in vitro studies are done in designed models that do not incorporate all the necessary biological partners that modify the final phenotype.

In chapter 4 we describe an EBS case with remarkable Mottled Pigmentation (MP) phenotype in association with autosomal recessive EXPH5 mutations. EXPH5 encoding exophilin-5 (also known as Slac2-b, an effector protein involved in intracellular vesicle trafficking and exosome secretion) has only recently been implicated in the pathophysiology of EB. We illustrate a clinically well-documented, mottled
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pigmentation phenotype related to a novel \textit{EXPH5} mutation. The only previous mention of pigmentary changes in EBS associated with \textit{EXPH5} mutations was in the original article of McGrath et al.\textsuperscript{13} which described subtle diffuse pigmentary skin mottling, similar (but less marked) to the pigment changes seen in Griscelli syndrome (GS). The absence of photo documentation of the pigmentary changes does not allow for comparison with the MP phenotype in our patient. Given that in GS there is a pigmentary dilution in skin and hair,\textsuperscript{14} we find the pigmentation abnormality in our patient to be phenotypically different from those in GS. Considering the novelty of the phenotype, we outline the arguments that support indirectly the association between \textit{EXPH5} mutations and the mottled pigmentation phenotype.

- Our patient is one of the oldest reported in the literature, which possibly allowed for the characterization of the pigmentary changes, a late onset feature, which started developing at 10 years of age.

- Mottled pigmentation segregates with the recessive EB phenotype: her parents, two brothers and one sister do not have mottled pigmentation.

- Skin mottling, mentioned in the original article implicating \textit{EXPH5} mutations in EB pathophysiology might, in fact, have resembled the mottled pigmentation in our patient.

- The possibility of EBS-MP was excluded through our extended EB gene panel test as no mutations in \textit{KRT5} and \textit{KRT14} were found.
The level of cleavage plane, as well as the electron microscopy findings were consistent with previously reported cases of EBS resulting from EXPH5 mutations.

Ultrastructural examination of our patient’s skin revealed an accumulation of mature melanosomes in the keratinocytes, thus providing evidence for successful transfer from melanocytes to the keratinocytes. The melanosomes showed, however, a disseminated distribution throughout the cell, and not predominantly supranuclear. We also noted a disruption of keratin filament network and keratin filament aggregation in basal to some extent suprabasal layer. Such finding was also reported by McGrath et al13 in keratinocytes from an affected individual, but also in those with Slac2-b knockdown. This underscores the role of Slac2-b in the maintenance of keratin filament network integrity. The question arises whether disruption in keratin filament network is related to the mottled pigmentation in our patient? Keratins have certainly been previously implicated in pigmentary abnormalities when:

- Melanosomes and mitochondria showed an accumulation and aberrant distribution in keratinocytes of patients with EBS-MP resulting from KRT5 and KRT14 mutations. 15

- Haploinsufficiency of KRT5 responsible for Dowling-Degos disease (DDD) affected melanosomes distribution in keratinocytes and led to reticulate hyperpigmentation. 16

- Autosomal dominant genodermatoses Naegelli-Franceschetti-Jadasson syndrome (NFJS) and dermatopathia pigmentosa reticularis (DPR) are associated with pigmentary disturbances and mutations predicted to cause KRT14 haploinsufficiency. 17

- A disturbed keratin 14 filament network in autosomal dominant EB simplex due to KLHL24 is accompanied by hyper- and hypopigmentation 4 and (V. Yenamandra, personal communication).

The next question would be: How do EXPH5 mutations lead to keratin filament network alteration? Very little is known about slac2-b and its previously investigated function in exosome traffic does not provide yet clear answers. One of the first steps would be to identify interaction partners for slac2-b and study their role in controlling keratinocyte behavior and function. We could start with an in silico analysis for binding domains and perform an unbiased screen to identify novel binding partners depending
on the presence of a binding motif. Then peptides mimicking the binding motif would be incubated with lysates from human keratinocytes. Bound protein will be separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) and the resolved bands containing binding partners would be sequenced by mass spectrometry. Next, the co-localization of novel binding partners and slac2-b would be analyzed using confocal microscopy.

As a side note in relation to the mottled pigmentation phenomenon in EBS-MP (KRT5): Bchetnia et al. 18 screened for candidate genes that may contribute to the pathogenesis of EBS-MP by comparing gene expression profile in EBS-MP and healthy volunteers using microarray analysis. The most remarkable difference, with suggestive connection to the skin mottled pigmentation, is a two-fold increase in tyrosine (TYR) gene expression level in EBS-MP skin tissue compared to controls. TYR is known as the major enzyme of melanin biosynthesis. The authors hypothesize that the higher expression of TYR in the microarray data could explain the observed ultrastructural features of EBS-MP phenotype including the accumulation of mature melanosomes in basal keratinocytes. 18 This insight raises the query whether TYR gene expression is also altered in EBS cases resulting from EXPH5 mutations with mottled pigmentation.

Chapter 5 reports another rare EB phenotype (generalized intermediate with prurigo papules) caused by DST mutations. To our knowledge, this is the first case in the literature with such an extent of skin involvement related to DST mutations. The results of the mutation analysis, immunofluorescence and immunoblot studies implicate mutations in the DST gene, encoding the hemidesmosomal component BPAG1-e, as etiology of EBS in our patient. The identified homozygous c.6559 C>T, p.Gln2187* mutation causes a distal truncation of the BPAG1-e protein. We considered the following arguments when assessing the association between the generalized intermediate with prurigo papules phenotype and the identified DST mutations:

- An affected sister with reportedly similar clinical phenotype.
- Exclusion of an autoimmune bullous disease and other differential diagnoses.
- Exclusion of other EB genes, including COL7A1, through our next generation sequencing gene panel test.
- Novel homozygous nonsense mutation not found in the Genome of the Netherlands 7, 1000 genomes, or the ExAc Browser databases and, to our knowledge, not earlier described in the literature.
- Mutation identified at novel site, in the last exon 24 of the epidermal isoform of *DST*. Previously reported homozygous nonsense mutations were all located in exon 23 and led to absence of BPAG1-e protein.
- Truncated BPAG1-e protein with a still present immunodominant region (red square) that has been implicated in the pathophysiology of bullous pemphigoid, a disorder inherently associated with tense blisters and severe pruritus. 19

**Figure 3.** Schematic representation of the BPAG1-e protein where the previously reported DST mutations are indicated above the schematic protein structure with grey arrows. The homozygous p. Gln2187* mutation is located in the intermediate filament binding domain (IFBD) and indicated with a red arrow. The immunodominant region is indicated by the red square.

Of note, in relation to the association between a BPAG1-e peptide and inflammation, Hall et al. 20 reported that rabbits immunized with a peptide encoded by the 230-kD bullous pemphigoid antigen cDNA develop an enhanced inflammatory response after epithelial injury. This raises the question whether exposure to a C-terminus truncated BPAG1-e molecule might promote an inflammatory response against remaining epitopes and elicit pruritus in the host.

In chapter 6 we present a Dutch pedigree with a surprising combination of two novel nonsense DST mutations and a heterozygous PLEC mutation. The findings imply that EBS caused by DST mutations is a dominantly inherited condition of reduced penetrance; PLEC haploinsufficiency may be a genetic determinant for penetrance and suggest that PLEC might function as a genetic modifier for DST. Through our next generation sequencing gene panel test we identified novel homozygous nonsense mutation (c. 4978delG; p.Val1660*) in exon 23 of the DST gene (Figure 4, red m). Sanger sequencing of genomic DNA confirmed the presence of this mutation in the index patient and the heterozygous status in all three sons. In the middle son, an additional heterozygous DST nonsense mutation (c.6298C>T; p.Gln2100*) was identified (Figure
4, blue m). Also, a heterozygous nonsense mutation c.6292C>T, p.Gln2098* in exon 31 of PLEC gene was found in the index patient and her youngest two sons (Figure 4, green m).

**Figure 4. Clinical pedigree and the unique genetic constellation of various family members.**

Following we highlight the arguments that led to the hypothesis that EBS resulting from DST mutations is a skin fragility disorder of variable heritability.

- Variation in the severity of the skin phenotype is contingent to the biallelic or heterozygous status for a particular pathogenic DST mutation; the index patient and her middle son (III:2) had the most severe phenotype, her youngest son (III:3) a milder but a continuously present phenotype, and her oldest son (III:1) only a mild, transient phenotype.

- The heterozygous status for c. 4978delG; p.Val1660* DST mutation (Figure 4, red m) led to an, albeit transient, clinical phenotype while the heterozygous status for c.6298C>T; p.Gln2100* DST mutation (Figure 4, blue m) did not result in a phenotype.

- Presence of PLEC haploinsufficiency influences phenotype severity and might explain the difference between a continuously present phenotype in the youngest son and a transient phenotype in the oldest son. In relation to our
study, the question arises whether the change in the amount of plectin might have a modifying role in conjunction with the DST mutation. Of note, the immunoblot staining against the rod domain of plectin was reduced in both the index patient and the middle son.

- BPAG1-e binds specifically to keratin filaments, and in conjunction with plectin, tethers them to hemidesmosomes (Kunzli et al, 2016, Michael et al, 2014). The DST mutations are expected thus to affect BPAG1-e’s ability to bind IF proteins. In a previously reported EBS case caused by DST mutations\textsuperscript{21}, the staining of plectin was brighter along the EMBZ. This resulted from an increased plectin expression which was quantified in keratinocytes to be 250% of control in a Western blot. Such observations underline the functional interplay between plectin and BPAG1-e and suggest that plectin may play the role of a genetic modifier for BPAG1-e.

Considering the presence of a disease phenotype in a monoallelic disease form in the oldest and youngest son, in conjunction with previously reported pedigrees (a Kuwaiti father who was heterozygous for the a nonsense mutation p.Gln1124* in DST with transient blistering in childhood \textsuperscript{22}; an Iranian pedigree with two children with heterozygous status and mild skin blistering, less severe than their parent who was homozygous for the p.Arg1249* mutation in DST\textsuperscript{23}), the term EBS-autosomal recessive is not accurate, as it implies the necessity of biallelic mutations for the development of a disease phenotype.

With the evolution of next generation sequencing techniques, the main focus is placed on molecular diagnosis instead of fluorescence antigen mapping on patient’s skin biopsies. Even though a simple blood test can provide an EB diagnosis in many cases, a skin biopsy still remains useful as it may offer the quickest answers and suggest candidate genes. Analyzing protein expression and the level of cleavage formation can have important prognostic function in genetic counseling. Finally, the value of skin biopsies is underscored in dermatological research where, for example, in chapter 5 an overexpression of plectin in the EB resulting DST mutations skin provided extra support for the data in chapter 6, which suggests that PLEC may function as a genetic modifier for DST.

In chapter 7 we aimed to fine tune the understanding of the level of cleavage formation in junctional epidermolysis bullosa (JEB) skin through fluorescence antigen mapping. Analysis of immunofluorescence patterns revealed two types of ‘lucidolytic’ cleavage: a low lamina lucida cleavage and a high lamina lucida cleavage. Mutations in genes
encoding for type XVII collagen and integrin α6β4 subunits cause a high junctional cleavage, while mutations in genes encoding for laminin-332 lead to a low junctional cleavage. By means of immune-electron microscopy, laminin-332 in the blister roof was shown to co-localize with the hemidesmosomes; we hypothesized that laminin is drawn to the blister roof by its binding strength with the hemidesmosomal molecules. The study provided an insight in the hierarchy of intermolecular binding in the lamina lucida of the basement membrane zone in human skin.

The application of the next generation sequencing gene panel test in our cold case EB population has provided unprecedented opportunities to discover rare genes and variants associated with unusual EB phenotypes. Throughout the discussion we assessed the evidence for the particular gene implication in a succinct way, combining genetic, informatic and literature support for the individual case. In phenotypes which are extremely rare it may be impossible to implicate with absolute certainty a specific gene or phenotype with the available sample size. Nevertheless, suggestive implication can be valuable and provide a starting point for future clinical and research investigations. Underneath we present de updated Figure 5. of the phenotype spectrum associated with basal EBS genes, including phenotypes in this thesis.
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**Figure 5. Phenotypic spectrum of basal epidermolysis bullosa and its discovery timeline.** The light green units indicate phenotypes previously reported by our research group at the Centre for Blistering Diseases, Department of Dermatology, University of Groningen, University Medical Centre Groningen, Groningen, The Netherlands. The pink units indicate the phenotypes investigated in this thesis.
References


