Mutation Analyses of *Keratin 5* and *Keratin 14* in Korean Patients with Epidermolysis Bullosa Simplex

Tae-Won Kang

Department of Medicine

The Graduate School, Yonsei University

Mutation Analyses of *Keratin 5* and *Keratin 14* in Korean Patients with Epidermolysis Bullosa Simplex

Directed by Professor Soo-Chan Kim

The Master's Thesis

Submitted to the Department of Medicine,

the Graduate School of Yonsei University

in partial fulfillment of the requirements for the

degree of Master of Medical Science

Tae-Won Kang

December 2008

This certifies that the Master's Thesis of

Tae-Won Kang is approved.

Thesis Supervisor: ***Soo-Chan Kim***

Thesis Committee Member: ***Wook Lew***

Thesis Committee Member: ***Man-Wook Hur***

The Graduate School

Yonsei University

December 2008

Acknowledgements

First of all, I would like to thank my thesis supervisor, Prof. Soo-Chan Kim for his outstanding guidance and encouragement to study this subject. I also appreciate Prof. Wook Lew and Prof. Man-Wook Hur who gave me expert advices and warm support. I wish to thank researcher Jeong Seon Lee for her bright ideas and unremitting efforts. She made it possible to complete this complicating research. And thanks to all members of my department, especially Dr. Se-Woong Oh, for their helpful assistance. Finally, I would like to thank my family - my father, mother, and sister- for their endless encouragements.

Table of Contents

ABSTRACT·····1
I. INTRODUCTION·····3
II. MATERIALS AND METHODS·····5
1. Subjects·····5
2. Immunofluorescence study·····5
3. Electron microscopy·····5
4. Mutational analysis·····6
A. DNA samples·····6
B. Polymerase chain reaction (PCR) amplification·····6
C. DNA sequence analysis·····8
III. RESULTS·····9
1. Clinical findings·····9
2. Mutational analysis·····14
A. *KRT5* and *KRT14* mutation survey·····14
IV. DISCUSSION·····17
V. CONCLUSION·····25
REFERENCES·····27
ABSTRACT IN KOREAN·····31

List of Figures

Figure 1. Clinical appearance of patient 14·····12

Figure 2. Clinical appearance of patient 16·····12

Figure 3. Clinical appearance of patient 6·····13

Figure 4. Schematic representation of mutation locations and EBS subtypes·····16

Figure 5. Histological examination, electron microscopic findings and sequence analysis of *KRT5* results (TAA deletion at nucleotide 531-533, resulting in p.Asn177del) for patient 14·····18

Figure 6. The characteristic heptad structure, (abcdefg)n of the α-helical rod domain·····19

Figure 7. Results of the electron microscopic findings and sequence analysis of *KRT5* (c.1429G>A substitution, p.E477K) for patient 16·····19

Figure 8. Results of the histological examination, electron microscopic findings and sequence analysis of *KRT14* (homozygous c.1174G>T, resulting in p.E392X/p.E392X) for patient 6·····21

List of Tables

Table 1. The sequences of PCR primers of *KRT5*·····6

Table 2. The sequences of PCR primers of *KRT14*·····7

Table 3. Clinical features of 16 Korean patients with EBS ·····10

Table 4. EBS subtypes and the mutation spectrum of 16 EBS patients·····15

Table 5. Changes in amino acid side chain polarity in 16 EBS patients·····24

<Abstract>

Mutation Analyses of *Keratin 5* and *Keratin 14* in Korean Patients with Epidermolysis Bullosa Simplex

Tae-Won Kang

Department of Medicine

The Graduate School, Yonsei University

(Directed by Professor Soo-Chan Kim)

Epidermolysis bullosa simplex (EBS) is a group of hereditary bullous disorders caused by mutations of the keratin genes *KRT5* and *KRT14*. Patients present with widely varying severity and are classified into three main subtypes, EBS Weber–Cockayne (EBS-WC), EBS Koebner (EBS-K), and EBS Dowling–Meara (EBS-DM), on the basis of the distribution and severity of blisters. Detailed knowledge of the spectrum of EBS mutations and their genotype-phenotype correlation is essential for accurate genetic counseling, prenatal diagnosis and prediction of prognosis. To date, about 130 mutations associated with EBS have been identified in *KRT5* and *KRT14*. A significant correlation between the position of the mutations within these proteins and the clinical severity of EBS has been noted.

In this study, we investigated 16 Korean EBS patients and their families by

performing a sequence analysis of the entire coding sequences of *KRT5* and *KRT14*. Pathogenic mutations were found in all cases. We have identified seven novel mutations: four mutations on the *KRT5* (p.V143F, p.R265P, p.C479X and p.Asn177del), and three on the *KRT14* (p.R125L, p.E392X and p.L401P). Nine mutations that have previously been reported were found within the *KRT5* and *KRT14* genes. Specifically, thirteen missense, two nonsense, and one small deletion mutation were found. Five of sixteen EBS patients were diagnosed with EBS-DM, and all mutations (K14-p.R125H, K14-p.R125L, K5-E477K, K5-p.C479X, K5-p.Asn177del) were located on the highly conserved ends of the alpha-helical rod domain, the helix initiation (HIP), or helix termination (HTP) peptides of *KRT5* and *KRT14*. Eight and three mutations were reported in EBS-K and EBS-WC, respectively. The positions of mutation in both subtypes are more widely distributed within the rod domains and in the L12 linker domains of both keratin genes. Interestingly, K14-p.E392X/p.E392X had a homozygous recessive profile (c.1174G>T), and the patient showed a EBS-K phenotype. This study shows possible implications of the novel mutations on protein structure, keratin intermediate filament (KIF) formation and the corresponding phenotypes.

Keywords : epidermolysis bullosa simplex, keratin 5, keratin 14, mutation

Mutation Analyses of *Keratin 5* and *Keratin 14* in Korean Patients with Epidermolysis Bullosa Simplex

Tae-Won Kang

Department of Medicine
The Graduate School, Yonsei University

(Directed by Professor Soo-Chan Kim)

I. INTRODUCTION

Epidermolysis bullosa (EB) comprises a group of inherited blistering disorders classified into three main subtypes on the basis of the ultramicroscopic cleavage level of blistering: simplex, junctional and dystrophic[1]. The disease encompasses a wide variety of clinical phenotypes, from mild blistering of the hands and feet to widespread and lethal blistering. Epidermolysis bullosa simplex (EBS) is the most common subtype of EB, and is predominantly inherited as an autosomal dominant trait, arising from mutations in the Keratin 5 (*KRT5*) or Keratin 14 (*KRT14*) genes[2]. These keratins are very important proteins in basal keratinocytes, maintaining the mechanical integrity of the epidermis against frictional forces. Recently, a number of additional functions of intermediate filaments have been discovered. These include the regulation of key signaling pathways that control cell survival, cell growth, and vectorial processes, which are associated with protein targeting, vesicle transport, and cell adhesion[3,4].

Keratins are intermediate filament cytoskeleton proteins in epithelial cells; K14 (a type I keratin) and K5 (its type II partner) are specifically expressed in

the basal cells of stratified epithelia. The heteropolymeric keratin filament network is assembled in the cytoplasm from type I and type II keratin proteins, which have a common structure with a discontinuous α-helical rod domain flanked by non-helical head and tail domains[5]. Electron microscopy and immunofluorescence mapping have shown that the blistering occurs in the basal keratinocytes and thus heals without scarring. Keratin filament bundle formation in basal keratinocytes depends on the correct interaction between the keratin 5 and keratin 14 proteins, and thus mutations in either gene can lead to cutaneous blistering after mechanical trauma.

EBS is traditionally classified into three main subtypes[2,6]: Weber–Cockayne is the mildest form of EBS, in which blistering mainly affects the hands and feet; Koebner is the intermediate type, with a more generalized pattern of blistering; and the third and most severe form of EBS is the Dowling–Meara type, in which extensive and severe blistering occurs in clusters (herpetiform-like). However, a few reports of recessive EBS subtypes, representing about 5% of all EBS mutations[7], have been published.

More than 130 different pathogenic mutations have now been documented in K5 and K14 (see http://www.interfil.org, Szeverenyi I., et al. 2008), clustered in specific regions or hotspots in the protein molecule[5]. It has been noted that a close correlation exists between the position and nature of the mutation and the severity of the disease. The helix boundary motifs at the beginning and end of the rod domain are critical for normal filament formation[8], and mutations in these areas cause the severest form of EBS (EBS-DM). The phenotype-genotype correlation for EBS-WC and EBS-K is less specific.

Although many mutations in EBS have been reported in Western countries[9-14], a study of the mutation spectrum in Korea has not been conducted. To identify additional EBS mutations for genotype and phenotype correlation in Korean patients, we performed mutational analysis of 16 EBS patients of Korean origin by performing a sequence analysis of the entire coding sequences of *KRT5* and *KRT14*.

II. MATERIALS AND METHODS

1. Subjects

Sixteen unrelated Korean EBS patients were studied. Patients were clinically diagnosed with EBS and classified to each subtypes. And, these diagnoses were confirmed by immunofluorescence antigen mapping and electron microscopy.

2. Immunofluorescence study

Direct immunofluorescence studies were performed using frozen sections of the margin of fresh blisters to rule out autoimmune blistering diseases. For immunofluorescence mapping studies, we used anti-cytokeratin 14 monoclonal antibody (Sigma-Aldrich, St Louis, MO), anti-cytokeratin 5/6 monoclonal antibody (Novocastra, Newcastle, UK), anti-type IV collagen monoclonal antibody (Sigma-Aldrich, St Louis, MO), and anti-type VII collagen antibody (Sigma-Aldrich, St Louis, MO). The secondary antibody was a rabbit anti-mouse IgG FITC conjugated (1:40, Dako, Glostrup, Denmark).

3. Electron microscopy

Three-millimeter punch biopsies from the lesional skin were fixed in 2.5% glutaraldehyde, post-fixed in 1% osmiumtetroxyde, dehydrated in a graded ethanol series, and embedded in Epon 812. Semithin sections were stained with toluidin blue. Ultrathin sections were selected, contrasted with uranyl acetate and lead citrate, and examined with a Philips CM 10 transmission electron microscope.

4. Mutational analysis

A. DNA samples

After obtaining informed consent from the patients, genomic DNA was extracted from the peripheral blood lymphocytes of the patients and their families using a DNA extraction kit (QIAamp DNA Blood Midi kit, Qiagen, Hilden, Germany).

B. Polymerase chain reaction (PCR) amplification

Total DNA was used as a template for the amplification of the genomic sequences of *KRT5* and *KRT14* (GenBank NM_000424.2 and NM_000526.3 respectively). *KRT5* segments, including all nine exons, and all exon-intron borders, were amplified by PCR using pairs of oligonucleotide primers synthesized on the basis of intronic sequences. *KRT14* segments, including all eight exons, and all exon-intron borders, were amplified by PCR using pairs of oligonucleotide primers synthesized on the basis of intronic sequences. The primer sequences are described in Table 1 and 2.

Amplification conditions were designed under the following protocol: 95°C for 1 minute followed by 35 cycles of 95°C for 40 seconds, 54-64°C for 40 seconds, and 72°C for 1 minute. The final extension was at 72°C for 3 minutes.

Table 1.The sequences of PCR primers of *KRT5*

Exon	Forward primer	Reverse primer
1	gagctctgttctctccagca *new: gaggatatccatcagcacta	ctccaccgccgaaaccaa *new: ccttctttctctctctttggc
2	ctagtgctttctgtgccctggatagg	ccatctggtaccaagaagac

3	tggccagagttcatgctac	tcaaccttggcctccagctcc
4	gagaaccagcagcctgcag	gaggtgtcagagacatgc
5	atgagattaacttcatgaagatg * new: tttcacatgggtgggttttt	ccattcttagtgtcgtcatg *new: gagccccattcttagtgtcg
6	tgacgacactaagaatgggg	tttagaactcaggccccttc
7	gagagccgagattgacaatg	tagagcagcttcgctttatc
8	tcgaatcatgaggatgggag	gaggaacactgcttgtga
9	agtagagtgctccaacaccag	gaggaggaggtggtggagac

Table 2.The sequences of PCR primers of *KRT14*

Exon	Forward primer	Reverse primer
1	cagctccatgaagggctcc	gagctagctggaatggtgcc
2	gacaaattacctgtgccttt *new: ttgacaaattacctgtgcct	gcccaagagtcttattcttt *new: ttttcatgcacctatcctggt
3	atcaggatgggtgtctcata	gtgttcaagtgatcctcctg
4	caggcctaaggaacaccaat *new: ggcctaaggaacaccaatcc	gagaatgccattcacaccag *new: tgccattcacaccagaagg
5	ggatgccgaggaatggttct *new: ccttctggtgtgaatggca	acgattagtgagtgtggccg *new: cctgggtgcaccaccctt
6	gaacggccacactcactaat *new: aagggtggtgcacccagg	cattagatacatggtggggc *new: ggaggagaggctgtgaaaat
7	gaggacgcccagtgagtctt *new: tcccctagtccgcccccc	atttggcggctggaggaggt *new: ggatctgccacagacaccac
8	ccaggtgtgagctcttaggaagc *new: aatcaggggagagggcaatg	gaggggatcttccagtgggatct *new gtccagctgtgaagtgcttg

* If two or more kinds of primer were used, they are described as ‘new’.

C. DNA sequence analysis

Sequence analyses were performed using Big Dye terminator technology (ABI 3100 Perkin-Elmer, Warrington, UK). Genomic DNA samples from 50 normal, healthy Koreans were used as a control.

III. RESULTS

1. Clinical findings

Sixteen unrelated Korean EBS patients were studied. At first, we classified the patients into each EBS subtypes on the basis of the diagnostic criteria described below[2].

(a) EBS Weber-Cockayne (EBS-WC): Blistering is limited to the hands and feet. The onset of blistering in patients with EBS-WC is usually early childhood, when the child begins to walk. As with several other keratin disorders, the disease can be temperature-dependent, such that symptoms worsen in the summer months.

(b) EBS Koebner (EBS-K): Blisters usually occur on the hands and feet and limbs within the first week of life, and occasionally become more widespread on the trunk. Blistering is usually limited to the hands and feet in adulthood, but blistering may still occur at sites of friction or trauma. As with EBS-WC, severity varies markedly by season.

(c) EBS Dowling-Meara (EBS-DM): The most severe EBS subtype. Blisters are found in clusters anywhere on the body, usually within the first few days of life. The hands and feet are the most severely affected. Patients with EBS-DM often also have palmoplantar keratoderma, nail dystrophy, and oral ulceration. Extensive involvement occasionally occurs, and can be fatal in neonates. As with other forms of EBS, the blistering tends to improve in adolescence and adulthood. However, the palmoplantar keratoderma tends to become more marked over time. Unlike EBS-K and EBS-WC patients, EBS-DM patients do not usually display significant seasonal variation. The diagnosis of EBS-DM is confirmed by observing its typical appearance on the electron microscopy of an intraepidermal cleavage plane and electron-dense keratin clumpings within the cytoplasm of basal cells.

The clinical features of the 16 Korean EBS patients are summarized in Table 3. Seven of the thirteen EBS patients had autosomal-dominant family histories. The other nine patients had no family history of skin blistering. All EBS-DM patients were further confirmed by electron microscopy which showed cytolysis in the basal keratinocytes and keratin clumpings within the cytoplasm of basal cells (data not shown). The clinical features of the two EBS-DM patients, patient 14 (Figure 1) who was a 5-month-old female and patient 16 (Figure 2) who was a 1-month-old female, were remarkable in that they showed severe generalized blisters, widespread skin infection, loss of nails, milia formation, and general health problems such as feeding difficulty and malnutrition. Patient 6 (Figure 3) showed generalized blisters with reticulated hyperpigmentation and developed advanced squamous cell carcinoma (SCC) on the tongue. He underwent a surgical excision and post-op radiotherapy. But, unfortunately, he died several months later after surgery.

Table 3. Clinical features of 16 Korean patients with EBS.

Patient No.	**Age/Sex**	**Family History**	**Age of onset**	**Clinical features**	**EBS subtype**
1	16Y/M	no	infancy	blisters on hands and feet	W-C
2	25Y/M	familial	10 years	blisters on hands and feet	W-C
3	18Y/M	familial	4 years	blisters on hands and feet	W-C
4	19Y/M	familial	infancy	generalized blisters, plantar hyperkeratosis	K
5	21Y/M	familial	infancy	generalized blisters	K
6	41Y/M	no	infancy	generalized blisters, reticulated hyperpigmentation,	K

				palmoplantar keratoderma, SCC on the tongue	
7	2mo/M	no	infancy	generalized blisters on the trunk, extremities	K
8	27Y/F	no	infancy	generalized blisters on the trunk, extremities	K
9	21Y/F	familial	infancy	generalized blisters on the trunk, extremities	K
10	2Y/F	familial	infancy	generalized blisters on the trunk, extremities	K
11	23Y/F	no	infancy	generalized blisters on the trunk, extremities, hyperpigmentation	K
12	16Y/M	no	neonate	severe generalized blister, palmoplantar keratoderma	D-M
13	47Y/M	no	neonate	severe generalized blister, palmoplantar keratoderma	D-M
14	5mo/F	no	neonate	severe generalized blister, oral mucosal erosion, nail loss	D-M
15	19Y/M	familial	neonate	severe generalized blister, palmoplantar keratoderma	D-M
16	1mo/F	no	neonate	severe generalized blister, oral mucosal erosion, nail loss	D-M

Y; year, mo; month, M; male, F; female ,

W-C; Weber-Cockayne, K; Koebner, D-M; Dowling-Meara

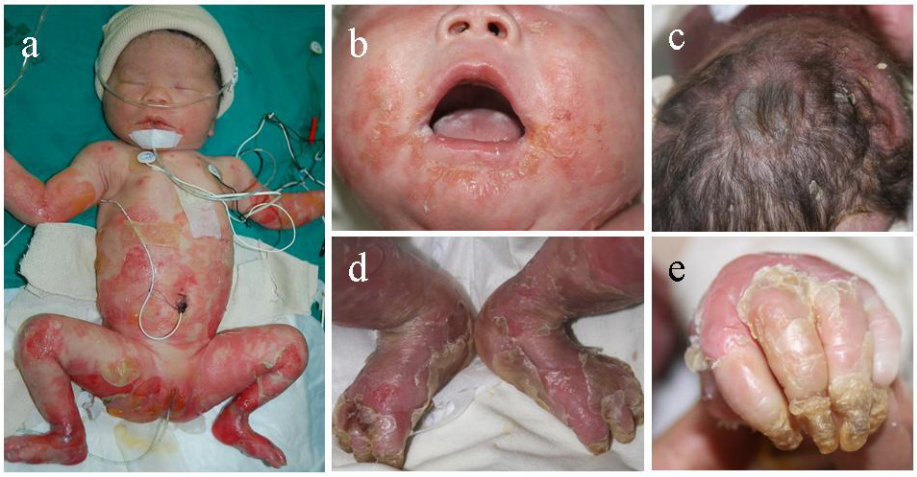

Figure 1. Clinical appearance of patient 14 shortly after birth. Widespread blisters and erosions are noted (a). At five-months, oral mucosal erosions (b) diffuse hair loss, and tense bullae on the scalp (c), severe blisters, erosions, confluent scales, and loss of nails are seen on the feet and hands (d, e).

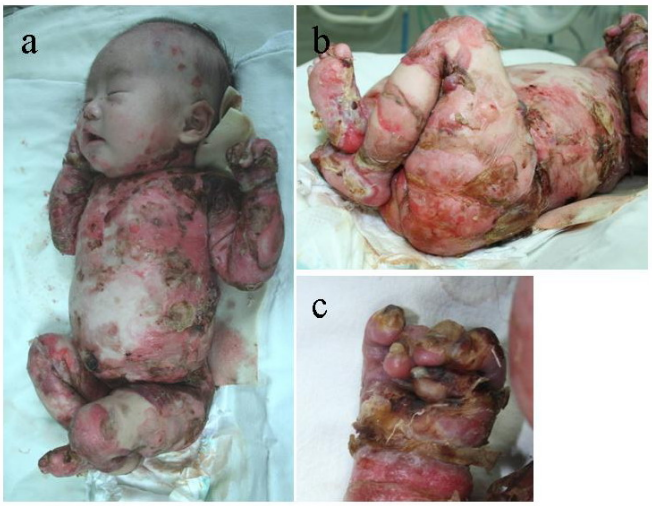

Figure 2. Clinical appearance of patient 16 at 1 month. Severe generalized blisters and erosions and widespread skin infection are seen on the whole body (a, b). Nail dystrophy and nail loss are seen on the hands (c).

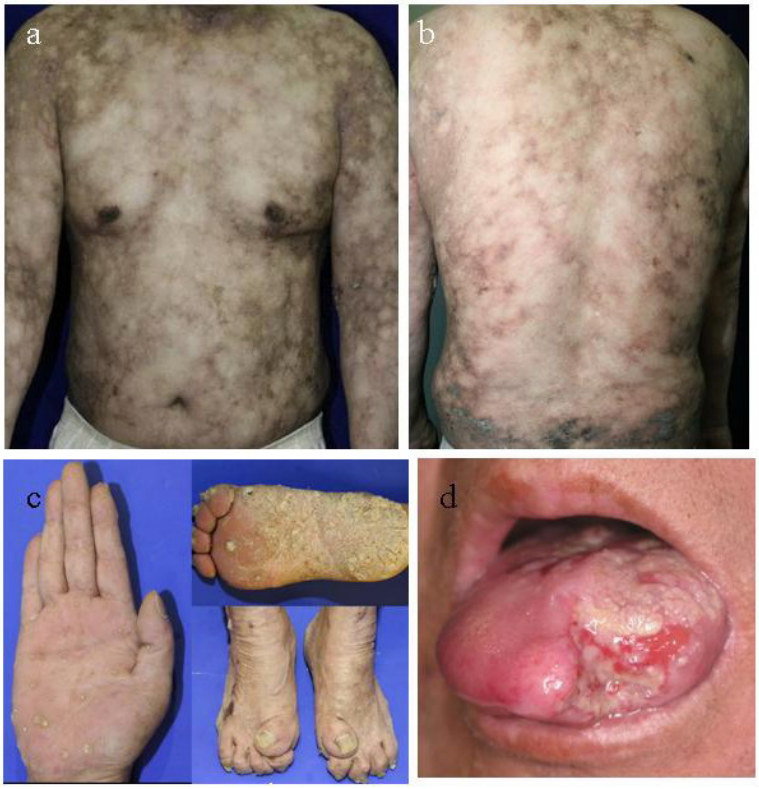

Figure 3. Clinical appearance of patient 6. Superficial erosions and reticulated hyperpigmented streaks are seen on the trunk (a, b), punctuate keratoderma of the palms and soles and dystrophic toes and toenails are observed (c), a painful ulcerative mass on his tongue was diagnosed as a squamous cell carcinoma (d).

2. Mutational analysis

A. *KRT5* and *KRT14* mutation survey

We investigated sixteen EBS patients and families of Korean origin by performing a sequence analysis of the entire coding sequences of *KRT5* and *KRT14*. Pathogenic mutations were identified in all patients (Table 4). These mutations included thirteen missense, two nonsense, and one small deletion mutation. Seven novel mutations were identified, specifically, four mutations on the *KRT5* (p.V143F, p.R265P, p.C479X and p.Asn177del), and three on the *KRT14* (p.R125L, p.E392X and p.L401P). These mutations were not found in the fifty unrelated healthy controls. The other nine patients had mutations that have been reported previously. Five of sixteen EBS patients were diagnosed as EBS-DM, and all mutations (K14-p.R125L, K-14p.R125H, K5-p.C479X, K5-p.E477K, K5-p.Asn177del) were located on the highly conserved ends of the alpha-helical rod domain, the helix initiation (HIP) or helix termination (HTP) peptides, respectively. Eight and three mutations are reported as EBS-K and EBS-WC. The positions of the mutations in both subtypes are widely distributed within the rod domains and in the L12 linker domains of both keratin genes. One patient (patient 6) had a novel homozygous c.1174G>T mutation in *KRT14*, p.E392X/p.E392X, and was diagnosed with autosomal recessive EBS. Figure 4 depicts the mutational position on both keratin genes.

Table 4. EBS subtypes and the mutation spectrum for 16 EBS patients.

Patient No.	**EBS subtype**	**Keratin**	**Exon**	**Domain**	**Nucleotide change**	**Mutation protein**	**Novel/ recurrent**
1	W-C	K14	exon 6	2B	**c.1202T>C**	**p.L401P**	**novel**
2	W-C	K14	exon 6	2B	c.1162C>T	p.R388C	reported[15]
3	W-C	K5	exon 5	L 12	c.992G>A	p.R331H	reported[12]
4	K	K5	exon 1	H1	**c.427G>T**	**p.V143F**	**novel**
5	K	K5	exon 3	1B	**c.794G>C**	**p.R265P**	**novel**
6	K	K14	exon 6	2B	**c.1174G>T c.1174G>T**	**p.E392X/ p.E392X**	**novel *recessive**
7	K	K5	exon 1	H1	c.449T>C	p.L150P	reported[16]
8	K	K5	exon 7	2B	c.1398G>C	p.E466D	reported[16]
9	K	K5	exon 2	1A	c.572A>C	p.Q191P	reported[17]
10	K	K14	exon 1	1A	c.428T>C	p.L143P	reported[18]
11	K	K14	exon 6	2B, HTP	c.1243T>C	p.Y415H	reported[19]
12	D-M	K14	exon 1	1A, HIP	**c.374G>T**	**p.R125L**	**novel**
13	D-M	K5	exon 7	2B, HTP	**c.1437delC**	**p.C479X**	**novel**
14	D-M	K5	exon 1	1A, HIP	**c.531_533delTAA**	**p.177delAsn**	**novel**
15	D-M	K14	exon 1	1A, HIP	c.374G>A	p.R125H	reported[20]
16	D-M	K5	exon 7	2B, HTP	c.1429G>A	p.E477K	reported[12,14]

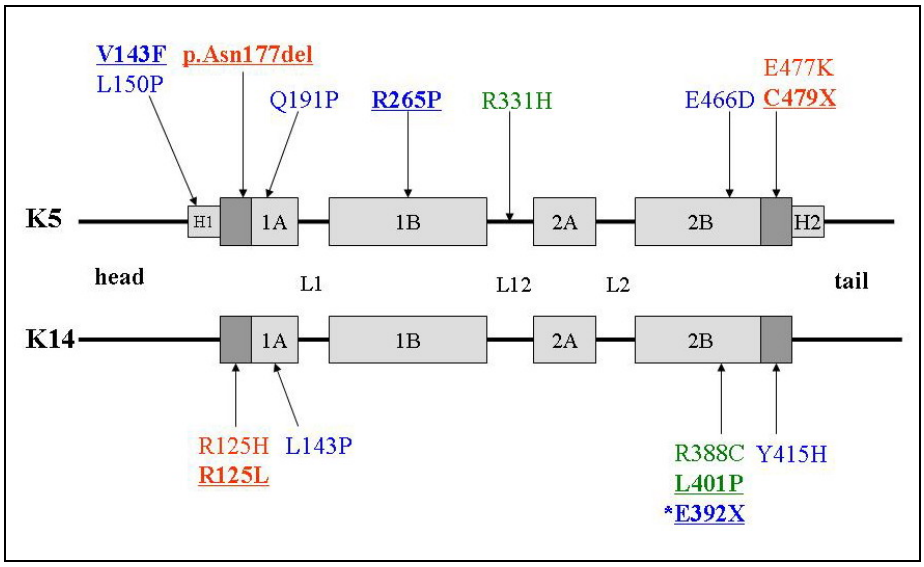

Figure 4. Schematic representation of mutation locations and EBS subtypes. Mutations in the EBS-WC are indicated by green, mutations in the EBS-K are indicated by blue, and mutations in the EBS-DM are indicated by red. Novel mutations are underlined.

* indicate homozygous recessive mutation.

IV. DISCUSSION

Mutation analysis of *KRT5* and *KRT14* in EBS is necessary to ensure precise diagnosis, prognostication, genetic counseling, prenatal diagnosis, and identification of future gene therapy trials that might benefit patients. So far, mutational analyses of *KRT5* and *KRT14* have been published only in certain ethnic groups in Western countries[9-14] and Japan[17]. Although there have been a few sporadic case reports of EBS in Korea, this is the first report of an ethnic mutation in Korean EBS patients.

Pathogenic mutations were identified in all sixteen EBS cases. These include thirteen missense mutations, two nonsense mutations, and one small deletion mutation. Overall, a single amino acid substitution is the predominant mutational event, which is consistent with previous data[21]. The frequencies of K5 and K14 mutations are reportedly similar. However, Yasukawa et al.[17] showed that the proportion of K5 mutations in Japanese patients with EBS is higher than that of K14 mutations (78% vs 21%, respectively). They proposed that it might reflect a characteristic ethnic feature in Japanese EBS patients. In this study, nine mutations were K5 mutations and, seven were K14. The frequency of a K5 mutation is slightly higher than that of a K14 (56% vs 44%, respectively), but the significance of this difference has not been determined. All EBS-DM mutations in this study are located on the helix boundary motifs of K5 and K14. This result is consistent with that of previous reports showing that the mutations that lie within the highly conserved end of the rod domain of K5 and K14 are critical for filament assembly and show a distinct clinical phenotype for EBS.

Patient 14 had c.531_533delTAA in exon 1 of *KRT5*, resulting in single amino acid deletion, K5-p.177delAsn. The patient showed very distinct clinical features such as generalized blisters, oral erosion, nail dystrophy, malnutrition due to poor oral intake, and widespread skin and systemic infection. Histopathologic examination of lesional skin showed

intracytoplasmic separation of basal keratinocytes. Electron microscopic study showed keratin clumpings in the cytoplasm of the basal keratinocytes (Figure 5). Liovic et al. reported that a mutation, K5-p.N177S (with the same residue as that of patient 14), in the structurally conserved helix initiation peptide motif causes a mild EBS-WC phenotype[22]. They suggested that the location of the protein defect might be of secondary importance to the type of amino acid change. Within the helix heptad repeat, N177(N9) is in position **f** (outward looking residue) of the helix heptad repeat and not involved in the process of heterodimerization (Figure 6). We can speculate that K5-p.177delAsn causes the structural deformity in the helix heptad repeat structure and is unable to form a stable heterodimer between mutant K5 and normal K14. Patient 13 had c.1437delC, K5-p.C479X, on the helix termination motif, and this novel mutation resulted in the EBS-DM phenotype.

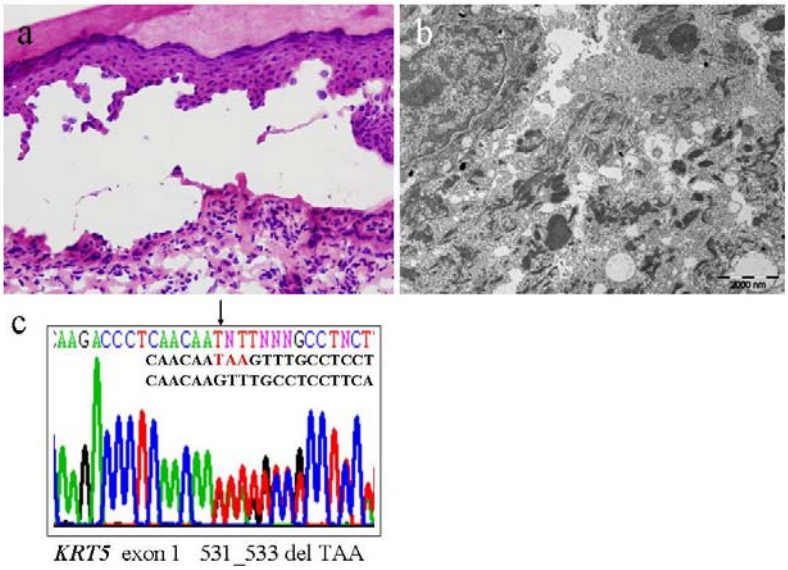

Figure 5. H&E stain of a lesional skin biopsy from patient 14. Suprabasal separation of the epidermis is apparent (a), electron microscopic examination showed keratin clumpings in the cytoplasm of the basal keratinocytes (b). Genomic DNA sequence analysis of *KRT5* showed TAA deletion at nucleotide 531-533, p.Asn177del, in the helix initiation peptide of *KRT5* (c).

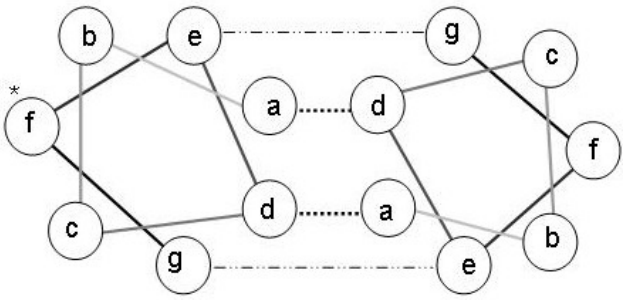

Figure 6. The α -helical rod domain possesses the characteristic heptad structure (abcdefg)n, which has apolar interactions between positions e and g, and salt bridges between positions a and d. In the case of N177, the position is located at ***f**.

Patient 16, K5-p.E477K, diagnosed with EBS-DM, showed remarkably similar clinical features to those of patient 14. Electron microscopic study revealed keratin clumpings in the cytoplasm of the basal cell (Figure 7). Previously reported cases of patients afflicted with K5-p.E477K showed exclusively severe phenotype since birth[12,14].

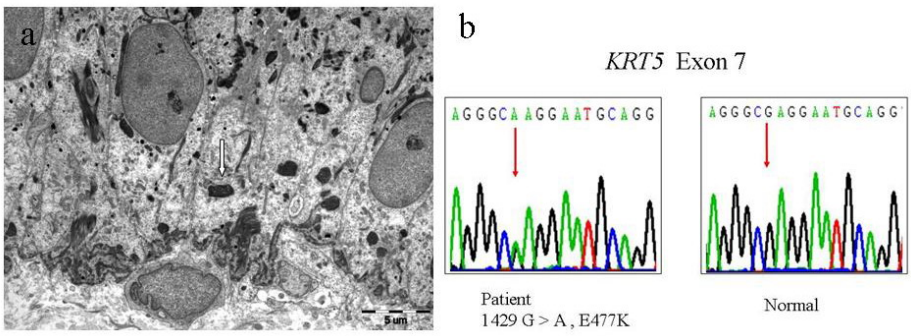

Figure 7. In patient 16, electron microscopic examination showed keratin clumpings (indicated by white arrow) in the cytoplasm of the basal keratinocytes (a). Sequence analysis of *KRT5* exon 7 showed 1429 G>A substitution, resulting in p.E477K in the helix initiation peptide of *KRT5* (b).

K14-p.R125 is a peculiar mutagenic codon that includes a highly mutable CpG dinucleotide in the DNA sequence, which accounts for the high prevalence of mutations. In this residue, p.R125C, p.R125G, p.R125H, and p.R125P have been reported previously[20,23,24]. All mutations in this residue have resulted in the severe phenotype of EBS-DM. We found two patients with this residue, and both of which were diagnosed with EBS-DM. One case is recurrent, p.R125H (patient 15). The other is novel, p.R125L (patient 12). Thus, the mutation in this residue was found to be more diverse.

Interestingly, Patient 6, who had a novel homozygous c.1174G>T mutation in *KRT14*, p.E392X/p.E392X, showed autosomal recessive EBS and squamous cell carcinoma of the tongue. He showed generalized scattered blisters and superficial erosions on sites of trauma followed by brownish reticulated patches, punctuate keratoderma of the palms and soles, and dystrophic toes and toenails. The ulcerative mass on his tongue arising from a site of frequent blistering was diagnosed as a squamous cell carcinoma. Histopathologic examination showed a subepidermal blister. Electron microscopic findings revealed reduced keratin tonofilament bundles in basal keratinocytes and the lysis of basal cells (Fig 8). Previous reports of recessive EBS have been more frequent in geographical regions with a high prevalence of consanguineous unions[10]. Overall, the majority of mutations result in the premature termination of translation and the absence of K14, which are caused by the functional knockout of type I keratin K14[25]. It was proved experimentally that K14 mRNA was undetectable because of the nonsense-mediated decay of the message. The reason that autosomal recessive EBS is almost exclusively associated with the K14 mutation is not clearly understood. However, the other type I keratin, K15, is regarded as a candidate to compensate for the loss of K14[26]. The clinical phenotype of recessive EBS varies markedly. Some researchers have proposed that no correlation between the position of the premature termination codon and the severity of the phenotype is obvious. patient 6 presented one of the most severe clinical

phenotype in reported recessive EBS[27] and unusual malignancy on his tongue and it is unclear whether there was an association between the development of tongue cancer and EBS.

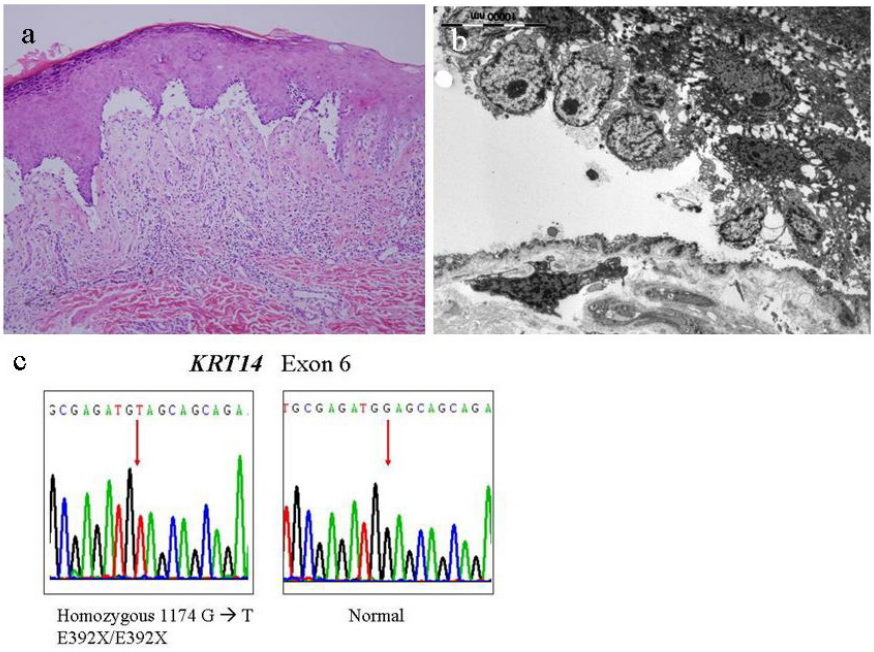

Figure 8. In patient 6, histopathologic examination revealed a subepidermal blister (a), electron microscopic study showed lysis of the basal layer, and reduced of tonofilament bundles in basal keratinocytes (b), genomic DNA sequence analysis of *KRT14* showed a homozygous mutant, c.1174G>T, resulting in p.E392X (c).

Two cases, p.V143F and p.L150P (patient 4 and 7 respectively), are mutations on the head domain of K5, which usually results in EBS-WC. It is an unusual finding that these two mutations in our study resulted in EBS-K phenotype.

The p.E466D on the 2B domain of K5 (patient 8) has previously been reported as a case of EBS-K[16].

A p.R265P (patient 5) is a novel mutation on the 1B domain of K5. The patient showed generalized blisters since infancy, which were diagnosed as EBS-K. Mutation of the 1B domain of K5 is quite rare, and only one case (K5-p.L311R) has been reported by Ciubotaru et al[10]. Previous data show that a mutation occurring on the 1B, 2A rod domain of K5, or the 2A domain of K14 is quite rare or absent.

A genomic DNA sequence analysis of K14 of patient 10 revealed two mutations, K14-p.L143P and K14-p.A413T. The former was a pathogenic mutation, and the latter was a polymorphic variant. Chao et al[28] reported the case of K14-p.A413T as a causative pathogenic mutation of an EBS-K patient. However, Hattori et al[29] reported an EBS-K patient with two mutations, K14-p.A413T and K5-p.V186M, and a sequence analysis of 56 normal healthy samples showed that the K14-p.A413T was a polymorphic variant found in six normal control samples. We also found a K14-p.A413T on fifty normal controls in sample study.

K14-p.Y415H (found in patient 11) is located on the helix termination peptide of the 2B domain. According to a previous report, K14-p.Y415H showed phenotypic variation. An EBS-K patient who presented with a mild phenotype and an EBS-DM patient were reported by two groups, repectively[19,30]. Our patient, with EBS-K, presented with moderate clinical severity.

Patient 3, who is carrying p.R311H of K5 in the L12 domain, showed a mild phenotype which was compatible with EBS-WC. Almost all mutations in the L12 domain of K5 have been reported as EBS-WC. Our case (patient 3) is compatible with this result. Patient 2, who was diagnosed with EBS-WC, had K14-p.R388C. Previously, K14-p.R388C and K-14-p.R388H were reported, and all patients showed mild phenotypes consistent with those of EBS-WC[12,13].

From this finding, we can assume that the mutational location on K5 and K14 is very informative in predicting the clinical outcome and prognosis of EBS patients.

Not only the site of the mutation but also the type of amino acid substitution may be important in determining the clinical phenotype[7,31]. In general, if no change in polarity or acidity occurs in a mutant protein, the resulting phenotype might be less severe than in the opposite situation. For example, p.Y415C and p.Y415H are reported mutations of the same site on K14. However, patients showed profoundly different clinical phenotypes. A patient carrying p.Y415C was diagnosed with EBS-WC[10], but the other patient carrying p.Y415H showed severe clinical phenotype consistent with EBS-DM[30]. This discrepancy may be explained by the type of amino acid that is substituted for the tyrosine residue at 415. The p.Y415C substitution results in the replacement of a neutral tyrosine by neutral cysteine. In contrast, the p.Y415H substitution results in the replacement of a neutral tyrosine to a polar basic histidine, and this is likely to interfere more seriously with keratin filament assembly and lead to a more severe phenotype. Interestingly, our case (Patient 11) who had p.Y415C on K14 showed moderate severity as EBS-K. We investigated the changes in the polarity of amino acid substitution (Table 5). We could not elucidate a clear relationship from the changes in the polarity of amino acid substitution. Further studies are needed to clarify this issue.

In summary, we performed the first large mutational analysis of 16 unrelated Korean EBS patients. Our data showed that patients with EBS phenotype harbor a spectrum of mutations in the *KRT5* or *KRT14*. Our data were similar to those of previous studies. Our results suggest that phenotype severity is ultimately determined by a combination of variables, which include the location of mutation, the nature of the amino acid change, the mode of inheritance, and other genetic modifiers, some of which are poorly understood. This study enhances the understanding of the EBS genotype-phenotype relationship and provides useful data for genetic counseling, prediction of prognosis, and attempting future gene therapeutic trials.

Table 5. Changes in amino acid side chain polarity in 16 EBS patients.

Patient No.	**EBS subtype**	**Keratin**	**Mutation protein**	**Changes in side chain polarity**
1	W-C	K14	p.L401P	nonpolar neutral → nonpolar neutral
2	W-C	K14	p.R388C	polar basic → polar neutral
3	W-C	K5	p.R331H	polar basic → polar basic
4	K	K5	p.V143F	nonpolar neutral → nonpolar neutral
5	K	K5	p.R265P	polar basic → nonpolar neutral
6	K	K14	p.E392X/ p.E392X	polar acidic → premature termination codon
7	K	K5	p.L150P	nonpolar neutral → nonpolar neutral
8	K	K5	p.E466D	polar acidic → polar acidic
9	K	K5	p.Q191P	polar neutral → nonpolar neutral
10	K	K14	p.L143P	nonpolar neutral → nonpolar neutral
11	K	K14	p.Y415H	nonpolar neutral → polar neutral
12	D-M	K14	p.R125L	polar basic → nonpolar neutral
13	D-M	K5	p.C479X	polar neutral → premature termination codon
14	D-M	K5	p.177delAsn	single amino acid deletion
15	D-M	K14	p.R125H	polar basic → polar basic
16	D-M	K5	p.E477K	polar acidic → polar basic

V. CONCLUSION

We investigated sixteen EBS patients and families of Korean origin by sequence analysis of the entire coding sequences of *KRT5* and *KRT14* as described above, and demonstrated pathogenic mutations in all cases. The summary of the results are described below.

1. Sixteen Korean EBS patients were classified in the each subtypes on the basis of diagnostic criteria as describe above. : Three patients were diagnosed with EBS-WC, eight were EBS-K, and five were EBS-DM.
2. Mutational analysis demonstrated 16 pathogenic *KRT5* and *KRT14* mutations (nine on *KRT5* and seven on *KRT14*). These include 13 missense, two nonsense, and one small deletion mutation.
3. Seven novel mutations were identified that four mutations on the *KRT5* (p.V143F, p.R265P, p.C479X and p.Asn177del), and three on the *KRT14* (p.R125L, p.E392X and p.L401P).
4. Five of 16 EBS patients were EBS-DM and all mutations (K14-p.R125L, K-14p.R125H, K5-p.C479X, K5-p.E477K, K5-p.Asn177del) located on the highly conserved ends of the alpha-helical rod domain, the helix initiation (HIP) or helix termination (HTP) peptides, respectively. Eight and three mutations are reported as EBS-K and EBS-WC, respectively.

The positions of mutations in both subtypes are widely distributed within the rod domains and in the L12 linker domains of both keratin genes.

5. One patient had a novel homozygous mutation, c.1174G>T/c.1174G>T on *KRT14* resulting in p.E392X/p.E392X, and was consistent with autosomal recessive EBS.

In conclusion, this study is the first large scale mutational analysis of EBS patients of Korean origin. This study should enhance our understanding of EBS genotype-phenotype relationship and provide useful data for genetic counseling, prediction of prognosis, future gene therapeutic trial in EBS patients.

REFERENCES

1 Uitto J, Richard G. Progress in epidermolysis bullosa: from eponyms to molecular genetic classification. *Clin Dermatol* 2005; **23**: 33-40.

2 Horn HM, Tidman MJ. The clinical spectrum of epidermolysis bullosa simplex. *Br J Dermatol* 2000; **142**: 468-72.

3 Kim S, Coulombe PA. Intermediate filament scaffolds fulfill mechanical, organizational, and signaling functions in the cytoplasm. *Genes Dev* 2007; **21**: 1581-97.

4 Gu LH, Coulombe PA. Keratin function in skin epithelia: a broadening palette with surprising shades. *Curr Opin Cell Biol* 2007; **19**: 13-23.

5 Lane EB, McLean WH. Keratins and skin disorders. *J Pathol* 2004; **204**: 355-66.

6 Irvine AD, McLean WH. Human keratin diseases: the increasing spectrum of disease and subtlety of the phenotype-genotype correlation. *Br J Dermatol* 1999; **140**: 815-28.

7 Porter RM, Lane EB. Phenotypes, genotypes and their contribution to understanding keratin function. *Trends Genet* 2003; **19**: 278-85.

8 Hatzfeld M, Weber K. Modulation of keratin intermediate filament assembly by single amino acid exchanges in the consensus sequence at the C-terminal end of the rod domain. *J Cell Sci* 1991; **99 (Pt 2)**: 351-62.

9 Sorensen CB, Ladekjaer-Mikkelsen AS, Andresen BS *et al.* [Epidermolysis bullosa simplex: genotype-phenotype correlation in Danish patients]. *Ugeskr Laeger* 2000; **162**: 1873-6.

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

11 Hamada T, Kawano Y, Szczecinska W *et al.* Novel keratin 5 and 14 gene mutations in patients with epidermolysis bullosa simplex from Poland. *Arch Dermatol Res* 2005; **296**: 577-9.

12 Muller FB, Kuster W, Wodecki K *et al.* Novel and recurrent mutations in keratin KRT5 and KRT14 genes in epidermolysis bullosa simplex: implications for disease phenotype and keratin filament assembly. *Hum Mutat* 2006; **27**: 719-20.

13 Rugg EL, Horn HM, Smith FJ *et al.* Epidermolysis bullosa simplex in Scotland caused by a spectrum of keratin mutations. *J Invest Dermatol* 2007; **127**: 574-80.

14 Pfendner EG, Sadowski SG, Uitto J. Epidermolysis bullosa simplex: recurrent and de novo mutations in the KRT5 and KRT14 genes, phenotype/genotype correlations, and implications for genetic counseling and prenatal diagnosis. *J Invest Dermatol* 2005; **125**: 239-43.

15 Chen H, Bonifas JM, Matsumura K *et al.* Keratin 14 gene mutations in patients with epidermolysis bullosa simplex. *J Invest Dermatol* 1995; **105**: 629-32.

16 Oh SW, Lee JS, Kim MY *et al.* Novel keratin 5 mutations in epidermolysis bullosa simplex: cases with unusual genotype-phenotype correlation. *J Dermatol Sci* 2007; **48**: 229-32.

17 Yasukawa K, Sawamura D, Goto M *et al.* Epidermolysis bullosa simplex in Japanese and Korean patients: genetic studies in 19 cases. *Br J Dermatol* 2006; **155**: 313-7.

18 Sorensen CB, Ladekjaer-Mikkelsen AS, Andresen BS *et al.* Identification of novel and known mutations in the genes for keratin 5 and 14 in Danish patients with epidermolysis bullosa simplex: correlation between genotype and phenotype. *J Invest Dermatol* 1999; **112**: 184-90.

19 Hut PH, v d Vlies P, Jonkman MF *et al.* Exempting homologous pseudogene sequences from polymerase chain reaction amplification allows genomic keratin 14 hotspot mutation analysis. *J Invest Dermatol* 2000; **114**: 616-9.

20 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.

21 Rugg EL, Leigh IM. The keratins and their disorders. *Am J Med Genet C Semin Med Genet* 2004; **131C**: 4-11.

22 Liovic M, Bowden PE, Marks R *et al.* A mutation (N177S) in the structurally conserved helix initiation peptide motif of keratin 5 causes a mild EBS phenotype. *Exp Dermatol* 2004; **13**: 332-4.

23 Csikos M, Szalai Z, Becker K *et al.* Novel keratin 14 gene mutations in patients from Hungary with epidermolysis bullosa simplex. *Exp Dermatol* 2004; **13**: 185-91.

24 Morley SM, D'Alessandro M, Sexton C *et al.* Generation and characterization of epidermolysis bullosa simplex cell lines: scratch assays show faster migration with disruptive keratin mutations. *Br J Dermatol* 2003; **149**: 46-58.

25 Has C, Chang YR, Volz A *et al.* Novel keratin 14 mutations in patients with severe recessive epidermolysis bullosa simplex. *J Invest Dermatol* 2006; **126**: 1912-4.

26 Jonkman MF, Heeres K, Pas HH *et al.* Effects of keratin 14 ablation on the clinical and cellular phenotype in a kindred with recessive epidermolysis bullosa simplex. *J Invest Dermatol* 1996; **107**: 764-9.

27 Yiasemides E, Trisnowati N, Su J *et al.* Clinical heterogeneity in recessive epidermolysis bullosa due to mutations in the keratin 14 gene, KRT14. *Clin Exp Dermatol* 2008.

28 Chao SC, Yang MH, Lee SF. Novel KRT14 mutation in a Taiwanese patient with epidermolysis bullosa simplex (Kobner type). *J Formos Med Assoc* 2002; **101**: 287-90.

29 Hattori N, Komine M, Kaneko T *et al.* A case of epidermolysis bullosa simplex with a newly found missense mutation and polymorphism in the highly conserved helix termination motif among type I keratins, which was previously reported as a pathogenic missense mutation. *Br J Dermatol* 2006; **155**: 1062-3.

30 Rugg EL, Baty D, Shemanko CS *et al.* DNA based prenatal testing for the skin blistering disorder epidermolysis bullosa simplex. *Prenat Diagn* 2000; **20**: 371-7.

31 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.

Abstract (In Korean)

한국인 단순형 수포성 표피박리증 환자에서의 케라틴 5 번 및 케라틴 14 번 돌연변이 분석

<지도교수 김수찬>

연세대학교 대학원 의학과

강태원

단순형 수포성 표피박리증(Epidermolysis bullosa simplex, 이하 EBS)은 표피 세포내의 케라틴 5번 또는 14번 유전자의 돌연변이에 의해 발생하는 유전성 수포성 질환으로 대개 상염색체 우성형태로 유전된다. 본 질환은 세포 골격을 유지하는데 중요한 단백질인 케라틴의 형성 이상으로 가벼운 외부 자극에도 쉽게 수포가 생성되며 조직 소견상 특징적으로 기저 세포의 세포질 내에서 세포의 분리가 나타난다. 환자는 임상적 중등도에 따라 심한 병변 양상을 띄는 Dowling-Meara형(EBS-DM), 전신적인 양상을 보이는 Koebner형(EBS-K), 그리고 국한성 병변 분포와 경한 양상을 나타내는 Weber–Cockayne형(EBS-WC)으로 분류하며 이전의 연구에 의하면 이러한 임상적인 중등도와 케라틴 단백 유전자의 이상 부위와 밀접한 상관 관계가 있음이 알려지고 있다. Dowling-Meara형의 경우 케라틴 단백의 말단 부위에 존재하는 매우 보존된 막대 도메인의 위치에서 대부분 변이가 발견되는 것으로 보고되고 있으며, Köbner형과 Weber–Cockayne형의 경우는 상기 부위 이외의

위치에서 다수 발견되는 것으로 알려져 있다. 현재까지 130여 개의 케라틴 5번 및 14번 유전자의 이상이 알려져 있으나 국내의 EBS에 대한 연구는 아직 제대로 이루어지지 않고 있는 상태이다.

본 연구는 16명의 한국인 EBS환자 및 그 가계를 대상으로 하였고, 돌연 변이 분석 결과 모든 환자에서 돌연변이가 발견되었다. 총 16건은 13개의 missene, 2개의 nonsense, 1 개의 small deletion 돌연변이로 밝혀졌다. 이중 기존에 보고되지 않은 7건의 새로운 돌연변이를 발견하였으며, 케라틴 5번에서 4개(p.V143F, p.R264P, p.C479X and p.Asn177del), 케라틴 14번에서 3개(p.R125L, p.E392X and p.L401P)가 각각 발견되었다. Dowling-Meara형으로 진단된 5명(K14-p.R125L, K-14p.R125H, K5-p.C479X, K5-p.E477K, K5-p.Asn177del)의 분석 결과 돌연변이의 위치가 모두 케라틴 단백질의 매우 보존된 막대 도메인의 말단 부위에 위치함을 확인하였다. Köbner형과 Weber–Cockayne형으로 진단된 환자의 돌연변이는 일부 예외가 있으나 주로 상기 부위 이외의 위치에서 발견되었다. 또한 한 환자의 경우(homozygous c.1174G>T, K14-p.E392X/p.E392X)는 드문 것으로 알려진 상염색체 열성 형태의 EBS로 확인되었다. 이상의 연구 결과로 국내의 EBS환자에서 비교적 특징적인 유전형과 표현형의 상관관계가 나타남을 확인하였고 이는 EBS에 대한 우리의 이해를 넓히고, 추후 산전 진단 및 예후 예측, 유전자 치료의 개발에 도움이 되는 자료로 이용될 수 있을 것으로 여겨진다.

핵심되는 말 : 단순형 수포성 표피박리증, 케라틴 5번, 케라틴 14번, 돌연변이