The practical diagnostic approach for hereditary epidermolysis bullosa in the era of next generation sequencing

Bryan Edgar K. Guevara[a,b], Wei-Ting Tua, Ping-Chen Hou[c], Hsin-Yu Huangc, Jessica Chen[d], Chao-Kai Hsu[a,d,e]

The new era of molecular diagnostics has provided new insights in both routine clinical work and research in hereditary epidermolysis bullosa (EB). Several different approaches and techniques have provided significant advantages in terms of diagnostic accuracy, predict- ing prognoses, clarifying the pathogenesis, and developing new therapies. In many developing countries, however, modern laboratory techniques remain inaccessible. Therefore, a practical diagnostic matrix has been developed to predict the diagnosis and subtype of EB. In this review, we highlight the molecular and practical techniques in diagnosing hereditary EB.

Keywords: Epidermolysis Bullosa

INTRODUCTION

Epidermolysis bullosa (EB) is a clinically heterogeneous group of rare inherited disorders characterized by marked skin fra- gility with blistering following minor trauma. It is classified into four main types (simplex, junctional, dystrophic, and Kindler syn- drome) with more than 30 subtypes. [1] This classification system follows an "onion approach" which considers the EB type, mode of inheritance, phenotype, immunofluorescence antigen map- ping, and mutation(s) in a patient with suspected EB. According to the 2014 consensus report on EB, there are at least 33 phe- notypic variants with 18 candidate genes.[1] KLHL24 and CD151 are two additional candidate genes that have been identified in recent years.[2-5] Therefore, at least 20 candidate genes are now re- sponsible for the pathogenesis of EB. Furthermore, we will expect new developments in EB classification and pathogenesis from the incoming consensus report in 2019.

Molecular techniques have enriched the atlas of derma- tology by directly aiding in discovering new diseases, defining dis- ease mechanisms, establishing disease models, and testing novel therapies. One of the most impactful developments of molecular dermatology has been Next Generation Sequencing (NGS). NGS has broadened our understanding of EB and improved our ability to diagnose it accurately. In resource-limited settings, this technol- ogy remains inaccessible due to its high cost, lack of facilities, and deficient training opportunities. Therefore, diagnosis of EB still relies on clinical acumen and use of histopathology, which is fre- quently inaccurate due to the heterogenic nature of EB. The cost of NGS is currently plummeting and will continue to become more affordable as the technology matures and as new techniques are developed in the future. Consequently, it will become an essential component of the dermatologist's arsenal for diagnosing difficult and rare dermatologic conditions.

Herein, we review the molecular and practical approaches for di- agnosing EB.

a. Department of Dermatology, National Cheng Kung University Hospital, College of Medicine, National Cheng Kung University, Tainan, Taiwan
b. Department of Dermatology, Southern Philippines Medical Center, Davao, Philippines
c. School of Medicine, College of Medicine, National Cheng Kung University, Tainan, Taiwan
d. Institute of Clinical Medicine, College of Medicine, National Cheng Kung University, Tainan, Taiwan.
e. International Research Center of Wound Repair and Regeneration (iWRR), National Cheng Kung University, Tainan, Taiwan.

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Corresponding author: Chao-Kai Hsu, MD PhD Email: kylehsu@mail.ncku.edu.tw

Next generation sequencing

The Human Genome Project (HGP) was an international collaborative research program designed to sequence the entire human genome. It was finally completed after more than a de- cade, using a first-generation sequencing technique known as Sanger sequencing.[6] Its success is regarded as one of the most critical accomplishments in medicine and scientific research. Sub- sequently, the second-generation sequencing method known as NGS was developed. NGS utilizes a high-throughput approach to sequencing called Massively Parallel Sequencing, which allows the whole genome to be completely sequenced in less than one day.[7] This technology has enabled the identification of new caus- ative genes in previously identified diseases and novel diseases alike. The "exome" refers to the component of the genome that predominantly encodes proteins. In the human genome, there are 180,000 exons arranged in approximately 22,000 genes. Al- though the exome comprises approximately 1-2% of the whole genome, the most interpretable mutations that alter phenotypes are located in the exome. Therefore, DNA sequencing that targets the exons within all genes of the genome – i.e., whole exome se- quencing (WES) – is thought to be an efficient method of analyz- ing a patient's DNA to discover the genetic basis of diseases.[8] The advantages that WES confers not only enables identification of novel genes with greater efficiency, but also enhances diagnostic accuracy and is contributing towards the development of person- alized medicine.

Novel genes for hereditary epidermolysis bullosa

New genes and clinical phenotypes of EB have been characterized since the last classification published in 2014.[1] In 2016, two separate groups reported that KLHL24 mutations in- duced human skin fragility.[2,3] Furthermore, mutations in this new candidate gene was also identified in seven cases out of the 183 cases of EB simplex with no other identified pathogenic muta- tion.[4] KLHL24 encodes for Kelch-like protein 24 (KLHL24), which

becomes over-stabilized through abolished autoubiquitination. This leads to excessive ubiquitination and degradation of Keratin 14, compromising the intermediate filaments of basal keratino- cytes.[2] Varying clinical features secondary to KLHL24 mutations were described.[2-4] The patients with KLHL24 mutations have atendency to produce trauma-induced blistering and atrophic scarring, which occur particularly on the lower legs and persist throughout childhood after minor trauma. The severity of blister- ing tends to lessen with increasing age. Nail defects and oral ulcer- ation are common findings as well, while dyspigmentation is not a prominent feature. These reports describe contradictory findings regarding hair phenotype.[2-4] KLHL24 also plays a crucial role in car- diac development and function[9], with several reports discovering that KLHL24 mutations may induce hypertrophic cardiomyopathy in humans.[10,11] Therefore, close clinical follow-up and functional studies of the cardiac status EBS patients with KLHL24 mutations is warranted.

Recently, mutations in CD151 was identified using WES in a patient with skin fragility and nephropathy.[5] The proband mani- fested with a Kindler syndrome-like phenotype with generalized blistering, particularly on pretibial areas, poikiloderma, acrogeria, nail dystrophy, alopecia, esophageal webbing, and strictures, as well as nephropathy manifesting with proteinuria. Tetraspanin CD151 is an endogenous component of the basement membrane, which forms laminin-binding complexes with α3β1 and α6β4 inte- grins of the skin and kidneys.[12] This suggests CD151 could be the 20th gene implicated in hereditary EB.

Uncovering these novel findings, including those from previously unsolved cases of EB (up to 20%), was only possible due to NGS.[4] NGS – particularly WES – further expands our understanding of the molecular basis of EB and adds new genetic subtypes in the current classification ofEB.

Clinical diagnostic matrix

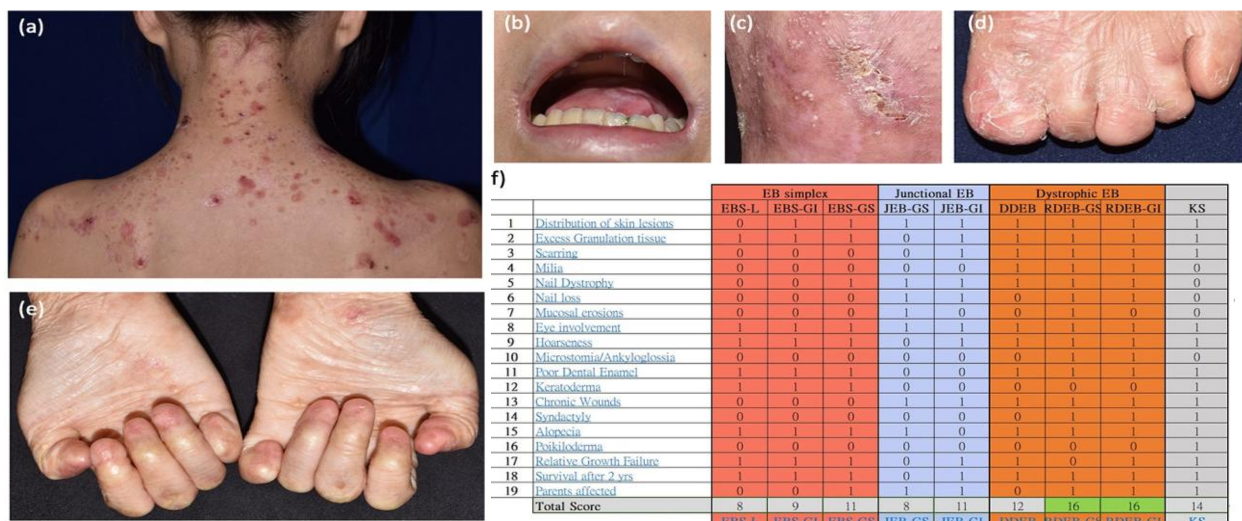
The clinical manifestations of EB are diverse, with at least 33 variants (9 major) and more being discovered at present. As a result, accurately diagnosing EB and the correct subtype is dif- ficult, but critical for management and genetic counseling. This usually requires expert clinicians and laboratories performing im- munofluorescence mapping, electron microscopy, and NGS with multi-gene panels. It is clear that a more practical approach is re- quired for accurately diagnosing EB in resource-limited countries.

A simple diagnostic tool was developed by Yenamandra et al. in 2017[13], which accurately predicts and classifies a patient to one of the nine major subtypes of EB. It quantifies 19 clini- cal features of EB against a scale, which can then be formulated into a single score via a matrix. This score is then used to pre- dict the most likely EB subtype displayed by the patient. This tool utilizes illustrations to help clinicians identify the relevant clinical features with greater ease. It has been demonstrated that use of this diagnostic matrix tool is an effective way to improve diagnos- tic accuracy in clinics with limited facilities. It was tested against 74 patients with a molecular diagnosis of EB, and demonstrated a concordance of 91.9%.[13] However, its diagnostic accuracy de- creases when used on patients less than six months of age and with individuals expressing mild manifestations where clinical assessment would be less precise. This diagnostic matrix tool is available freely online and has proven to be useful among derma- tologists and EB nurses all around the world.

Case study

A 14-year-old female in a non-consanguineous family presented with generalized blisters, erosions, and atrophic skin since birth. She also displayed microstomia, milia, syndactyly, and anonychia (Figure 1a-e).

After her clinical data was recorded in the diagnostic matrix tool, a score of 16 was determined in both recessive dystrophic-gen- eralized severe (RD-GS) EB and recessive dystrophic-generalized intermediate (RD-GI) EB (Figure 1f). The family pedigree demon- strates that other family members did not exhibit similar clini- cal findings with the index patient (Figure 2a). An ultrastructural



		EB simplex			Junctional EB		Dystrophic EB			
		EBS-L	EBS-GI	EBS-GS	JEB-GS	JEB-GI	DDEB	RDEB-GS	RDEB-GI	KS
1	Distribution of skin lesions	0	1	1	1	1	1	1	1	1
2	Excess Granulation tissue	1	1	1	0	1	1	1	1	1
3	Scarring	0	0	0	0	1	1	1	1	1
4	Milia	0	0	0	0	0	1	1	1	0
5	Nail Dystrophy	0	0	1	1	1	1	1	1	0
6	Nail loss	0	0	0	1	1	0	1	1	0
7	Mucosal erosions	0	0	0	1	0	0	1	0	0
8	Eye involvement	1	1	1	1	1	1	1	1	1
9	Hoarseness	1	1	1	0	1	1	1	1	1
10	Microstomia/Ankyloglossia	0	0	0	0	0	0	1	1	0
11	Poor Dental Enamel	1	1	1	0	0	1	1	1	1
12	Keratoderma	1	1	1	0	0	0	0	0	1
13	Chronic Wounds	0	0	0	1	1	1	1	1	1
14	Syndactyly	0	0	0	0	0	0	1	1	1
15	Alopecia	1	1	1	1	0	1	1	1	1
16	Poikiloderma	0	0	0	0	0	0	0	0	1
17	Relative Growth Failure	1	1	1	0	1	1	0	1	1
18	Survival after 2 yrs	1	1	1	0	1	1	1	1	1
19	Parents affected	0	0	1	1	1	0	1	1	1
	Total Score	8	9	11	8	11	12	16	16	14
		EBS-L	EBS-GI	EBS-GS	JEB-GS	JEB-GI	DDEB	RDEB-GS	RDEB-GI	KS

Figure 1. The patient manifesting with multiple blisters, erosions, and atrophic scars (a), microstomia and dental caries (b), milia formation (c), syndactyly and anonychia (d,e); Clinical diagnostic matrix revealing a total score of 16 for both Recessive Dystrophic EB-Generalized Severe and Intermediate EB (f).

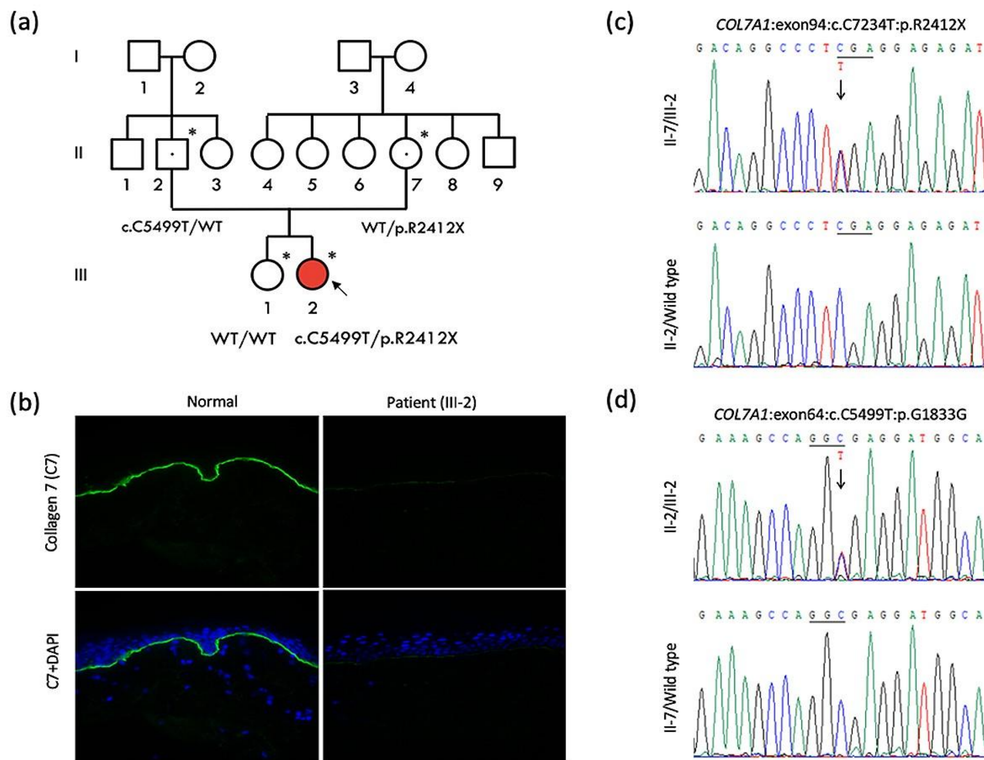


Figure 2. Family pedigree highlighting that other family members do not exhibit similar phenotypic manifestations. However, the father (II-2) and mother (II-7) each harbors a heterozygous mutation in COL7A1, while the proband (III-2) carried both mutations. (a); The staining of type VII collagen is reduced compared with the control skin, consistent with recessive dystrophic EB (40X; green: type VII collagen; blue: 4',6-diamidino-2-phenylindole, DAPI) (b); Sanger sequencing reveals the mutation, c.7234C>T (p.Arg2412Ter), on exon 94 of the COL7A1 gene is present in III-2 (proband) and II-7 (mother) (c); while the mutation, c.5499C>T (p.Gly1833Gly), on exon 64 of the COL7A1 is present in III-2 (proband) and II-2 (father) (d).

study of the patient's skin sample revealed poorly formed and sparse anchoring fibrils, while immunofluorescence mapping revealed reduced protein levels of type VII collagen (Figure 2b). Whole exome sequencing identified two heterozygous mutations in COL7A1 – a previously reported nonsense mutation, c.7234C>T (p.Arg2412Ter)14, and a previously reported splice-site mutation, c.5499C>T (p.Gly1834Gly). This splice-site mutation results in the deletion of 35 bases and a subsequent frameshift, ultimate- ly producing a premature stop codon 25 amino acids upstream (p.E1834RfsX25).15 One of these mutations was identified in each parent using sanger sequencing (Figure 2c,d). In conclusion, the clinical, histopathological, and genetic findings were consis- tent with RDEB, as predicted by the diagnostic matrix tool. There- fore, we propose the use of this tool as a practical approach for the diagnosis of hereditary EB (Figure 3).

Conclusion

New molecular techniques in dermatology have further elucidated the pathogenesis of EB and discovered novel findings in addition to the already established classifications of EB. New technologies such as NGS/WES have made the accurate diagnosis of EB possible. Although these technologies are not yet accessible to all, a practical diagnostic tool that can accurately classify clinical presentations of EB currently does exist and is freely available on- line. Greater international collaboration and research is required to fully understand this debilitating disease, which is impacting patients on a global scale.

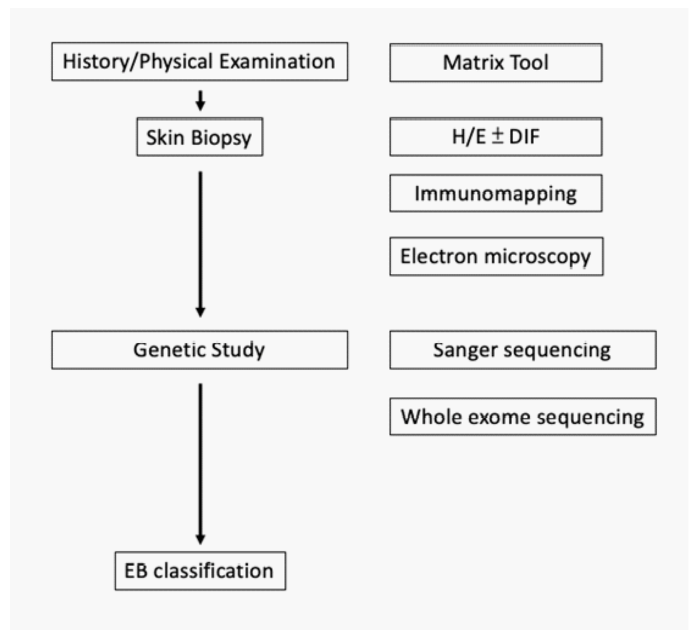


Figure 3. Diagnostic algorithm for hereditary EB

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