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Multigene Next-Generation Sequencing Panel Identifies Pathogenic Variants in Patients with Unknown Subtype of Epidermolysis Bullosa: Subclassification with Prognostic Implications

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TO THE EDITOR

The heritable forms of epidermolysis bullosa (EB) make up a phenotypically and genotypically heterogeneous group of blistering disorders. EB was initially divided into three broad categories, namely, simplex (EBS), junctional (JEB), and dystrophic (DEB), based on the topographic location of tissue separation within the dermal-epidermal basement membrane zone. More recently, Kindler syndrome, depicting multiple levels of tissue separation, was added as the fourth major subtype of EB (Fine et al., 2014). Subsequently, however, a number of clinical variants with refined phenotypic features were noted. The phenotypic variability encountered in EB was subsequently shown to reflect genotypic heterogeneity, and the current database consists of mutations in a total of 19 distinct genes within the EB spectrum (Lee et al., 2017; Uitto et al., 2017).

We have recently developed a disease-specific, gene-targeted panel next-generation sequencing for identification of pathogenic variants in EB. This panel covers 21 distinct genes, 18 of them shown to harbor causative mutations in EB and three of them involved in differential diagnosis of skin fragility disorders. We tested this panel in a group of patients representative of a large multiethnic cohort of EB patients in Iran. Most patients had EB of undefined subtype, and in some cases only archived DNA was available. This study was approved by the institutional review board of the Pasteur Institute of Iran, and all subjects and parents of underage patients gave written informed consent to participate in research and gave permission to publish their images. DNA was extracted from peripheral blood samples from patients, their parents, and other clinically affected and unaffected family members, including siblings (if available), using a QIAamp DNA Blood Mini Kit (Qiagen, Valencia, CA) or by salting-out method. For DNA, target enrichment was performed using the TruSeq Custom Amplicon kit (Illumina Inc., San Diego, CA). DesignStudio (Illumina Inc.) was used for library design. All exons, at least 20 base pairs of the intron at each intron-exon boundary, and up to 50 base pairs of 3′- and 5′-UTRs were targeted. The designed library contained the genes *CD151, CDSN, CHST8, COL17A1, COL7A1, DSP, DST, EXPH5, FERMT1, ITGA3, ITGA6, ITGB4, JUP, KRT5, KRT14, LAMA3, LAMB3, LAMC2, PKP1, PLEC,* and *TGM5,* divided into 421 targets covered by 968 amplicon probes, which were designed to cover 99% of targeted bases. A total of 36,724,892 reads were aligned to the human genome, with the mean coverage of the target region being ×424.

A total of 91 probands representing 91 families with a total of 133 affected individuals were studied; 43% of patients were female. Patients' ages at the time of the study ranged from 4 days to 55 years. Among them, 38 were infants (<1 year of age), and seven had already died from complications of the disease at the time of the study, but DNA was available for analysis. All patients had a tentative diagnosis of EB, based on neonatal blistering and erosions of the skin and oral mucous membranes, associated in some cases with aplasia cutis congenita and developing scarring alopecia, paronychia, and dental abnormalities (see Supplementary Figure S1 online). Although EB was suspected based on clinical presentation, no definitive subclassification could be made in 87 patients. Among the families, 79 showed parental consanguinity, accounting for 87% of all patients. This is significantly higher than the general rate of consanguinity in Iran, 39% (Saadat et al., 2004), suggesting that most causative mutations in these families would be homozygous.

Bioinformatics analysis of the sequencing results, followed by manual interrogation of the original binary alignment map files with assistance of Integrative Genomics Viewer, version 2.3.93 (Broad Institute, Boston, MA), identified a

Abbreviations: EB, epidermolysis bullosa; EBS, epidermolysis bullosa simplex; JEB, junctional epidermolysis bullosa; DEB, dystrophic epidermolysis bullosa

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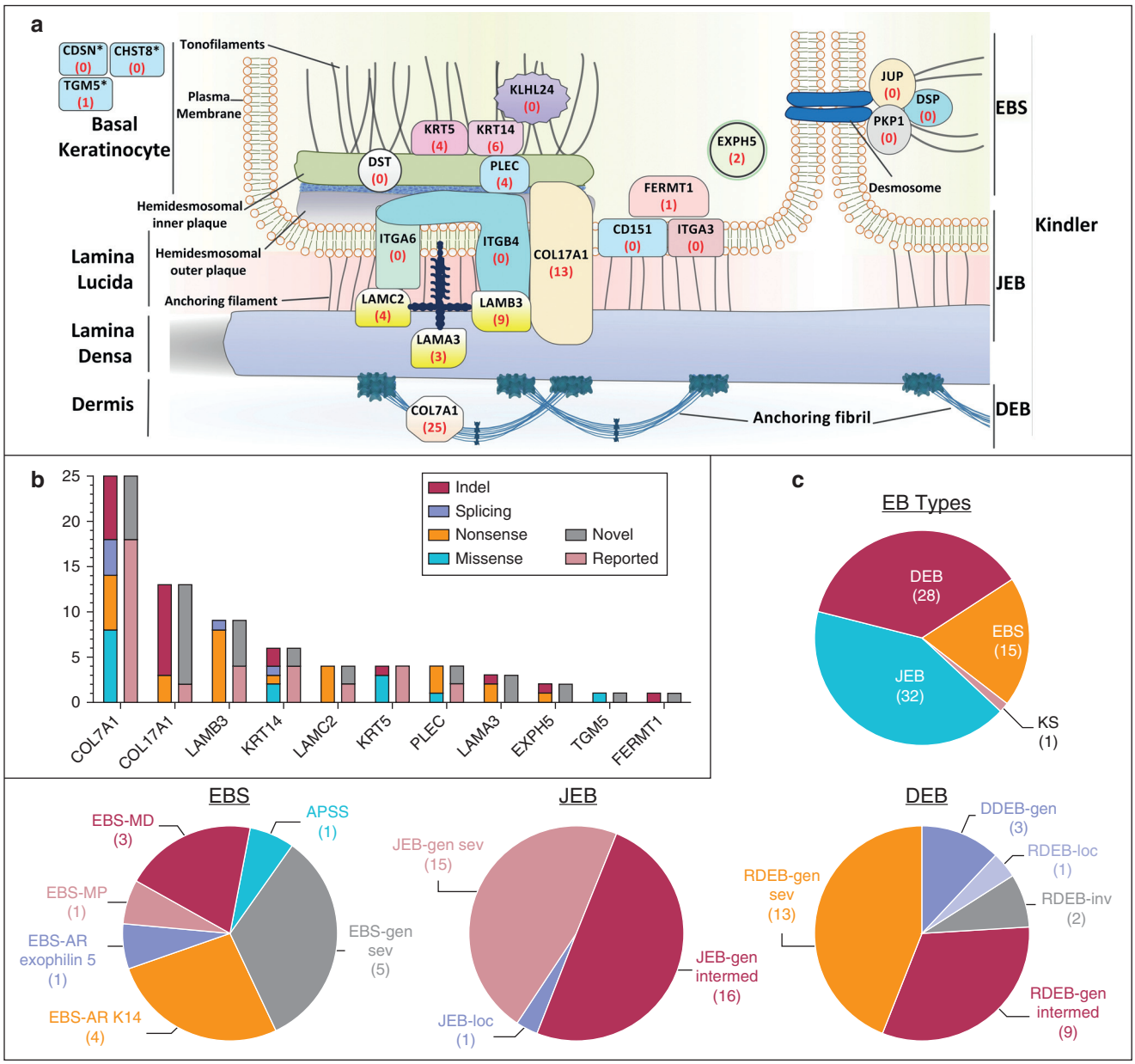

Figure 1. Schematic presentation of the protein products of EB-related genes within the cutaneous basement membrane zone, characteristics of the mutations and the predicted subtypes of EB. (**a**) The number of causative mutations identified in each gene in the patients in this study are included in parentheses. Note that *CDSN*, *CHST8,* and *TGM5* are expressed primarily in the stratum granulosum and stratum corneum and are associated with acral peeling skin syndrome, which is in the differential diagnosis of EB. (**b**) Distribution of causative mutations identified in patients with EB. For each gene, the bar graph on the left displays the breakdown of types of mutations identified, and the bar graph on the right displays the number of novel versus previously reported mutations in each gene. (**c**) Diagnosis of EB subtypes in patients included in the panel, using a combination of the types of mutations and consistent phenotypic characteristics. The top right pie chart displays classification by major types of EB, and the bottom three charts represent the number of patients diagnosed with subtypes within each major category (EBS, JEB, and DEB, from left to right). DEB, dystrophic epidermolysis bullosa; EB, epidermolysis bullosa; EBS, epidermolysis bullosa simplex; JEB, junctional epidermolysis bullosa.

number of sequence variants in all 21 genes. After classification of variants and their confirmation by Sanger sequencing in each patient and in his/her relatives as appropriate, 72 pathogenic or likely pathogenic variants, according to American College of Medical Genetics and Genomics criteria (Richards et al., 2015), were identified in 68 families in 11 distinct genes (Figure 1a, Supplementary Table S1 online). Among these 72 mutations, 35 (48.6%) were previously unreported to our knowledge, based on analysis of the EB literature and the human gene mutation database (http://www.hgmd.cf.ac.uk). The distribution of novel versus previously reported mutations, along with the types of mutations identified in each gene, is indicated in Figure 1b. Identification of mutations in the genes previously associated with EB, including eight variants of unknown significance, in combination with clinical evaluation and segregation studies, allowed us to predict the subtype of EB in 76 out of 91 families, with an efficiency of 83.5% (Figure 1b).

In case of EBS, six patients harbored pathogenic *KRT14* mutations. Autosomal recessive EBS due to homozygous *KRT14*

mutations was found in four families, and two heterozygous de novo mutations in autosomal dominant families were encountered. The phenotype in all *KRT14* patients was characterized by generalized trauma-induced blistering with minimal nail dystrophy. Heterozygous pathogenic variants in the *KRT5* gene were identified in four patients, three of which were determined to be de novo. In three of these patients, the identified variants in *KRT5* were previously associated with EBS, and when combined with a phenotype of generalized blistering and moderate nail dystrophy without extracutaneous findings, a diagnosis of EBS generalized severe was made. The fourth patient had a phenotype of blistering along with a reticulated pattern of hyperpigmented macules and patches. The variant, c.1649delG in *KRT5*, has previously been associated with EBS with mottled pigmentation. This variant has additionally been associated with EBS with migratory circinate erythema (Gu et al., 2003), but such a phenotype was not present in our patient. Combining the patient's phenotype with the genetic findings, a final diagnosis of EBS with mottled pigmentation was made. Two of the three patients with de novo *KRT5* mutations were born to consanguineous parents.

Mutations in other EBS-related genes were also found in *EXPH5* (two families), *TGM5* (one family), and *PLEC* (three families). The mutation in *TGM5* was found in a patient with mild blistering of the hands, consistent with a diagnosis of acral peeling skin syndrome. In three families, *PLEC* mutations were noted, and in two of them, examination of the probands at the ages of 8 and 6 years, respectively, showed evidence of muscle weakness of the upper extremities, consistent with EBS with muscular dystrophy. In another family with *PLEC* mutations, the proband was only 28 days old, apparently with no evidence of muscle weakness. Given the strong association between *PLEC* mutations and muscular dystrophy, as well as reports of delayed presentation of the muscle findings as late as the fourth decade of life (Pulkkinen et al., 1996), the patient was considered to have EBS with muscular dystrophy and was referred for additional workup.

Out of 32 patients with JEB, 15 patients had a diagnosis of generalized severe subtype, based on homozygous loss-of-function mutations either in *LAMA3*, *LAMB3*, or *LAMC2*, which encode the subunits of laminin 332. In fact, five of these patients had died before 1 year of age. Seventeen patients with JEB generalized intermediate were characterized by mutations in either the corresponding genes encoding laminin 332 subunit polypeptides or in *COL17A1*, most of them being previously unreported to our knowledge (Figure 1b).

In patients with DEB, 25 pathogenic/likely pathogenic mutations were identified in *COL7A1*, seven of them previously unreported (Figure 1b). Thirteen families were classified as generalized severe because of homozygous premature termination codon-causing mutations (five nonsense, five splicing, and three indel) in *COL7A1*. These patients are predicted to develop severe mutilating scarring because of extensive skin fibrosis, frequently associated with development of aggressive squamous cell carcinomas. A milder phenotype, recessive DEB generalized intermediate, was predicted in nine patients because of the presence of missense, indel, nonsense, or splicing mutations in one or both *COL7A1* alleles. Those patients with frameshift or nonsense mutations with an intermediate phenotype are predicted to show a more severe phenotype as they age. In three families, the phenotype was classified as dominant DEB localized, accompanied by heterozygous de novo *COL7A1* mutations. In one family, a large homozygous deletion mutation in the *FERMT1* gene was noted, consistent with the diagnosis of Kindler syndrome (Figure 1b).

Since the development of this panel, an additional causative gene, *KLHL24*, has been identified in EBS (Has, 2017; He et al., 2016; Lee et al., 2017; Lin et al., 2016). All causative mutations identified in this gene have been located on the initiation codon of translation. Therefore, in the 15 patients in this panel in whom no mutations were found, sequencing of this site on the *KLHL24* gene was performed. None of the 15 patients harbored a mutation at this site.

The importance of identifying specific mutations in families with EB is emphasized by several considerations. First, the information can be used for early prognostication of the progression and the overall outcome of the disease. Second, identification of the mutant genes and specific mutations can be used for prenatal testing and preimplantation genetic diagnosis (Pfendner et al., 2003). Third, knowledge of the mutations allows screening for heterozygous carriers in large consanguineous families, with implications for genetic counseling. Finally, although EB is currently an intractable disorder, recent preclinical studies have suggested new avenues for potential treatment, some of which are dependent on the knowledge of the types of mutations (Atanasova et al., 2017; Woodley et al., 2017).

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

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Nuclear Proteins and Apoptotic Bodies Are Found in the Lupus Band of Patients with Cutaneous Lupus Erythematosus

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TO THE EDITOR

The lupus band (LB) of deposited immunoglobulins and complement at the dermoepidermal junction (DEJ) detected by direct immunofluorescence can aid to the diagnosis of cutaneous lupus erythematosus (Burnham et al., 1963; Harrist and Mihm, 1980). However, little is known about the pathogenesis. Nuclear autoantigens from apoptotic cells may drive the immunological response in systemic lupus erythematosus, and several antinuclear antibodies are associated with systemic lupus erythematosus. Assessing apoptosis at the DEJ and identifying the antigens in the LB might provide valuable insight into the pathogenesis.

We retrospectively selected biopsies from patients collected between 2002 and 2014 in the biobank of the Groningen Center for Blistering Diseases with a positive LB on sun-exposed lesional or nonlesional skin (Harrist and Mihm, 1980) and clinical features compatible with cutaneous lupus erythematosus and/or systemic lupus erythematosus (Supplementary Table S1 online).

To investigate apoptotic DNA fragments at the DEJ, the sections were stained by the deoxynucleotidyl transferase fluorescein-dUTP nick end labeling. Sections were double stained with IgM or IgG and antibodies against the nuclear antigens dsDNA, Ro/SSA (Ro52), La/SSB, U1 RNP, and Smith (Supplementary Table S2 online). Skin biopsies of sun-exposed normal skin as well as skin biopsies of three patients with pemphigus erythematosus and a pseudo "LB" were included as control specimens (Oktarina et al., 2012). In addition, three randomly selected biopsies were double stained for deposited IgM together with monoclonal antibodies against desmoglein 1, desmoglein 3, type XVII collagen, type IV collagen, and type VII collagen.

In 10 of 19 skin biopsies (53%), deoxynucleotidyl transferase fluorescein-dUTP nick end labeling positive cell fragments were found at the DEJ (Supplementary Table S1). In 9 of 19 skin biopsies (47%), one or more nuclear antigens were demonstrated at the DEJ and showed colocalization

Abbreviations: DEJ, dermoepidermal junction; LB, lupus band

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