Epidermolysis bullosa simplex in Oriental; genetic studies in 19 patients.

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mutations changed amino acids that were evolutionarily. While we could not detect mutations in 5 EBS-K cases without familial history. There are similar phenotype/genotype correlations to the patients from Western countries. The proportion of *KRT5* mutation (11/14; 78.5%) is higher than that of the *KRT14* mutation rate (3/14; 21.4%) in Oriental EBS patients.

Epidermolysis bullosa (EB) encompasses a group of heterogeneous genetic skin diseases characterized by mechanical stress-induced blistering of skin. EB is classified in 3 major groups according to the level of dermoepidermal separation at the basement membranes zone. Epidermolysis bullosa simplex (EBS) results from intra-epidermal blister formation due to cytolysis of the basal keratinocytes. EBS affects approximately 1 in 30000 to 50000 of the population.[1] EBS can be subdivided into three major subtypes based on the severity of the clinical findings. The mildest subtype is the Weber-Cockayne type EBS (EBS-WC; OMIM 131800) with blistering restricted to the hands and feet, while the moderately severe subtype, the Koebner type (EBS-K; OMIM13190) showing more generalized blister formation, and the most severe subtype, Dowling-Meara (EBS-DM; OMIM 131760) which is characterized by severe herpetiform blistering.[2] In EBS-DM, the keratin intermediate filaments (KIF), major components of cytoskeletal network within keratinocytes, are clumped, a finding that serves as a diagnostic feature in electron microscopy.[2][3]

EBS is mostly inherited in an autosomal dominant fashion and caused by a

single mutation in either keratin genes, *KRT5* or *KRT14*.[2][3] The majority of mutations are nucleotide substitutions that lead to missense mutations. These genes encode keratin5 (K5) and keratin14 (K14) proteins which then form heterodimers that assemble into KIF of the basal cells in the epidermis.[4]Autosomal dominant mutations in *KRT5* or *KRT14* act in a dominant negative manner, i.e., the abnormal protein produced by the mutated allele interferes with the normal protein produced by the normal allele in the process of keratin filament assembly.[3] Both keratin proteins have a similar basic molecular structure as other intermediate filaments (IF), consisting of a central α-helical rod domain of about 310 amino acids, responsible for dimerization and higher order polymerization. This domain consists of four segments (1A, 1B, 2A and 2B) and is interrupted by three non-helical linkers (L1, L12 and L2).[4]

There is a correlation between the EBS phenotype and the K5 or K14 functional domain in which the mutation occurs. The mutations responsible for EBS-DM lie within the highly conserved ends of the rod domain, which are critical for K5 and K14 assembly. In contrast, the majority of the EBS-K mutations also lie within the rod domain but are more centrally located, whereas the EBS-WC mutations are mostly in the non-helical regions.[3][5][6]

Despite many reported mutations in EBS in the western countries,[3][6] the precise mutation spectrum in Oriental EBS patients has not been examined. To identify additional EBS mutations for genotype/phenotype correlation studies in Orientals, we performed *KRT5* and *KRT14* mutation analysis by direct sequencing in 17 Japanese and 2 Korean EBS families and compared them to the previously reported mutations in Western patients.

MATERIALS AND METHODS

Patients

The clinical phenotypes of the 17 Japanese and 2 Korean (Case 6 & 7) EBS cases are summarized in Table. EBS was at first clinically diagnosed and later confirmed by transmission electron microscopic examination of a skin biopsy obtained from the leading edge of a fresh blister that reveals splitting just above the basal cell layer and/or by immunohistochemical antibody/antigen mapping.

Mutation analyses

Genomic DNA extracted from whole blood was used as a template. The 9 exons of *KRT5* and the 8 exons of *KRT14* as well as each of intron-exon boundaries were amplified by the methods previously reported.[7][8] DNA sequencing of the PCR products was carried out with an ABI 3100 sequencer. For all novel mutations, their presence in 50 unrelated ethnically matched control individuals has been excluded.

RESULTS

Pathogenic mutations were identified in 14 EBS cases out of 19(Table).[7], [9]-[14] In EBS-WC patients , 2 novel missense mutations (473A>T; D158V, 1054C>T; R352S) were detected in *KRT5*. The D158V mutation (Case1) lies within the non-helical V1 head domain, while the R322S mutation (Case2) is in the 2A rod domain. Four out of five *KRT5* missense mutations were novel in EBS-K patients (428T>A; V143D, 558G>A; V186M, 573C>A; Q191P, 1550G>A; G517D). The V143D mutation (Case4) was in the V1 head domain and the G517D mutation (Case5) was in the non-helical tail V2 domain of K5. The other mutations (Case 6,7) were in the 1A rod domain. We could not find any mutations in five EBS-K patients (Case 15-19)who did not have family history of disease. All mutations found in EBS-DM in this study has been previously reported.[7], [11] -[12] Case 14 was given the diagnosis of EBS with mottled pigmentation (EBS-MP) that is characterized by mottled pigmentation, punctate or warty keratoses in addition to an EBS-WC type blistering pattern.[13] The P25L mutation in KRT5 which was specific for EBS-MP was again confirmed in Case 14.

DISCUSSION

The ethnic and geographic features in this study of EBS patients are important. A recent report revealed EBS types in Israel that have a unique mutation spectrums and different patterns of inheritance including a high incidence of recessive cases compared with European or US associated families.[14] Six Japanese EBS mutations have been previously reported[15]-[20], which are not enough to reveal the precise mutation spectrum. To assess the possibility that EBS may present with certain specific features in Orientals, which also comprise a closed ethnic group, 17 Japanese and 2 Korean EBS patients were examined in this study. We detected 14 mutations, six of which are novel, and have summarized them together with all previously reported *KRT5* and *KRT14* mutations in English literature (Figure 1).

Oriental EBS patients showed very similar phenotype/genotype correlations as patients from Western countries. All EBS-DM mutations in this study, were the same as in previous reports, and mutations lay within the highly conserved ends of the rod domain (helix initiation peptide and helix termination peptide) that are critical for K5 and K14 filament assembly.[5] The E477K mutation in *KRT5* and R125C or H mutation

in *KRT14*, which accounts for about 70% of EBS-DM mutations [8] were also detected in Oriental EBS-DM patients. While EBS-K and EBS-WC mutations were located within the rod domain more centrally or within the non-helical regions. It is remarkable that the novel R352S mutation in *KRT5* is the first mutation found in the 2A rod domain. Two novel *KRT5* mutations associated with EBS-K lay within the head or tail domains where EBS-K mutations are rarely identified. We could not find any clear distinctions between the EBS-WC and EBS-K mutation positions. The fact that the M119V mutation in *KRT14* was detected in both EBS-K [9] and EBS-WC (Case 3) supports the fact that the clinical manifestations of EBS-K and EBS-WC may overlap and the disease severity is likely to be affected by other factors.

Six novel *KRT5* missense mutations were identified. The V143D and D158V mutation are sited in the H1 region defined known to be sub-domains with a high degree of sequence homology within all intermediate filaments and are located immediately adjacent to the ends of the rod domain. Since the lengths of the H1 domains are different among individual intermediate filaments (IF),[15][16] we compared the corresponding amino acids of residue 143 and 158 with other type II keratins. They are

highly conserved (Figure 2). The positions of other mutations (valine at residue 186, glutamine at residue 191 in the 1A domain and arginine at residue 352 in the 2A domain) were also well-conserved among not only type II keratins but also all other types of IF (Figure2).[21][22] We could not determine whether the glycine residue at 517 was the evolutionarily conservation because of the diversity of tail domains in other type II keratins.

All mutations except the V186M mutation resulted in the alteration of the polarity of the affected amino acids. However an alteration at residue 186 in *KRT5* (V186L) has been previously described in association with EBS-K.[10] These findings suggest that all the novel mutations affect the important, conserved IF amino acids, suggesting that they may interfere the keratin heterodimer formation and protein-protein interactions and are therefore likely to be pathogenic.

Mutation detection rates in patients with EBS was reported to be 85-90%.[3] We also failed to detect any mutations in five de novo EBS-K cases . They might have mutations in the regulatory regions including promoter regions where we did not examine or in genes other than *KRT5* and *KRT14*.

The remarkable finding is that proportion of *KRT5* mutation(11/14; 78.5%) is

higher than that of the *KRT14* mutation rate (3/14; 21.4%) in this study. In general, the ratio of *KRT5* and *KRT14* mutations have been reported to be approximately equal. [3][7]. Whether this higher portion of *KRT5* mutations is characteristic of Oriental patients with EBS or not, needs further research into Oriental mutation reports.

Table: Clinical phenotypes and mutations found in Oriental EBS cases included in this study

Case	Age/Sex	Phenotype	Inheritance	Gene	Mutation	Effect	Reference
1.	8/M	WC	familial	*KRT5*	473A>T	D158V	novel
2.	24/M	WC	familial	*KRT5*	1054C>T	R352S	novel
3.	38/F	WC	familial	*KRT14*	415A>G	M119V	[9]
4.	1/M	K	de novo	*KRT5*	428T>A	V143D	novel
5.	54/M	K	familial	*KRT5*	558G>A	V186M	novel
6.	23/F	K	familial	*KRT5*	573C>A	Q191P	novel
7.	27/M	K	familial	*KRT5*	1550G>A	G517D	novel
8.	0/M	K	de novo	*KRT5*	558G>T	V186L	[10]
9.	0/M	DM	de novo	*KRT5*	527A>G	N176S	[11]
10.	42/M	DM	de novo	*KRT5*	1423G>A	E475K	[7]
11.	1/F	DM	de novo	*KRT5*	1429G>A	E477K	[12]
12.	8/F	DM	familial	*KRT14*	434G>A	R125H	[12]
13.	1/M	DM	de novo	*KRT14*	433C>T	R125C	[12]
14.	30/M	MP	familial	*KRT5*	465C>T	P25L	[13]
15.	1/F	K	de novo	ND			
16.	1/F	K	de novo	ND			
17.	1/M	K	de novo	ND			
18.	30/F	K	de novo	ND			
19.	35/F	K	de novo	ND			

EBS-WC, Weber-Cockayne type EBS; EBS-K, Koebner type EBS; EBS-DM, Dowling-Meara type EBS; EBS-MP, EBS with mottled pigmentation; ND, not detected

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REFERENCE

1 Fine JD. The epidemiology of inherited epidermolysis bullosa. In: Fine JD, Bauer EA, McGuire J et al eds. *Epidermolysis bullosa.* Bartimore: The John Hopkins University Press 1999:101-13

2 Fine JD, Eady RAJ, Bauer EA, et al. Revised classification system for inherited epidermolysis bullosa: Report of the Second International Consensus Meeting on diagnosis and classification of epidermolysis bullosa. *J Am Acad Dermatol* 2000; 42:1051-66.

3 Fuchs EV. The molecular biology of epidermolysis bullosa simplex. In: Fine JD, Bauer EA, McGuire J et al eds. *Epidermolysis bullosa.* Bartimore: The John Hopkins University Press 1999:280-99

4 Steinert PM. The two-chain coiled-coil molecule of native epidermal keratin intermediate filaments is a type I-type II heterodimer. *J Biol Chem* 1990;265:8766-74

5 Coulombe PA, Hutton ME, Vassar R. A function for keratins and a common thread among different types of epidermolysis bullosa simplex diseases. *J Cell Biol*

1991;115:1661-74

6 Irvine AD, McLean WHI. The molecular genetics of the genodermatoses: progress to date and future directions. *Br J Dermatol* 2003;148:1-13

7 Schuilenga-Hut PH, Vlies P, Jonkman MF, et al. Mutation analysis of the entire keratin 5 and 14 genes in patients with epidermolysis bullosa simplex and identification of novel mutations. *Hum Mutat* 2003;21:447

8 Stephens K, Ehrlich P, Weaver M, et al. Primers for exon-specific amplification of the KRT5 gene: identification of novel and recurrent mutations in epidermolysis bullosa simplex patients. *J Invest Dermatol* 1997;108:349-53

9 Cummins RE, Klingberg S, Wesley J, et al. Keratin 14 point mutations at codon 119 of helix 1A resulting in different epidermolysis bullosa simplex phenotypes. *J Invest Dermatol* 2001;117:1103-7

10 Liovic M, Stojan J, Bowden PE, et al. A novel keratin 5 mutation (K5V186L) in a family with EBS-K: a conservative substitution can lead to development of different disease phenotypes. *J Invest Dermatol* 2001;116:964-9

11 Stephens K, Ehrlich P, Weaver M, et al. Primers for exon-specific amplification of the KRT5 gene: identification of novel and recurrent mutations in epidermolysis bullosa simplex patients. *J Invest Dermatol* 1997;**108**:349-53

12 Coulombe PA, Hutton ME, Letai A, et al. Point mutations in human keratin 14 genes of epidermolysis bullosa simplex patients: genetic and functional analyses. *Cell* 1991;**66**:1301-11

13 Uttam J, Hutton E, Coulombe PA, Anton-Lamprecht I, et al. The genetic basis of epidermolysis bullosa simplex with mottled pigmentation. *Proc Natl Acad Sci U S A* 1996;**93**:9079-84

14 Ciubotaru D, Bergman R, Baty D, et al. Epidermolysis bullosa simplex in Israel: clinical and genetic features. *Arch Dermatol* 2003;**139**:498-505

15 Nomura K, Shimizu H, Meng X, et al. A novel keratin K5 gene mutation in Dowling-Meara epidermolysis bullosa simplex. *J Invest Dermatol*. 1996;**107**:253-4.

16 Matsuki M, Hashimoto K, Yoshikawa K, et al. Epidermolysis bullosa simplex (Weber-Cockayne) associated with a novel missense mutation of Asp328 to Val in Linker 12 domain of keratin 5. *Hum Mol Genet*. 1995;**4**:1999-2000.

17 Nomura K, Umeki K, Meng X, Tamai K, et al. A keratin K5 mutation (Leu 463-->Pro) in a family with the Weber-Cockayne type of epidermolysis bullosa simplex. *Arch Dermatol Res*. 1997;289:493-5.

18 Yamanishi K, Matsuki M, Konishi K, et al. A novel mutation of Leu122 to Phe at a highly conserved hydrophobic residue in the helix initiation motif of keratin 14 in epidermolysis bullosa simplex. *Hum Mol Genet*. 1994;3:1171-2

19 Umeki K, Nomura K, Harada K, et al. A keratin K14 gene mutation in a Japanese patient with the Dowling-Meara type of epidermolysis bullosa simplex. *J Dermatol Sci*. 1996;11:64-9.

20 Gu LH, Ichiki Y, Sato M, et al. A novel nonsense mutation at E106 of the 2B rod domain of keratin 14 causes dominant epidermolysis bullosa simplex.*J Dermatol*. 2002;29:136-45.

21 Conway JF, Parry DAD.Intermediate filament structure: 3. Analysis of sequence information. *Int J Biol Macromol* 1988;10:79-98

22 Fuchs E, Weber K. Intermediate filaments: structure, dynamics, function, and disease. *Annu Rev Biochem* 1994;63:345-82

APPENDICES

LEGEND

Figure 1: Correlation between previously reported and novel EBS mutation locations and disease severity. Mutations identified in EBS-WC were indicated in green, in EBS-K by a blue color and in EBS-DM by red. Novel mutations were shown by a large text font size. Mutations detected in Orientals were underlined ([] is reference number) and a cross means mutations that were not novel but were detected in this study.

Figure 2: Schematic demonstration of the V143, D158, V186, Q191 and R352 residue in K5. All of them are highly conserved among other type II keratins or intermediate filaments. Shown are amino acid sequences of the H1 region, the 1A and 2A rod domain segment along with a selected group of other type II IF keratins, type III IF vimentin, type IV IF neurofilament subunit M (NF-M), type V IF lamin. Sequences were obtained from Genbank. GenBank Accession Numbers as follows (K1; AF237621, K2e; AF019084, K3; X05418 X05421, K4; X61028, K5; NM 000424, K6a; L42583, vimentin; BC000163, NF-M; Y00067, lamin; M13451)

Abbreviations

EB, epidermolysis bullosa; EBS, epidermolysis bullosa simplex; EBS-DM, Dowling-Meara type EBS; KIF, keratin intermediate filaments; EBS-K, Koebner type EBS; EBS-WC, Weber-Cockayne type EBS; EBS-MP, EBS with mottled pigmentation; HIP, Helix initiation peptide; HTP, Helix termination peptide; IF, intermediate filaments; KIF, keratin intermediate filaments; K5, keratin 5; K14, keratin 14

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ARTICLE

Colocalization of Multiple Laminin Isoforms Predominantly beneath Hemidesmosomes in the Upper Lamina Densa of the Epidermal Basement Membrane

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SUMMARY Multiple laminin isoforms including laminins 5 (α3 β3 γ2), 6 (α3 β1 γ1), 10 (α5 β1 γ1), and possibly laminins 7 (α3 β2 γ1) and 11 (α5 β2 γ1) are present in the epidermal basement membrane. However, only the precise epidermal ultrastructural localization of laminin 5 (α3 β3 γ2) has been elucidated. We therefore determined the precise expression and ultrastructural localization of the α5, β1, β2, and γ1 chains in the epidermis. The expression of laminin chains in skin samples was analyzed from patients with epidermolysis bullosa (EB, $n=15$) that harbor defects in specific hemidesmosome (HD)-associated components. The expression of the α5, β1, and γ1 chains (present in laminins 10/11) and β2 chain (laminins 7/11) was unaffected in all intact (unseparated) skin of EB patients including Herlitz junctional EB with laminin-5 defects ($n=6$). In the basement membrane of human epidermis, the α5, β1, β2, and γ1 chains were expressed but also localized to the dermal vessels. Immunogold electron microscopy of normal human epidermis localized the α5, β1, β2, and γ1 chains to the upper lamina densa, with between 84% and 92% of labeling restricted to beneath the HDs, similar to laminin 5 ($n\geq200$ gold particles per sample, sample number $n=4$) but distinct from collagen IV labeling (with only 63% labeling beneath HDs, $p<0.001$). Taken together, the majority of the α5β1/β2γ1 laminin chains are located beneath HDs. This suggests that laminin-10-associated chains have specific functions or molecular interactions beneath HDs in the epidermal basement membrane.

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KEY WORDS
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In skin, laminins are present in the epidermal basement membrane, around blood vessels, nerves, and adnexal structures. It is generally thought that laminin 5 (α3 β3 γ2), possibly laminin 6 (α3 β1 γ1), and laminin 10 (α5 β1 γ1) are expressed in the human epidermal basement membrane (Aumailley and Rousselle 1999) (see Figure 1). The expression of laminins 7 (α3 β2 γ1) and 11 (α5 β2 γ1) has yet to be confirmed in the adult human epidermis (Aumailley and Rousselle 1999). Of these, laminin 5 (α3 β3 γ2) is the most well-studied epidermal isoform (Nishiyama et al. 2000; Mercurio et al. 2001; Geuijen and Sonnenberg 2002; McMillan et al. 2003b). Laminin 5 is thought to be crucial for the correct assembly and adhesion of hemidesmosomes (HDs) via the receptor, the α6β4 integrin (Niessen et al. 1994).

The distinct roles of laminin isoforms in the processes of cutaneous morphogenesis are poorly understood. Laminin 10 (α5 β1 γ1), however, has recently been implicated in several functions including hair follicle development (Li et al. 2003). In an α5 chain (laminin 10/11) knockout mouse model, the addition of exogenous laminin 10 was used to correct follicular development (Li et al. 2003). Laminin 10 is therefore implicated in hair follicle cell growth and adhesion (Gu et al. 2001; Pouliot et al. 2002; Li et al. 2003). Cell adhesion assays have demonstrated that multiple laminins (including laminins 5, 10, and 11) can act as adhesive substrates for keratinocytes and that this adhesion is mediated by the integrins α3β1 and α6β4

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