Whole-exome sequencing improves mutation detection in a diagnostic epidermolysis bullosa laboratory

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Whole-exome sequencing improves mutation detection in a diagnostic epidermolysis bullosa laboratory

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Key words: whole-exome sequencing, diagnosis, mutation, epidermolysis bullosa

What's already known about this topic?

- Skin microscopy and Sanger sequencing are useful techniques for the accurate diagnosis of specific subtypes of EB.
- The specificity and sensitivity of these current diagnostic tools is good although some cases of EB elude a precise laboratory diagnosis and the work involved is often time-consuming, labour-intensive and expensive.
- There is a need to refine and improve diagnostics for EB.

What does this study add?

- Whole-exome sequencing with bioinformatics support can identify mutations in cases of EB for which current diagnostic techniques fall short.
- Whole-exome sequencing has the potential to lessen the need for diagnostic skin biopsies in EB, as well as reducing laboratory costs.

- The adoption of whole-exome sequencing into routine laboratory EB diagnostics, however, still requires a reduction in the time needed for both sample processing and data interrogation to match current diagnostic tests for EB.

ABSTRACT

Background: Subtypes of inherited epidermolysis bullosa (EB) vary significantly in their clinical presentation and prognosis. Establishing an accurate diagnosis is important for genetic counselling and patient management. Current approaches in EB diagnostics involve skin biopsy for immunohistochemistry and transmission electron microscopy, as well as Sanger sequencing of candidate genes. Although informative in most cases, this approach can be expensive, laborious and may fail to identify pathogenic mutations in ~15% of cases.

Objective: Next generation DNA sequencing (NGS) technologies offer a fast and efficient complementary diagnostic strategy, but the value of NGS in EB diagnostics has yet to be explored. The aim of this study was to undertake whole-exome sequencing (WES) in 9 cases of EB in which established diagnostic methods failed to make a genetic diagnosis.

Methods: Whole-exome capture was performed using genomic DNA from each case of EB followed by massively parallel sequencing. Resulting reads were mapped to the human genome reference hg19. Potentially pathogenic mutations were subsequently confirmed by Sanger sequencing.

Results: Analysis of WES data disclosed biallelic pathogenic mutations in each case, with all mutations occurring in known EB genes (*LAMB3*, *PLEC*, *KIND1* and *COL7A1*). This study demonstrates that NGS can improve diagnostic sensitivity in EB compared to current laboratory practice.

Conclusions: With appropriate diagnostic platforms and bioinformatics support, WES is likely to increase mutation detection in cases of EB and improve EB diagnostic services, although skin biopsy remains an important diagnostic investigation in current clinical practice.

INTRODUCTION

Epidermolysis bullosa (EB) constitutes a diverse group of genodermatoses characterized by trauma-induced skin fragility, blisters and erosions.[1] Currently divided into 4 main subtypes (EB simplex, junctional EB, dystrophic EB and Kindler syndrome) based on the level of blister formation at or close to the dermal-epidermal junction (DEJ), the molecular pathology of EB now involves mutations in 18 genes.[1,2] Over the last 20 years, international consensus group meetings have been held, most recently in 2013,[1] to revise and update the diagnosis and classification of EB. New forms of EB have been added, some disease names have been changed, and recommendations have been made about the laboratory diagnosis of EB.[1]

Currently, the diagnosis of most cases of EB involves a skin biopsy. Typically, skin sections are stained with a panel of basement membrane zone antibodies and viewed by immunofluorescence microscopy (IFM). In many autosomal recessive forms of EB, the inherent loss-of-function mutations are likely to lead to a reduction or absence of immunolabelling for one particular protein, thus identifying the candidate gene for Sanger sequencing (SS).[3-5] In autosomal dominant forms of EB (and some autosomal recessive cases), however, IFM may not show clear differences from normal control skin, and further clues might be sought from transmission electron microscopy (TEM).[6] The overall objective is to determine which gene(s) to then investigate by SS. For the known EB genes, pairs of primers are designed to amplify individual exons and flanking introns. Polymerase chain reaction (PCR) products (typically 200-350 base pairs in size) are then individually examined by SS. PCR amplification protocols and primer pair sequences have been published for all known EB genes and similar protocols and reagents have been adopted into laboratory practice for diagnosing EB throughout the world. Several of the EB genes contain numerous

exons: for example, *COL7A1* (encoding type VII collagen, which is mutated in DEB) contains 118 exons and >70 PCR primer pairs are necessary for amplification of all exons and flanking introns.[7,8] To amplify all 18 genes implicated in the different forms of EB currently requires >400 primer pairs for genomic DNA analysis, hence the almost inevitable need for candidate gene clues from skin biopsies.

In 2004, we established a national diagnostic service for EB in the UK (designated The Robin Eady National Diagnostic Epidermolysis Bullosa Laboratory, and based at St Thomas' Hospital, London, UK). The approach to diagnosis in cases of EB has involved IFM, TEM and SS, as outlined above, but in a number of cases (perhaps ~15% of >1500 cases) these methods have failed to reveal any pathogenic mutations. Some of these cases may reflect erroneous clinical diagnoses by the referring clinicians but undoubtedly, technical limitations have also contributed to the sensitivity of current diagnostic methods.

One new technology that could potentially improve sensitivity in EB diagnostics is next-generation sequencing (NGS), in which the whole genome or a portion thereof is sequenced.[9,10] NGS has proven very useful for identifying novel genetic variants responsible for Mendelian disorders, including a new form of EBS.[11] However, although informative in a research setting, the diagnostic utility of NGS remains unclear.[12-14] To assess the potential impact of NGS on EB diagnostics, we undertook WES and bioinformatic data analysis on 9 autosomal recessive cases of EB in which current diagnostic strategies had failed to identify one or both of the pathogenic mutations.

Methodology

Cases for study

The 9 cases, with a clinical diagnosis of EB, that were selected for study were all routine diagnostic cases referred to the National Diagnostic EB Laboratory between 2008 and 2013 for which skin biopsy analysis and SS had failed to identify pathogenic mutations.

Whole-exome sequencing

Whole-exome capture was performed by in-solution hybridization using the SureSelect All Exon 50Mb Version 4.0 (Agilent) followed by massively parallel sequencing with 100-bp paired-end reads on the HiSeq2000 platform (Illumina). Resulting reads were mapped to the human genome reference hg19 using the novoalign (Novocraft Technologies Sdn Bhd, Selangor, Malaysia) alignment tool. Variant calling was undertaken at the individual sample level with the Samtools mpileup utility.[15] Resulting variant calls were filtered with the bcftools, filtered for a minimum coverage (calls with <4 reads filtered) and hard filtered for quality (variant with quality <20 filtered from further analysis). This high quality call set was then annotated with respect to the genes, and consequences on protein sequence and/or splicing with the Annovar tool.[16] Further annotation regarding previously reported observation of specific variants and estimated population frequencies was achieved through further rounds of Annovar annotation against dbSNP137, population frequency estimates from 1000 Genomes project http://www.1000genomes.org/, NHLBI GO Exome Sequencing Project (ESP) (https://esp.gs.washington.edu/drupal/), and ~1000 control exomes that have been processed through the same bioinformatics analysis pipeline. The exome data on all cases was deemed to be of very high quality. Over 8.6 gigabases of mappable sequence data was generated, such that >90% of the coding bases of the exome defined by the GENCODE Project were represented by at least 20 reads.

RESULTS

Case 1: Generalized intermediate JEB with elusive mutation in LAMB3

A 58-year-old white male had a clinical diagnosis of generalized intermediate JEB (previously known as non-Herlitz JEB)[1] characterized by life-long trauma-induced blistering and erosions (illustrated on the lower limbs in Figure 1), nail loss/dystrophy and some hair loss, along with two separate squamous cell carcinomas. Previous skin biopsy had shown a lamina lucida plane of cleavage by antigen mapping and markedly reduced intensity immunolabelling for laminin-332. SS of *LAMA3*, *LAMB3*, *LAMC2*, and subsequently *COL17A1*, *ITGA6* and *ITGB4*, however, did not reveal any mutations. By WES, we identified 236 novel heterozygous and 20 novel homozygous variants in his genomic DNA (see Table 1), including a homozygous 2-bp deletion mutation c.1587_1588delAG (p.Thr529Thrfs*6) in exon 13 of *LAMB3*, which was then verified by repeat SS. This deletion occurred within a ~4.7MB block of homozygosity. The mutation has been reported previously and shown to result in skipping of downstream exon 14,[17] which we were also able to verify by reverse-transcriptase PCR using RNA extracted from the patient's skin (data not shown). Typically, skipping of exon 14 would be out-of-frame but with the addition of the 2-bp deletion in exon 13, the combined deletion is in-frame. This case shows that WES can offer a more sensitive approach in identifying mutations in *LAMB3*, but in addition, the findings question the current paradigm for genotype-phenotype correlation as this mutation would usually be expected to result in severe generalised JEB (previously referred to as Herlitz JEB).[1] The WES

findings also stress the need for further studies at RNA and protein levels in some cases of EB.

Case 2:* *Mild acral blistering, nail dystrophy and hypotonia with a mutation in a different plectin isoform

An 18-year-old Pakistani male presented with mild blistering, developmental delay and hypotonia. A history of consanguinity supported autosomal recessive inheritance. IFM revealed a complete absence of plectin immunostaining, supporting bialleleic loss-of-function mutations in *PLEC*. However, no mutation in *PLEC* was identified on SS. Given the proposed mode of inheritance and rarity of this phenotype our hypothesis was that the disease causing variant would be novel and homozygous. By WES we identified 454 heterozygous and 54 homozygous novel variants (Table 1). The filtered variant list generated after WES included a novel frameshift mutation (c.92_93insG) in exon 1 of a plectin isoform previously unlinked with EB (transcript accession number: AF330791). Notably, only the U53204 plectin transcript is routinely sequenced during our mutation screening practice. We made new genomic primers to span this mutation and confirmed its presence on SS. The mutation identified by WES was located in a plectin isoform not previously thought to be expressed in skin; we have since modified our *PLEC* screening protocol accordingly.

Cases 3 and 4:* *Kindler syndrome with elusive KIND1 mutations

An 18-year-old Turkish male (case 3) had a history of trauma-induced blisters from early life with the development of some photosensitivity and poikiloderma in light-exposed sites

during infancy and childhood. An unrelated case, an 8-year-old Indian boy (case 4), had acral blistering from the first week of life, as well as photosensitivity, palmoplantar skin thickening, poikiloderma and a urethral stricture/stenosis. SS of *KIND1* failed to reveal any mutations in either subject. However, in the Turkish case, WES revealed 398 novel variants (393 heterozygous and 5 homozygous; see Table 1), including compound heterozygous mutations in *KIND1* (c.1811G>A, p.Trp604*; c.614G>A, p.Trp205*). The mutation p.Trp604* is novel, whereas p.Trp205* has been reported previously.[18] SS of *KIND1* failed to reveal any mutations in case 4, but WES revealed 553 novel variants (541 heterozygous and 12 homozygous; see Table 1), which included compound known heterozygous donor splice site mutations, c.1718+2T>C, and c.384_385+2del4 in *KIND1*.[19,20] All 4 mutations were confirmed in genomic DNA by repeat SS using new primers spanning the mutations. WES was therefore shown to be a sensitive means of identifying existing and new *KIND1*/*FERMT1* mutations in Kindler syndrome.

Case 5: Clinically mild blistering with DST-e identified as incorrect candidate gene by IFM

A 21-year-old white male had mild generalized skin fragility since early life: most of the blistering was acral. IFM showed a complete absence of immunostaining for the 230-kDa bullous pemphigoid antigen (BP230) but no other major differences from control skin. SS of *DST-e* (that encodes BP230), however, failed to identify any pathogenic mutations. Surprisingly, among 181 heterozygous and 3 homozygous novel variants identified by WES (Table 1), we identified compound heterozygous mutations in a different basement membrane zone gene, *COL7A1*. The mutations were c.793C>T (p.Gln265*) and c.6005G>A (p.Arg2002His). These mutations in *COL7A1* were confirmed by SS and both parents were

shown to be respective carriers of one of the two mutations identified. Neither of these two mutations has been reported previously. Compound heterozygosity for this nonsense/missense combination of mutations in type VII collagen would be expected to lead to recessive dystrophic epidermolysis bullosa (RDEB). In this case, WES revised the clinical diagnosis from EBS to RDEB, although why the IFM only revealed an abnormality in BP230 is not known.

Case 6: Missed COL7A1 mutation due to polymorphism in primer sequence

A new-born white British male presented with generalized skin blistering in whom skin biopsy revealed a complete absence of type VII collagen at the DEJ, consistent with a diagnosis of severe generalised RDEB (Figure 2). Screening of *COL7A1* by SS revealed one heterozygous mutation in exon 7, c.904G>T (p.Glu302*), but no second mutation. By WES, among 657 heterozygous and 24 homozygous novel variants (Table 1), however, a second mutant *COL7A1* allele was identified: a heterozygous glycine substitution, c.7505G>A (p.Gly2502Glu), in exon 99. Neither of these mutations has been reported previously but both are typical of the type of gene pathology that is found in RDEB. The reason why the second mutation was missed by SS was because we subsequently identified a single nucleotide polymorphism in intron 99-100, rs6781283, which is located within the reverse primer used to amplify this exon and flanking introns. This polymorphism is likely to have prevented primer annealing and, therefore, the deleterious allele would not have been amplified during PCR. New primers were designed and the heterozygous glycine substitution was subsequently detected by SS. This finding has important implications for the optimal design of working primers in gene amplification and SS.

Cases 7-9: Elusive second allele COL7A1 mutations in RDEB

A 27-year-old white British woman (case 7) with lifelong trauma-induced blistering and nail dystrophy had a clinical diagnosis of DEB, possibly localized RDEB or *de novo* dominant DEB, since no other family members were affected. Screening of *COL7A1* by SS revealed a known single heterozygous mutation, c.4027C>T (p.Arg1343*) in exon 34. This finding indicated a diagnosis of RDEB but no second mutation was found. However, WES identified 208 heterozygous and 9 homozygous novel variants (Table 1) that revealed a second heterozygous mutation, c.8676G>A (p.Trp2892*), in exon 117 of *COL7A1*. This second mutation, which has not been reported previously, occurs close to the 3' end of the gene and probably accounts for the relatively milder RDEB phenotype.

A 26-year-old Pakistani man (case 8) had clinical features of severe generalized RDEB, with marked trauma-induced skin fragility, hand contractures, neck scarring, and oesophageal stenoses. SS of *COL7A1* revealed a single previously unreported heterozygous mutation c.3630_3631insC (p.Gln1211Profs*8) but no identifiable second loss-of function mutation. However, WES disclosed 618 heterozygous and 20 homozygous novel variants (Table 1), including a second heterozygous mutation, c.520G>A (p.Gly174Arg) in exon 4 of *COL7A1*. This missense mutation within the NC-1 domain of type VII collagen has been reported previously in RDEB and shown to cause aberrant splicing. [21]

A 61-year-old white British woman (case 9) had lifelong mild, predominantly acral blistering. Two siblings were similarly affected and all were thought to have a mild form of RDEB. SS of *COL7A1* revealed a single heterozygous mutation, c.1732C>T (p.Arg578*) in exon 13. The two siblings were also carriers of this mutation, which is known to be a

common recurrent loss-of-function mutation in *COL7A1* in the UK and northern Europe.[22] No second mutation, however, was identified by SS. In contrast, WES identified 207 novel heterozygous and 7 homozygous variants, including a second mutant allele, c.2126T>C (p.Val709Ala), in exon 16 of *COL7A1*, which was also present in both siblings but not in the exome sequences from >900 ethnically matched controls. This mutation is likely to cause subtle disruption to the function of type VII collagen, given the mild phenotype in this family. The WES data thus expand genotype-phenotype correlation by implicating a further non-glycine missense mutation in the pathophysiology of RDEB.

DISCUSSION

In these 9 cases of EB, WES proved to be highly informative in identifying the pathogenic mutations. In 6 of these cases (1, 3, 4, 7, 8, 9), the mutations were missed by initial SS – not because of human error, but rather due to the well-known variable peak heights associated with current SS chemical labelling of DNA that can lead to imprecision in interpreting sequence traces. Review of the original sequence traces indicated either subtle changes in peak heights or equivocal findings, although repeat SS with close attention to the nucleotides implicated by WES did reveal the presence of the mutations. In case 5, it is uncertain why the IFM showed a lack of BP230 immunostaining when the pathogenic mutations were in type VII collagen – but this highlighted the potential fallibility of using IFM to determine the candidate gene for SS in cases of EB, even though this approach has proved to be highly informative, particularly in severe recessive forms of EB. In this case, the relatively mild clinical features were thought to be consistent with other cases that have demonstrated pathogenic mutations in *DST-e*,[23] and thus the combination of

clinicopathological information unfortunately led to erroneous sequencing of *DST-e*. Only the finding of *COL7A1* mutations using WES provided the true molecular pathology and helped correct the clinical diagnosis from autosomal recessive EB simplex to one of mild RDEB. For the other 2 cases, however, WES was fundamentally important in identifying the molecular basis of the EB. In case 2, the clinicopathological evidence pointed to plectin pathology but SS did not disclose any mutations in *PLEC*, at least using established sequencing protocols and primers. However, WES identified a recessive homozygous mutation in a different isoform of *PLEC*. We subsequently used reverse transcriptase PCR to confirm that this isoform was indeed expressed in skin (data not shown) and thereafter modified our PCR approach for SS of *PLEC* for screening future cases. In case 6, the failure of SS lay in the fact that there was a non-pathogenic polymorphism within one of the PCR primers that led to failure of amplification of that *COL7A1* allele, which in this case happened to contain one of the pathogenic mutations. We have previously encountered this failure to amplify one allele for exon 23 of *LAMB3* in cases of junctional EB which led to a redesign of the genomic DNA PCR primers,[24] and our new findings for *COL7A1* have also prompted us to redesign primers for exon 98 and 99 and flanking introns. The primers we recommend are: 5'-cgtatgtcttactccacagc (intron 97) and 5'-acccttttagtcctgcactc (intron 99). Given that the primer pair we were using was the same as that initially suggested in a widely cited *COL7A1* amplification protocol,[7] our data should encourage others involved in *COL7A1* mutation analysis by SS to review their choice of PCR primers for these particular exons.

Our study is not a direct comparison between our existing diagnostic approach for EB and NGS. Rather, this analysis was meant as a first step in determining whether WES might improve diagnostic sensitivity in EB in being able to identify mutations that have

proved elusive using current SS approaches. Unequivocally, WES is helpful in that regard,[25] although certain types of mutations may also be difficult of impossible to detect by WES, for example in non-coding regions.

From a practical perspective, the introduction of NGS into routine EB diagnostics requires further considerations of cost, facilities, staff, and time to report. In our institution (King's College London), current costs of WES (including bioinformatics analysis) are ~£900 (2014 prices) which compares favourably with SS of EB genes. Of note, it costs a similar amount just to sequence the *COL7A1* gene alone. Moreover, the cost of WES is likely to decrease further over the next few years, thus providing a strong economic argument for adoption of NGS into EB diagnostics. The challenge, however, lies in data interrogation and the bioinformatics analyses required to scrutinise the sequence variants and to determine causality. For EB diagnostics this would mean a realignment of technical wet lab skills (IFM and TEM) in favour of computer database and *in silico* work. The biggest challenge, however, lies in the time it takes to process and analyse a case. In EB diagnostics a rapid diagnosis is often very important to optimise clinical management, particularly in neonates with fragile skin. The current approach using skin biopsy assessment followed by SS of candidate genes (implicated by IFM and/or TEM) allows for possible diagnoses to be made within 2-3 days. In contrast, the quickest time that WES could be completed (at present) would be a minimum of 5 days. Being able to reduce the time it takes to make a diagnosis using WES, therefore, will be fundamental to its application in clinical service. New platforms to enable this are in development, but only when more rapid sample analysis is feasible in a diagnostic lab setting can one really begin to think about changing diagnostic practice. For now, however, skin biopsy remains an integral part of current EB diagnostics.

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REFERENCES

1. Fine J-D, Bruckner-Tuderman L, Eady RA *et al.* Inherited epidermolysis bullosa: updated recommendations on diagnosis and classification. *J Am Acad Dermatol* 2014; in press

2. Salam A, Proudfoot LE, McGrath JA. Inherited blistering skin diseases: underlying molecular mechanisms and emerging therapies. *Ann Med* 2014; **46**: 49-61

3. Hintner H, Stingl G, Schuler G *et al.* Immunofluorescence mapping of antigenic determinants within the dermal-epidermal junction in the mechanobullous diseases. *J Invest Dermatol* 1981; **76**: 113-118

4. Heagerty AH, Kennedy AR, Eady RA *et al.* GB3 monoclonal antibody for diagnosis of junctional epidermolysis bullosa. *Lancet* 1986; **1**: 860

5. Bruckner-Tuderman L, Mitsuhashi Y, Schnyder UW *et al.* Anchoring fibrils and type VII collagen are absent from skin in severe recessive dystrophic epidermolysis bullosa. *J Invest Dermatol* 1989; **93**: 3-9

6. Eady RA, Dopping-Hepenstal PJ. Transmission electron microscopy for the diagnosis of epidermolysis bullosa. *Dermatol Clin* 2010; **28**: 211-222

7. Christiano AM, Hoffman GG, Zhang X *et al.* Strategy for identification of sequence variants in *COL7A1* and a novel 2-bp deletion mutation in recessive dystrophic epidermolysis bullosa. *Hum Mutat* 1997; **10**: 408-414

8. Dang N, Murrell DF. Mutation analysis and characterization of *COL7A1* mutations in dystrophic epidermolysis bullosa. *Exp Dermatol* 2008; **17**: 553-568

9. Cho R, Simpson MA, McGrath JA. Next generation diagnostics for genodermatoses. *J Invest Dermatol* 2012; **132**: E26-E27

10. Salam A, Simpson MA, Stone KL *et al.* Next generation diagnostics of heritable connective tissue disorders. *Matrix Biol* 2013; **33C**: 35-40

11. McGrath JA, Stone KL, Begum R *et al.* Germline mutation in *EXPH5* implicates the Rab27B effector protein Slac2-b in inherited skin fragility. *Am J Hum Genet* 2012; **91**: 1115-1121.

12. Maddalena A, Bale S, Das S *et al.* Technical standards and guidelines: molecular genetic testing for ultra-rare disorders. *Genet Med* 2005; **7**: 571-583

13. Richards CS, Bale S, Bellissimo DB *et al.* ACMG recommendations for standards for interpretation and reporting of sequence variations: Revisions 2007. *Genet Med* 2008; **10**: 294-300

14. Biesecker LG. Exome sequencing makes medical genomics a reality. *Nat Genet* 2010; **42**: 13-14.

15. Li H, Handsaker B, Wysoker A *et al.* The Sequence Alignment/Map format and SAMtools. *Bioinformatics* 2009; **25**: 2078-2079

16. Wang K, Li M, Hakonarson H. ANNOVAR: functional annotation of genetic variants from high-throughput sequencing data. *Nucleic Acids Res* 2010; **38**: e164

17. Varki R, Sadowski S, Pfendner E *et al.* Epidermolysis bullosa. I. Molecular genetics of the junctional and hemidesmosomal variants. *J Med Genet* 2006; **43**: 641-52.

18. Siegel DH, Ashton GH, Penagos HG *et al.* Loss of kindlin-1, a human homolog of the Caenorhabditis elegans actin-extracellular-matrix linker protein UNC-112, causes Kindler syndrome. *Am J Hum Genet* 2003; **73**: 174-187

19. Techanukul T, Sethuraman G, Zlotogorski A *et al.* Novel and recurrent *FERMT1* gene mutations in Kindler syndrome. *Acta Derm Venereol* 2011; **91**: 267-270

20. Fassihi H, Wessagowit V, Jones C *et al.* Neonatal diagnosis of Kindler syndrome. *J Dermatol Sci* 2005; **39**: 183-185

21. Kern JS, Grüninger G, Imsak R *et al.* Forty-two novel COL7A1 mutations and the role of a frequent single nucleotide polymorphism in the MMP1 promoter in modulation of disease severity in a large European dystrophic epidermolysis bullosa cohort. *Br J Dermatol* 2009; **161**: 1089-1097

22. Mellerio JE, Dunnill MG, Allison W *et al.* Recurrent mutations in the type VII collagen gene (COL7A1) in patients with recessive dystrophic epidermolysis bullosa. *J Invest Dermatol* 1997; **109**: 246-249

23. Groves RW, Liu L, Dopping-Hepenstal PJ *et al.* A homozygous nonsense mutation within the dystonin gene coding for the coiled-coil domain of the epithelial isoform of BPAG1 underlies a new subtype of autosomal recessive epidermolysis bullosa simplex. *J Invest Dermatol* 2010; **130**: 1551-1557

24. Liu L, Choy YS, Wessagowit V *et al.* Single nucleotide polymorphism in a commonly utilized *LAMB3* primer sequence: implications for mutation detection and haplotype analysis in junctional epidermolysis bullosa. *J Dermatol Sci* 2006; **44**: 48-51

25. Takeichi T, Nanda A, Liu L *et al.* Impact of next generation sequencing on diagnostics in a genetic skin disease clinic. *Exp Dermatol* 2013; **22**: 825-831

TABLES

Table 1. WES data and impact on EB diagnostics for the 9 cases studied.

Patient no.	Clinical diagnosis	Gene	Mutation	Mutation type	PolyPhen-2: prediction, score	SIFT: prediction, score	Inheritance	ingle nucleotide substitutions in WES (number			Impact of WES
								Known	Novel		
									Heterozygous	Homozygous	
Case 1	GIJEB	*LAMB3*	c.1587_1588del2,PTC	Frameshift			AR	23488	256		Identification of an atypical mutation in *LAMB3* with implications for revising genotype-phenotype correlation.
									236	20	
Case 2	EBS	*PLEC*	c.92_93insG, PTC	Frameshift			AR	24257	508		Identification of a *PLEC* mutation in an isoform not thought to be expressed in skin with implications for expanding PLEC gene screening for diagnostic practice
									454	54	
Case 3	Kindler	*KIND1/FERMT1*	c.1811G>A, p.Trp604*	Nonsense			AR	24504	398		Improved detection of *KIND1/FERMT1* mutations indicating current SS primers are sub-optimal
			c.614G>A, p.Trp205*	Nonsense					393	5	
Case 4	Kindler	*KIND1/FERMT1*	c.1718+2T>C	Splice site			AR	24283	553		Improved detection of *KIND1/FERMT1* mutations indicating current SS primers are sub-optimal
			c.384_385+2del4	Splice site					541	12	
Case 5	EBS/RDEB	*COL7A1*	c.793C>T, p.Gln265*	Nonsense			AR	23649	184		Identification of mutations in a different, unsuspected EB gene providing a diagnosis that would otherwise have been clinically erroneous and missed in SS screening
			c.6005G>A, p.Arg2002Hi	Missense	Probably damaging, 1	Tolerated, 0.11			181	3	
Case 6	severe RDEB	*COL7A1*	c.904G>T, p.Glu302*	Nonsense			AR	24001	681		Improved detection of *COL7A1* mutations in exon 98 and 99 because of previous failure to amplify DNA due to a polymorphism within one of the PCR primers used for SS
			c.7505G>A, p.Gly2502Gl	Missense	Probably damaging, 0.999	Damaging, 0			657	24	
Case 7	mild RDEB	*COL7A1*	c.4027C>T, p.Arg1343*	Nonsense			AR	24260	217		Improved detection of *COL7A1* mutations indicating current SS primers are sub-optimal
			c.8676G>A, p.Trp2892*	Nonsense					208	9	
Case 8	severe RDEB	*COL7A1*	c.3630_3631insC, PTC	Frameshift			AR	24250	638		Improved detection of *COL7A1* mutations indicating current SS primers are sub-optimal
			c.520G>A, p.Gly174Arg	Missense	Probably damaging, 1	Damaging, 0.002			618	20	
Case 9	mild RDEB	*COL7A1*	c.1732C>T, p.Arg578*	Nonsense			AR	24231	214		Improved detection of *COL7A1* mutations indicating current SS primers are sub-optimal
			c.2126T>C, p.Val709Ala	Missense	Possibly damaging, 0.939	Damaging, 0			207	7	

GIJEB:generalised intermediate junctional epidermolysis bullosa; EBS: Epidermolysis bullosa simplex; RDEB: recessive dystrophic epidermolysis bullosa; SS: Sanger Sequencing; PTC: premature termination codon; WES: Whole Exome Sequencing; AR: autosomal recessive.