1

Introduction

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The cold cases in this thesis were extracted from the Dutch National Epidermolysis Bullosa Registry. These patients were diagnosed and treated at the Centre for Blistering Diseases, Department of Dermatology, University of Groningen, University Medical Centre Groningen, Groningen, which is also the national referral centre for blistering diseases, both acquired and inherited, in The Netherlands. This thesis resulted after fruitful collaborative work with The Department of Genetics, University of Groningen, University Medical Centre Groningen, Groningen.

Epidermolysis bullosa

EB comprises a group of clinically heterogeneous inherited blistering diseases that affect the skin and sometimes also the mucous membranes. The term was coined for the first time by Koebner in 1886. As insight in the molecular background of disease pathophysiology grew, the EB spectrum expanded and, to date, it includes more than 30 clinical subtypes resulting from mutations in at least 20 different genes. EB is classified into four major types (EB simplex, junctional EB, dystrophic EB, and Kindler syndrome) based on the level of cleavage formation in the skin (Figure 1). The level of cleavage is determined with immunofluorescence antigen mapping (IFM) and/or transmission electron microscopy (TEM) studies. Regardless the modern advancements in genetic analysis techniques, IFM and TEM are still valuable tools for the determination of EB type and identification of the candidate gene.

Figure 1. Schematic representation of the level of blister formation in the skin of major epidermolysis bullosa (EB) types; EB simplex (EBS), junctional EB (JEB), dystrophic EB (DEB) and Kindler syndrome. Genes involved in the respective subtype are shown underneath.
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Figure 1.

Schematic representation of the level of blister formation in the skin of major epidermolysis bullosa (EB) types; EB simplex (EBS), junctional EB (JEB), dystrophic EB (DEB) and Kindler syndrome. Genes involved in the respective subtype are shown underneath.

The EB classification was updated at the latest consensus meeting, held in London, in June 2013 where a new "onion skinning" algorithm was introduced that takes into account successively the major EB type present, phenotypic characteristics (distribution and severity of disease activity; specific extracutaneous features), mode of inheritance, targeted protein and its relative expression in skin, gene involved and type(s) of mutation present, and when possible specific mutation(s) and their location(s). Over the recent years a few new skin fragility entities were added to the EB spectrum. These blistering disorders are rare and often involve just a few patients. Nonetheless, accurate identification and characterization of these distinct conditions is essential in refining diagnosis and acquiring a thorough understanding of the pathophysiology of EB. Also, this knowledge helps gain insight into the macromolecular interplay necessary for the maintenance of mechanical integrity and signaling within healthy skin. Following is a concise introduction to basal EBS and JEB subtypes (Table 1); these are relevant to the investigations reported in this thesis.

<table>
<thead>
<tr>
<th>Targeted proteins</th>
<th>EB subtype</th>
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<tbody>
<tr>
<td><strong>Basal EB simplex</strong></td>
<td></td>
</tr>
<tr>
<td>Keratin 5, 14</td>
<td>EBS, localized (K5; K14) EBS, generalized severe (K5; K14) EBS, generalized intermediate (K5; K14) EBS with mottled pigmentation (K5; K14) EBS, migratory circinate (K5) EBS, autosomal recessive (K14)</td>
</tr>
<tr>
<td>Kelch-like 24</td>
<td>EBS, autosomal dominant</td>
</tr>
<tr>
<td>Exophilin 5 (slac2-b)</td>
<td>EBS, autosomal recessive</td>
</tr>
<tr>
<td>Plectin</td>
<td>EBS with muscular dystrophy EBS with pyloric atresia EBS-Ogna EBS-skin only</td>
</tr>
<tr>
<td>BPAG1-e (BP230)</td>
<td>EBS, autosomal recessive-BP230 deficiency</td>
</tr>
<tr>
<td>Type XVII collagen (BPAG2, BP180)</td>
<td>EBS, generalized intermediate</td>
</tr>
<tr>
<td>Integrin β4</td>
<td>EBS with pyloric atresia</td>
</tr>
<tr>
<td>Tetrascarpin CD151</td>
<td>Kindler syndrome-like EBS with multi-systemic manifestations including nephropathy</td>
</tr>
<tr>
<td><strong>Junctional EB</strong></td>
<td></td>
</tr>
<tr>
<td>Type XVII collagen (BPAG2, BP180)</td>
<td>JEB, generalized intermediate JEB, late onset JEB, localized</td>
</tr>
<tr>
<td>Integrin α6β4</td>
<td>JEB with pyloric atresia (integrin α6β4) JEB, localized (integrin β4)</td>
</tr>
<tr>
<td>Laminin 332 (α3,β3,γ2 chain)</td>
<td>JEB, generalized severe JEB, generalized intermediate JEB, localized JEB, inversa JEB laryngo-onycho-cutaneous syndrome (isoform α3 chain)</td>
</tr>
<tr>
<td>Integrin α3</td>
<td>JEB with respiratory and renal involvement</td>
</tr>
</tbody>
</table>
Basal epidermolysis bullosa simplex

This EBS subtype is characterized by a cleavage plane within the basal epidermal keratinocyte layer. Albeit not adopted by the official EB consensus, a separate ‘pseudojunctional’ cleavage plane has been noted by means of TEM; this term signifies a very low basal cleavage where basal keratinocytes’ fragments remain attached to the blister floor. Suprabasal EB is defined by a cleavage plane above the basal layer; its background will not be discussed here for it stands beyond the scope of this thesis.

Basal EBS is the most frequently encountered EB subtype; its prevalence is estimated 1/25,000 live births.\(^6,7\) Altogether, there are eight genes involved in the pathogenesis of basal EBS (Figure 1)\(^2,8,9\) This EB subtype has mainly an autosomal dominant inheritance pattern, where 75% of affected individuals harbor mutations in the KRT5 and KRT14 genes.\(^10\) Rare basal EBS subtypes implicate PLEC, DST, COL17A1, ITGB4, EXPH5, CD151 and the recently discovered KLHL24 gene. These genes code for plectin, BPAG1-e (also known as BP230), type XVII collagen (also known as Bp180 or BPAG2), integrin β4 subunit, exophillin-5 (also known as slac2-b) and kelch-like 24 proteins, respectively; together they play an essential role in the maintenance of cell adhesion, mechanical keratinocyte integrity, cellular signaling, vesicle transport or intracellular turnover of intermediate filaments.\(^9,11,12\)

Heterogeneity of basal EBS clinical phenotype

The basal EBS phenotypic spectrum is very heterogeneous, ranging from mild localized acral skin fragility to severe generalized blistering and sometimes mucous membrane involvement. Certain subtypes may exhibit nail dystrophy and pigmentedary changes of the skin. Finally, the respiratory, gastrointestinal and genito-urinary system may also be involved.\(^2\) A timeline of phenotype discovery pertaining to various basal EBS genes is illustrated in Figure 2. Clinical entities such as Dowling-Degos Disease (DDD), Naegelli-Franceschetti-Jadassohn syndrome (NFJS) and Dermatopathia Pigmentosa Reticularis (DPR) have been included (although not skin fragility disorders) because their phenotypes are associated with pigmentedary disturbances and the underlying pathogenic mechanism may be relevant for understanding the nature of a ‘mottled pigmentation’ phenotype in EBS resulting from EXPH5, KRT5 or KRT14 mutations. Pathogenic mutations in the less conserved non-helical head and tail domains of intermediate filament keratins 5 and 14, or mutations expected to cause haploinsufficiency are presumed to underlie the pigmentedary changes in the above mentioned non-EB genodermatoses.\(^13-16\)
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**History of clinical phenotypes associated with mutations in basal EBS genes**

KRT5, KRT14, PLEC, DST, COL17A1, ITGB4, EXPH5, CD151, KLHL24

<table>
<thead>
<tr>
<th>Year</th>
<th>Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>1886</td>
<td>Dowling-Degos Disease (DDD)</td>
</tr>
<tr>
<td>1926</td>
<td>PLEGr</td>
</tr>
<tr>
<td>1938</td>
<td>KRT5</td>
</tr>
<tr>
<td>1954</td>
<td>KRT14</td>
</tr>
<tr>
<td>1971</td>
<td>PLEC</td>
</tr>
<tr>
<td>1992</td>
<td>KRT5, KRT14</td>
</tr>
<tr>
<td>1993</td>
<td>PLEC</td>
</tr>
<tr>
<td>1996</td>
<td>COL17A1</td>
</tr>
<tr>
<td>2003</td>
<td>DST</td>
</tr>
<tr>
<td>2004</td>
<td>KLHL24</td>
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<td>2005</td>
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<td>2010</td>
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<td>2016</td>
<td>KRT5</td>
</tr>
<tr>
<td>2017</td>
<td>KRT5</td>
</tr>
</tbody>
</table>

**EBS-gen-intemed**
Mainly acral blistering, but also significant generalised skin fragility

**EBS-gen-severe**
Severe, generalised, grouped haemorrhagic blistering; frequent mucosal involvement; subungual blistering; palmoplantar keratoderma

**EBS-loc**
Mainly acral blistering

**Naegeli-Franceschetti-Jadassohn syndrome (NFJS)**
Reticular pigmentation; hypohidrosis; palmoplantar keratoderma; absent dermatoglyphics

**KRT5, KRT14**
Mainly acral skin blistering, may be widespread; onychogryphosis

**KRT14**
Dermatopathia pigmentosa reticularis (DPR)
Reticular pigmentation; alopecia; teeth abnormalities; nail dystrophy

**KRT5, KRT14**
EBS-MP
Mild skin blistering; meiotico pigmentation on trunk and extremities; punctate palmo-plantar keratoderma (variable); nail dystrophy (variable)

**COL17A1**
EBS-gen-intemed
Haemorrhagic blisters on hands, feet, face, waist and subungually; after two years localized distribution

**DST**
EBS-AR-BP230 defectivity
Predominantly acral skin blistering; post-inflammatory hyperpigmentation

**EXPH5**
EBS-AR
Scale crusts and intermittent blistering; weals/keloids improving later in life

**PEC**
EBS-Ogna
Mainly acral skin blistering, may be widespread; onychogryphosis

**PLEGr**
EBS-MD
Blisters as early as birth; acral predominance; muscular dystrophy in infancy to adulthood

**PLEC/ITGB4**
EBS-PA
Generalized skin fragility; congenital absence of skin; mucous membranes involvement; pyloric atresia

**PLEC exon1a**
EBS-AR-skin-only
Acral blistering at walking age, which became generalized at puberty; verrucous papules; excoriations and post-inflammatory hyperpigmentation

**KRT5**
EBS-nails only AD
Mildly dystrophic toenails, microchondra and thickening of the index toenail plate, horizontal ridging of the great toenail

**CD151**
EBS-AR
Kindler syndrome-like blistering, particularly on pretibial areas, pachyderma, nail dystrophy, loss of teeth, early onset alopecia, and esophageal webbing and strictures
Figure 2. 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.

Legend Figure 2

AR: autosomal recessive
AD: autosomal dominant
EBS-gen-intermed: epidermolysis bullosa simplex generalized intermediate
EBS-loc: epidermolysis bullosa simplex localized
EBS-gen-severe: epidermolysis bullosa simplex generalized severe
EBS-MP: epidermolysis bullosa simplex with mottled pigmentation
EBS-migr: epidermolysis bullosa simplex migratory circinate
EBS-MD: epidermolysis bullosa simplex with muscular dystrophy
EBS-PA: epidermolysis bullosa simplex with pyloric atresia

Junctional epidermolysis bullosa

According to the latest consensus, JEB has an autosomal recessive pattern of inheritance and is defined by a cleavage plane through the lamina lucida (Figure 1.). Only a single case of JEB, involving a heterozygous COL17A1 mutation, with skin blistering and abnormal dentition inherited in an autosomal dominant manner has been reported. To date, seven genes have been associated with JEB: LAMA3/3A, LAMB3, LAMC2, COL17A1, ITGA6, ITGB4 and ITGA3. These genes encode the chains of laminin-332, hemidesmosomal molecules (type XVII collagen, integrin α6β4), and a focal adhesion component (integrin α3 subunit), respectively (Figure 3). Corresponding EB subtypes contingent to the targeted protein are presented in Table 1. Generally, loss-of function mutations in genes encoding laminin-332 result in JEB generalized severe, formerly known as Herlitz JEB. The extensive and persistent damage to the skin and mucous membranes lead to severe extracutaneous complications such as shortness of breath, failure to thrive and vulnerability to infections. These complications are so overwhelming that they lead to death with an average life expectancy of 6 months. An additional, potentially lethal subtype is JEB with pyloric atresia caused by pathogenic mutation in ITGA6, ITGB4. This clinical entity is associated with generalized blistering, cutis aplasia, pyloric atresia and sometimes genito-urinary involvement; milder cases have also been reported. Laryngo-onycho-cutaneous syndrome (LOC) is another severe JEB subtype; its phenotype is characterized by chronic granulation tissue in the mucosa, larynx and eyes. In the other JEB subtypes, earlier known as non-Herlitz the genetic mutations are generally less disruptive and comprise symptoms such as: skin blistering, atrophic scarring, nail dystrophy, alopecia and enamel abnormalities. An interesting, lately discovered clinical entity is JEB with respiratory and renal involvement resulting from mutations in ITGA3. This gene encodes the focal adhesion polypeptide integrin α3 subunit (Figure 4). The affected patients were very sick neonates with nephrotic syndrome, pulmonary inflammation and minor or no skin blistering.

The epidermal basement membrane zone

The epidermal basement membrane zone (EBMZ) in the skin is a critical interface at the dermal-epidermal junction and represents a highly specialized structure that mediates the binding of basal keratinocytes to the underlying basement membrane. Its chief adhesion units are the hemidesmosomes. They are ultrastructurally identified as electron-dense structures at the base of the basal keratinocytes. Hemidesmosomes found in skin contain the following molecular components: BPAG1-e, BPAG2, integrin α6β4, tetraspanin CD151 and plectin. Focal adhesions (also known
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as integrin adhesomes) are additional specialized attachment structures located between hemidesmosomes (Figure 3). More than 150 proteins are involved in their composition. In relation to EB only integrin α3 subunit and kindlin-1 are relevant thus far. The integrity of the skin relies on well-assembled and functional hemidesmosomes and focal adhesions. These junctional complexes are not simply compounds of adhesion molecules; they also play a significant role in signaling pathways involved in the differentiation and migration of epithelial cells such as during wound healing and in tumor invasion. Chapter 2 provides a detailed review about the basement membrane zone, its constituents and their associated skin blistering disorder.

**Figure 3.** Schematic representation of the dermal-epidermal junction with its main adhesion units. Molecules or their subunits targeted by genetic mutations are shown in color (excluding grey).
Diagnostic algorithm for a ‘cold case’ from our National EB Registry

Clinical analysis

Patients with basal intraepidermal cleavage plane were the largest group in the ‘cold case’ population from our national EB registry. At the beginning of the research program there were 20 unsolved cases, representing approximately 15% of the EBS cohort. The patients were all clinically evaluated by the same expert (prof. M.F. Jonkman). Phenotype investigation included: age of onset, clinical course, exacerbating factors, detailed clinical pedigree and assessment of extracutaneous features (hair, nails, teeth, respiratory, gastro-intestinal, cardiologic, genito-urinary and neurologic systems).

Immunofluorescence antigen mapping (IFM) and transmission electron microscopy (TEM) studies

Routinely, 4-mm healthy and lesional punch biopsies (naturally occurring or artificially induced by the ‘mini skin rub test’*) were stained with monoclonal antibodies directed at different epidermal basement membrane components to determine protein expression (increased/4+; normal/3+; reduced (ranging 1+/2+); absent/-), and also to identify the cleavage plane. For TEM studies 2 mm punch biopsies were obtained from both healthy and lesional skin. Special attention was paid to features such as: level of cleavage, characterization of hemidesmosomes, aspect of intermediate filaments, distribution of cell organelles and presence of intracellular vesicles. IFM and TEM were executed as previously reported.\textsuperscript{35,36}

*For instructions please visit: YouTube. (2017). Mini skin rub test. Online: https://www.youtube.com/watch?v=fz8nW3z51Gw

Genetic analysis

Standard Sanger sequencing of most probable candidate genes revealed no genetic variants in known EB genes. Following, we applied our diagnostic next generation sequencing gene panel test consisting of a comprehensive set of 33 genes associated with/or mimicking EB. The EB panel includes the following genes: ATP2C1, CD151, CDSN, COL17A1, COL7A1, CSTA, DSP, DST, EXPH5, GJB6, FERMT1, ITGA3, ITGA6, ITGB4, JUP, KRT1, KRT10, KRT14, KRT16, KRT17, KRT5, KRT6A, KRT6B, KRT6C, KRT9, LAMA3, LAMB3, LAMC2, PKP1, PLEC, SPINK5, TGM5 and WNT10A. The test is based on targeted
SureSelect enrichment (Agilent Technologies Inc., Santa Clara, CA USA) and subsequent sequencing on a MiSeq sequencer (Illumina Inc., San Diego, CA, USA). Such a technique provides the advantage of analyzing in parallel all the EB genes with a single test. The obtained results were compared to known variants in the Genome of the Netherlands (Genome of the Netherlands Consortium, 2014), 1000 genomes (http://www.internationalgenome.org/1000-genomes-browsers/), and the ExAc Browser databases (http://exac.broadinstitute.org/). Finally, the found mutations were confirmed by Sanger sequencing.

**Aims and outline of the thesis**

The aim of current thesis was to characterize remarkable phenotypes of epidermolysis bullosa and identify the underlying genetic mutation in the unsolved cases in our national EB Registry. In chapter 2 we provide an introductory scientific review on the latest knowledge about blistering diseases related to the dermal-epidermal junction which gives a platform for deeper understanding of the proteins involved in EB. Chapter 3 illustrates a family were heterozygosity for a novel missense mutation in the ITGB4 gene resulted in an autosomal dominant epidermolysis bullosa. This represents, to our knowledge, the first dominant phenotype related to an ITGB4 mutation. Also, we propose a hypothesis as to why we consider the heterozygous ITGB4 mutation to be pathogenic. In chapter 4 we describe an EBS case with a remarkable Mottled Pigmentation (MP) phenotype in association with autosomal recessive EXPH5 mutations. By means of electron microscopy studies we propose a hypothesis behind the etiology of pigmentary changes in our patient. Chapter 5 reports another unusual EB phenotype (intermediate generalized with prurigo papules) caused by a distal truncation of the BPAG1-e protein. To our knowledge, this is the first case in the literature with such an extent of skin involvement related to DST mutations. In chapter 6 we present semi-dominant, pseudo-dominant and autosomal recessive heritability for the DST gene in a Dutch pedigree and suggest that PLEC might function as a genetic modifier for DST. Finally, in chapter 7 we concentrated on the analysis of lamina lucida cleavage pattern in junctional epidermolysis bullosa (JEB), a subtype of EB. The insights aim to facilitate the diagnosis of EB through faster identification of the candidate gene.
References:


