Mutation analysis of *KRT5* and *KRT14* genes with epidermolysis bullosa simplex

PhD thesis

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I. INTRODUCTION

Clinical phenotypes of EBS

Epidermolysis bullosa simplex (EBS) is a group of hereditary bullosus disorders with an estimated prevalence of 1:50 000. Patients suffer from recurrent intraepidermal blister formation within the skin caused by lysis of the basal keratinocytes induced by mild mechanical traumas. In the basal form of EBS, the blisters heal without scarring. The three major clinical subtypes of the basal form of EBS are the localized EBS (EBS-loc), EBS Dowling-Meara (EBS-DM) and other generalized EBS (EBS, gen-nonDM). Rare subtypes include EBS with muscular dystrophy (EBS-MD), EBS with pyloric atresisa (EBS-PA), migratory circinate EBS (EBS-migr), EBS Ogna (EBS-Og) and EBS with mottled pigmentation (EBS-MP).

The mildest and most common form of EBS is the EBS-loc type with blistering usually limited to the hands and/or feet. The first blisters develop already in early childhood, when the infant begins to walk. Similarly to the other EBS disorders, the symptoms of EBS-loc are temperature dependent worsening during summer.

The EBS, gen-nonDM is associated with generalized blistering from the first week of life and the symptoms may become later more widespread on the trunk with occasional involvement of the oral mucosa, teeth and nails as well. In some patients, the skin symptoms are associated with nail dystrophy and mild focal palmoplantar keratoderma. There is a marked seasonal variation of severity.

The most severe form of basal EBS is the EBS-DM, when patients have widespread herpetiform blistering on any site of the skin from the first days of life. Serous and haemorrhagic blisters occour most frequently on the palms and soles, as well as on the trunk and neck. Patient with EBS-MP often also have palmoplantar keratoderma, nail dystrophy and oral ulceration. Extensive

involvement occasionally occurs and can be fatal in neonates. The diagnosis of EBS-DM is confirmed by the typical appearance on electron microscopy of an intraepidermal cleavage plain and electron-dense aggregates within the cell cytoplasm.

EBS-MP is a rare form of basal EBS mild to moderate, intraepidermal blistering limited usually to young ages. The lysis of basal keratinocytes is followed by a progressive reticular hyperpigmentation extending to the trunk and extremities. In some patients, the skin symptoms are associated with nail dystrophy and mild focal palmoplantar keratoderma. EBS-MP appears at birth or shortly thereafter and the subsequent pigmentation may improve in adult ages. EBS-MP displays either the characteristics of EBS-loc (blistering limited to hands and feet) or EBS-gen (generalized blistering) sometimes with punctate or more severe palmoplantar keratoderma, as in EBS-DM. The characteristic mottled pigmentation appears as a reticular pattern of hypo- and hyperpigmented maculae.

EBS is most often expressed as an autosomal dominant trait but there have been a few report of recessive EBS. EBS patients are known to carry defects in either the keratin genes *KRT5* or *KRT14* expressed mostly in the basal layer of the epidermis.

Mutation analysis difficulties in *KRT14*

While the *KRT5* gene proved to be a rather easy target for mutation analysis. Analysis of the *KRT14* however showed to be more difficult due to the presence of a highly homologous pseudogene. Its exon and intron sequences are identical in 95% and 93% of the respective sequences of *KRT14*. Avoiding pseudogene amplification is a particular problem in the molecular diagnosis of keratin disorders since several keratins have multiple pseudogenes. Pseudogenes have been reported for *KRT6*, *KRT8*, *KRT14*, *KRT16*, *KRT17*, *KRT18* and

KRT19. Thus it is important to be aware of pseudogenes to ensure that mutation analysis is only performed on the functional gene.

The presence of a full and a partial *KRT14* pseudogene sequence was demonstrated earlier. Pseudogenes are functional defected copies of known genes, which have lost their protein-coding ability and are no longer expressed in the cell. Although pseudogenes harbour gene-like features, they are nonetheless non-functional, often due to genetic disablements. Their presence in the genome is often inhibits genetic analysis - sequences of pseudogenic origin contaminate data, since their appearance as contamination can be seen in the analysis results over the functional sequence. Amplification of *KRT14* pseudogene contaminating resources force geneticists carrying out EBS mutation analysis to use elimination methods, such as restriction digestion specific for pseudogenic DNA or by using RNA based analysis, creating cDNA obtained from skin biopsies. Another strategy is the long-range PCR for specific full-length amplification of the human *KRT14* gene.

We developed a new, simply-to-apply PCR method, where individual exons can be examined one at a time, allowing a simple mutation validation by PCR-RFLP.

II. AIM OF THE STUDY

By genetic and clinical analysis of the 10 EBS families the aim of this study was:

- To validate a new and easy way of mutation analysis strategy which efficiently excludes the *KRT14* pseudogene.

- To identify the genetic mutations and polymorphisms with genetic analysis in Hungarian EBS families.

- To verify new mutations and examine of their phenotype function, evaluate their importance.

- To determine genetic polymorphisms frequency of *KRT5/14* genes in Hungarian population.

- To reveal EBS genotype-phenotype correlations by coupling the results of the mutation analysis to the clinical data of the patients.

- To refine EBS classification by characteristics of the structural and backround of genetic

III. PATIENTS AND METHODS

Patients

Here, we present data collected in 10 families referred to us by DEBRA Hungary. Out of the index patients 2 had the clinical symptoms of EBS-DM, one had the features of EBS-gen-nonDM, 6 patients were diagnosed as EBS-loc and one had EBS-MP.

The families of the 10 Hungarian EBS patients were unrelated and non-consanguinous and presented with a de novo mutation (one patient) or dominantly inherited skin symptoms (9 families).

All participating family members were informed before the genetic studies and gave their written consent to the mutation analysis.

Mutation analysis

DNA isolation and PCR amplification

DNA was extracted from peripheral blood samples of the probands and from the patient's family members using standard protocol. Using genomic DNA isolates as templates, exons 1-9 of *KRT5* and exons 1-8 of *KRT14* were amplified. The *KRT5* gene proved to be a rather easy target for mutation analysis. All *KRT5* gene primers we used have been described previously. Analysis of the *KRT14* however showed to be more difficult due to presence of pseudogenes. New primers were designed for *KRT14* exons to avoid the amplification of the highly homologous full *KRT14* pseudogenes. Positions of the PCR primers have been selected manually with an emphasis of the dissimilar nucleotides at the primer's 3' end. In each primer set at least one/two nucleotides at the 3' end are different from the pseudogene. At least one primer in each pair is located several dozen nucleotides upstream from the intron-exon boundary, which enables detection of splice site mutations. The primers also do not bind to the regions of

the *KRT14* containing single nucleotide polymorphisms (SNPs) as verified by analyzing the SNP databases NCBI's. Primer specifity was re-proofed with Primer3 and VectorNTI, annealing temperatures were determined by *in silico* predictment and confirmed by rutine PCR runs. For amplification of the entire coding region of *KRT14*, containing eight exons, eight PCR primer pairs have been designed.

Conformation-sensitive gel electrophoresis (CSGE) and sequencing

PCR products were screened for sequence variations by heteroduplex analysis using conformation-sensitive gel electrophoresis (CSGE). Bands of altered modality were re-amplified from genomic DNA by PCR and the products were sequenced in forward and reserve orientation using ABI Prism 310 automated sequencing system. Sequencing of the *KRT14* amplicons demonstrated that they contain only sequences of KRT14 without co-amplification of the pseudogene.

Restriction Fragment Length Polymorpism (RFLP)

Mutations were confirmed by restriction analysis. To discount common polymorphisms we employed restriction enzymes that specifically cut either the mutated or the wild-type sequence. Restriction enzymes having an additional, control digestion site in the PCR product were preferred.

Controls

For all novel mutations, sequence analysis of unaffected family members (if available) and their presence in 50 unrelated healthy/ethnically matched control individuals has been excluded.

IV. RESULTS

- To examine *KRT14* mutations in Hungarian families with EBS we developed a simple allele-specific method to study the *KRT14* gene excluding pseudogene sequence contamination.

- In Hungarian patients with EBS's disease we found 6 different novel and 4 different previously described heterozygous missense mutations:

Detected novel missense mutations:
KRT14 (p.L136Q, p.E411K, p.I412N)
KRT5 (p.I183V, p.E190D, p.R331G)

Detected previously described missense mutations:
KRT5 (p.P25L, p.E170K, p.Q191P)
KRT14 (p.R388C)

- We further identified 7 different polymorphisms in both genes:

KRT5 (p.A52A, p.L117L, p.G128R, p.G138E, p.Q171Q, p.T210T)
KRT14 (p.N123N)

Detection of mutations in *KRT5*

EBS/1: In the present four generation EBS-MP pedigree, mutation index patient's DNA revealed a recurrent EBS-MP-associated heterozygous mutation p.P25L in the first exon of *KRT5*. This mutation is a T-to-C transition at nucleotide position 74 of *KRT5,* resulting in a proline to leucine exchange in codon 25. We further identified a non-synonymous, heterozygous sequence alteration p.G138E/c.G413A in the index patient which closely corresponded with the p.P25L in the pedigree. The p.P25L mutation was confirmed by re-sequencing in all affected patients. The non-affected family member carried

neither the p.P25L mutation nor the p.G138E polymorphism. We describe here the first reported EBS-MP case from Hungary.

EBS/2: In patient with EBS-loc disease we identified a previously identified mutation of *KRT5* gene (p.E170K).

EBS/3: DNA of EBS-loc **Patient 3**, showed a heterozygous A>G transition (c.547A>G) in exon 1 of *KRT5*, an Ileu>Val mutation in the 183th codon (p.I183V). The affected father is also a carrier. Interestingly, a different mutation (p.I183F) of the same codon has been previously described in association with EBS-DM. We hypothetize, that p.I183V should cause less intraproteic stress and steric disortion than p.I183F.

EBS/4: In a three-generation EBS-loc family, the affected members were found to carry a mutation in the exon 1 of *KRT5* gene. A heterozygous G>C transversion (c.570G>C), changed 190 codon Glu>Asp (p.E190D). This mutation was also found to distort a Tse I restriction site. A previously reported mutation in the same codon (p.E190K) was also described in association with the EBS-loc phenotype.

EBS/5: In patient with EBS-gen-nonDM disease we identified a previously identified mutation of *KRT5* gene (p.Q191P).

EBS/6: Exon 5 of *KRT5* was found to carry the C>G transversion (c.991C>G), causing an Arg>Gly substitution in codon 331 (p.R331G) in the EBS-loc family. The above described mutation also destorts an AciI restriction site. Two other mutations at codon 331 of *KRT5* (p.R331H, p.R331C) have been identified previously as causing EBS-loc.

Detection of mutations in *KRT14*

EBS/7: EBS-loc family presented with the novel *KRT14* mutation p.L136Q. This is the first alteration to be detected at codon 136 in a type I keratin (c.407T>A). The change in polarity of this residue could lead to defected keratin filament interactions and may therefore be pathogenic. p.L136Q also introduced

a Dde I. restriction site. It is remarkable that the novel mutation p.L136Q in *KRT14* is the first mutation found in this position.

EBS/8: In EBS-loc family was identified to carry the recurrent mutation p.R388C of *KRT14*.

EBS/9: The probands' sample DNA of the EBS-DM affected carried the heterozygous G>A transition (c.1231G>A) in exon 6 of *KRT14*. The deduced novel p.E411K mutation results in the change of an acidic to basic characterized residue. A new MboII restriction site is also generated. Interestingly, only the probands were affected, mutations were absent in the parents' DNA samples. Our hypothesis of a de novo mutation was confirmed. Gonadal mosaicism of the parents can not be excluded. Another alterations of codon 411 of the *KRT14* gene (p.E411X, Glu411del (c.1231_1233delGAG)) has been described previously in association with EBS-gen-nonDM and EBS-loc phenotypes.

EBS/10: In a three-generation EBS-DM family, the affected members were found to be heterozygous for a novel p.I412N mutation located in exon 6 of *KRT14*. Sequence analysis revealed a T>A transversion (c.1235T>A) which altered Ileu> Asn in codon 412. The mutation resulted in the loss of a Sau3AI restriction site. This novel mutation (p.I412N) is the first alteration found in this position of the sequence.

Polymorphisms in the *KRT5* and *KRT14* genes

In addition, six different polymorphisms in *KRT5* (c.156C>A/p.A52A; p.L117L/c.C351T; c.382G>C/p.G128R; c.413G>A/p.G138E; c.513G>A/p.Q171Q; c.630T>C/p.T210T;). and one polymorphism in *KRT14* (c.369T>C/p.N123N) gene were identified and their frequencies determined in the normal population.

V. CONCLUSION

- Our studies point out to establish the diagnosis it is essential to eliminate reliably the *KRT14* pseudogene, which is an extra step for the analyzers doing restriction enzyme-digestion mostly. Our results prove the restriction enzyme-digestion method is not a reliable approach for the elimination of the pseudogene. In our work we used a new method for the effective and fast elimination of *KRT14* gene and pseudogene. At the cutting out of the *KRT14* pseudogene and the mutation analysis of *KRT14* we developed a simple allele-specific method to add the safely, exact diagnosis-establishment of EBS patient. With this method not only the patients but their affected and non-affected family members may be examine easily, quickly, simply. This method made the mutation analysis of *KRT14* easier and more cost effective compared to previously described methods. The biggest advantage of this approach that it is non-invasive. Because of its simplicity it makes possibilities doing international population studies. After publishing our study two another studies came out about the same method. In the international literature these publications reinforce and confirm our approach of *KRT14* pseudogene elimination.
- Using this approach we were able to identify 6 novel and 4 previously described mutations as well as 8 polymorphisms in *KRT5* and *KRT14* genes. The detected known *KRT5* and *KRT14* mutations are suggested that they play an important role of the EBS pathomechanism confirming international experiences. The identified 6 new missense mutations add to different databases on mutation enlarging their information volume. Our report gives further knowledge about the correlation of protein-structure functions.

- In the case of all identified new mutations we checked the frequency of nucleotide changes in 100 unrelated, healthy control individuals. Because of all identified new mutations were detected only the probands, the results confirm and exclude the possibility of polymorphisms.

- Studying the *KRT5* and *KRT14* genes from the EBS families, several polymorphisms were identified both in the affected and in the unaffected individuals. The frequency of these allele variations was determined in 100 unrelated control individuals (200 alleles). From our results it is established that all the identified polymorphisms are common in the Hungarian population.
- The identified genetic differences contain unique mutation spectrum, which inland specifics contribute to the deeper understanding of pathomechanisms of the EBS background and the EBS genotype–phenotype relationship.

- The individual genetic examinations have diagnostic value, which help to establish correct diagnosis and do genetic counseling for affected families and as well as apply prevention. Our results may pave the way for gene therapy.

VI. PUBLICATIONS IN THE FIELD OF THE THESIS

VI.1. Published author articles related to the subject of the thesis

1. **Glász-Bóna A**, Medvecz M, Sajó R, Lepesi-Benkő R, Tulassay Z, Katona M, Hatvani Zs, Blazsek A, Kárpáti S. (2009) Easy method for keratin 14 gene amplification to exclude pseudogene sequences: new keratin 5 and 14 mutations in epidermolysis bullosa simplex. Journal of Investigative Dermatology, 129(1):229-31. **IF: 5,543**

2. **Glász-Bóna A**, Medvecz M, Virágh Zs, Hatvani Zs, Blazsek A, Kárpáti S. (2010) Epidermolysis bullosa simplex with mottled pigmentation – mutation analysis proved the diagnosis in a four-generation pedigree. European Journal of Dermatology **IF: 2,251** (in press)

3. Csikós M, Becker K, Rácz E, **Bóna A**, Benkő R, Czippán Á, Katona M, Bruckner-Tuderman L, Kárpáti S, Horváth A. (2004) Herediter epidermolysis bullosa molekuláris genetikai vizsgálata. Bőrgyógyászati és Venerológiai Szemle; 80: 195-202.

VI.2. Published abstracts related to the subject of the thesis

1. **Bóna A**, Csikós M, Sajó R, Horváth A, Kárpáti S. (2004) Mutation analysis of keratin 5 and keratin 14 genes in patients with Epidermolysis bullosa simplex. J Invest Dermatol, 123(2): 135.

2. **Bóna A**, Csikós M, Sajó R, Benkő R, Kárpáti S. (2004) Epidermolysis bullosa simplex genetikai hátterének vizsgálata. Bőrgyógyászati és Venerológiai Szemle, 80: 295.

3. **Bóna A**, Csikós M, Sajó R, Kárpáti S. (2005) Identification of Novel and Known Mutations in Keratin 5 and 14 Genes in Weber-Cockayne Subtype Epidermolysis Bullosa Simplex. J Invest Dermatol, 125(Supl.1s): 85.

4. **Bóna A,** Sajó R, Blazsek A, Lepesi-Benkő R, Medvecz M, Kárpáti S. (2007) Epidermolysis bullosa simplex: új mutációk a magyar populációban. Bőrgyógyászati és Venerológiai Szemle, 83: 214.

5. **Bóna A**, Medvecz M, Németh M, Hatvani Z, Sajó R, Lepesi-Benko R, Tulassay Z, Katona M, Blazsek A and Kárpáti S. (2008) Keratin 5 (*KRT5*) and keratin 14 (*KRT14*) mutation analysis by a novel approach. J Invest Dermatol, 128(Supl.1s): 733.

6. Blazsek A, Virágh Zs, Németh M, Hatvani Zs, Lepesi-Benkő R, **Bóna A**, Medvecz M, Kárpáti S. (2007) Mutációanalízis az epidermolysis bullosa simplex egy ritka altípusában: Epidermolysis bullosa simplex with mottled pigmentation Magyarországon. Bőrgyógyászati és Venerológiai Szemle, 83: 214.

7. Blazsek A, Virágh Zs, Németh M, Hatvani Zs, Barna K, Lepesi-Benkő R, **Bóna A**, Katona M, Medvecz M, Kárpáti S. (2008) Epidermolysis bullosa simplex with mottled pigmentation – analysis of a four-generation Hungarian case. J Invest Dermatol, 128(Supl.1s): 734.

VI.3. Published abstracts not related to the subject of the thesis

1. Csikós M, Rácz E, Benkő R, **Bóna A**, Lászik A, Szakács O, Horváth A, Kárpáti S. (2004) Maternal Germline Mosaicism, *LAMB3* hotspot mutation R635X and Prenatal Testing in Herlitz Junctional Epidermolysis Bullosa. J Invest Dermatol, 123(2): 133.

2. Becker K, **Bóna A**, Csikós M, Kárpáti S. (2004) Two Novel Mutations in the *EBP* gene in Conradi-Hünermann-Happle Syndrome. J Invest Dermatol, 123(2): 136.

3. Csikós M, Rácz E, Benkő R, **Bóna A**, Bégány Á, Horváth A, Kárpáti S. (2003) Sikeres DNS-alapú prenatális diagnózis epidermolysis bullosa junctionalis (Herlitz) variánsban. Bőrgyógyászati és Venerológiai Szemle, 79: 241.

4. Benkő R, Csikós M, Sárdy M, **Bóna A**, Kárpáti S. (2004) A SPINK5 gén mutációanalízise Comel-Netherton szindrómában. Bőrgyógyászati és Venerológiai Szemle, 80: 295.

5. Lepesi-Benő R, Medvecz M, Becker K, Sardy M, **Bóna A**, Blazsek A, Sajo R, Hatvani Zs, Kornsee Z, Kárpáti S. (2006) Mutation Analysis of Patients with Comel-Netherton Syndrome in Hungary. J Invest Dermatol, 126(Supl.3s): 221.

6. Blazsek A, Németh M, Hatvani Zs, Lepesi-Benkő R, **Bóna A**, Medvecz M, Kárpáti S. (2007) Multi-locus genetic analysis of monilethrix in a 3 generation Hungarian pedigree. J Invest Dermatol, 127(Supl.2s): 502.

7. Blazsek A, Virágh Zs, **Glász-Bóna A**, Wikonkál N, Hársing J, Mazán M, Hatvani Zs, Medvecz M, Kárpáti S. (2009) Dowling Degos Disease – Diagnosis confirmed by the identification of a recurrent *KRT5* mutation. J Invest Dermatol, 129(Supl.2s)

VI.4. Oral presentations and posters related to the subject of the thesis

1. **Bóna A**, Csikós M, Sajó R, Kárpáti S, Horváth A. Keratin 5 és kertain 14 gének mutációanalízise epidermolysis bullosa simplex kórképek hátterében. (poszter), IV. Magyar Sejtanalitikai Konferencia Budapest, 2004. május 6-8.

2. **Bóna A**, Csikós M, Sajó R, Kárpáti S, Horváth A. Novel keratin 5 and keratin 14 genes mutation in patients with Epidermolysis bullosa simplex. (előadás), 3th Congress of the German-Hungarian Dermatologic Society (DUDG), Pécs, 2004. augusztus 27-29.

3. **Bóna A**, Medvecz M, Németh M, Hatvani Zs, Sajó R, Lepesi-Benkő R, Tulassay Zs, Katona M, Blazsek A, Kárpáti S. Keratin 5 (*KRT5*) and keratin 14 (*KRT14*) mutation analysis by a novel approach. (poszter) - **I. díj,** 7th Congress of the German-Hungarian Dermatologic Society (DUDG), Budapest, 2008. június 19-21.

4. **Bóna A**, Sajó R, Blazsek A, Lepesi-Benkő R, Hatvani Zs, Virágh Zs, Németh M, Medvecz M, Kárpáti S. Epidermális keratin mutációk vizsgálata genodermatózisokban. (előadás), Magyar Humángenetikusok VII. Munkakonferenciája, Pécs, 2008. július 11-13.

5. **Glász-Bóna A,** Blazsek A, Hatvani Zs, Virágh Zs, Medvecz M, Kárpáti S. Epidermolysis bullosa simplex mottled pigmentációval (EBS-MP) – Az első magyarországi eset mutációanalízise. (poszter), Magyar Humángenetikusok VIII. Munkakonferenciája, Debrecen, 2010. szeptember 2-4.