COL7A1 Mutation Analysis in Korean Patients with Dystrophic Epidermolysis Bullosa

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Abstract

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Dystrophic epidermolysis bullosa (DEB) is characterized by mucocutaneous blistering, scarring and nail dystrophy following minor trauma. DEB is caused by mutations in the *COL7A1* encoding type VII collagen, which is the major component of the anchoring fibrils. DEB occurs as either autosomal dominant or recessive trait, usually recessive form having more severe clinical presentation. However, the phenotypical variability also depends on the different type of mutations in DEB alleles and their position within the gene. To date, more than 300 different mutations have been reported, but we cannot predict the exact genotype-phenotype correlation. Furthermore, no systematic study has thus far revealed detailed delineation of *COL7A1* mutations in Korean DEB patients except several case reports.

In this study, we performed mutational analysis of 18 distinct Korean DEB families (4 dominant and 14 recessive). The result demonstrated 30

pathogenic *COL7A1* mutations among total 33 alleles. It included total 24 kinds of *COL7A1* mutation: 8 premature termination codons (PTC), 6 insertion/deletion frameshift mutations, 6 glycine substitutions (GS), 4 alternative splicings. We found out 10 novel mutations and 5 different recurrent mutations, R669X, G798R, G2043R, G2204S and E2857X. A computer analysis was carried out to assess the potential for each mutation to affect the splicing of the *COL7A1* mRNA. It predicted information contents score (Ri) changes in 10 distinct mutations. Among the 10 mutations, 1094-G>C, 2392G>A (G798R), 6899A>G (Q2300R), 341G>T (G114V), 682+1G>A show significant Ri changes, which was confirmed to induce cryptic splicing in the previous reports or in this study. We classified the patients into four categories to investigate the genotype-phenotype correlation: dominant DEB; mild (mitis type), moderate severe, severe recessive DEB (Hallopeau-Siemens type). The mutations of DDEB families were 3 and 1 alternative splicing, and two patients of mild RDEB had compound heterozygosity of alternative splicing/GS and alternative splicing/PTC combinations. Most of mutations observed in moderate severe RDEB and HS-RDEB are PTC-causing mutations, which did not show any difference between two groups. This study also included 1 patient with rare variant of DEB, transient bullous dermolysis of newborn (TBDN), which was

compound heterozygote for G798R and 6246del27.

Key Words : dystrophic epidermolysis bullosa, *COL7A1*, mutation, Korean

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I. INTRODUCTION

One of the most important roles of skin is a protection from the external environment. The mechanical stability is provided by the epidermal basement membrane zone. This zone contains auxiliary structures, known as anchoring complexes, which consist of hemidesmosomes, anchoring filament and anchoring fibrils[1]. Type VII collagen is the major component of the anchoring fibrils that maintain adhesion of the epidermis to the dermis. Type VII collagen is a nonfibrillar collagen composed of three identical α1 (VII) chains. It is encoded by the COL7A1 gene on the chromosomal region 3p21, which consists of 118 exons and introns and spans over 30.5 kb[2]. Type VII collagen contains a central collagenous triple-helical domain and amino- and carboxy-terminal non-collagenous domains (NC1 and NC2, respectively). The collagenous domain consists of characteristic (Gly-X-Y)n repeat sequences, which are interrupted by the insertion or deletion of one or several amino acids on 19 occasions. The largest of theses is a 39-amino acid non-collagenous 'hinge' region near the approximate center of the collagenous

domain. The collagenous domain is flanked by amino- and carboxy-terminal non-collagenous domains[3].

Dystrophic epdermolysis bullosa is an inherited blistering skin disorder, characterized by mucocutaneous blistering, scarring and nail dystrophy following minor trauma. DEB is caused by mutations in the *COL7A1*, and occurs as either autosomal dominant or recessive trait, usually recessive form having more severe clinical presentation[4]. However, the phenotypical variability also depends on the different type of mutations in DEB alleles and their position within the gene. Typically, the most severe type of DEB, the recessive Hallopeau-Siemens variant (HS-RDEB), is frequently due to the presence of mutations leading to premature termination codon on both alleles[5], whereas autosomal dominant cases are frequently caused by heterozygous glycine substitutions within the collagenous triple helix[6]. However, heterozygous PTC on one allele usually does not cause any clinical abnormality. The accumulating database of other missense, splice site, or insertion/deletion frameshift mutations offers to increase the understanding of genotype-phenotype correlation.

Splicing mutations make a significant role to human genetic disease, since they account for approximately 15% of all mutations in human disease[7] and almost 17% in *COL7A1*[8]. Splice site mutations may result in exon skipping, activation of cryptic splice sites, creation of a pseudo-exon within an intron, or intron retention[9]. The effect of a specific mutation on the splicing can be predicted in silico by information theory[10]. Changes in the affinity of a protein for its binding site, such as splicing machinery, can be estimated from the individual information content of the natural and variant sequences[11]. The computational tool can calculate the individual information content (Ri) for any variant that occurs within a binding site, and can be used to predict whether genomic DNA sequences changes may affect splice site.

To date, more than 300 different mutations of the *COL7A1* have been reported, but we can not predict the exact genotype-phenotype correlation.

Furthermore, very few recurrent mutations or hot spot are known. Since the mutations are distributed over the entire gene, the mutation analysis of *COL7A1* is time consuming and expensive[12]. Especially, in Korean DEB patients, no systematic study has thus far revealed detailed delineation of *COL7A1* mutations patients except several case reports.

In this study, we performed mutational analysis of 18 distinct Korean DEB families (4 dominant and 14 recessive), as well as the computational study of each mutations, and have demonstrated the characteristic features of *COL7A1* mutations in Korean DEB patients.

II. MATERIALS AND METHODS

1. Subjects

Eighteen unrelated Korean DEB families were studied. DEB was first clinically diagnosed and later confirmed by immunofluorescence antigen mapping and electron microscopy if the diagnosis is not definite with clinical findings.

2. Mutational analysis

A. DNA samples

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

B. Polymerase chain reaction amplication

Total DNA was used as template for amplication of genomic sequences of *COL7A1* (GenBank L23982). *COL7A1* segments including all 118 exons, and all exon-intron borders were amplified by PCR using pairs of oligonucleotide primers synthesized on the basis of intronic sequences. The primers were designed by Primer3® program (http://cbr-rbc.nrc-cnrc.gc.ca/cgi-bin/primer3_www.cgi). The primer sequences were described in table 1.

Amplification conditions were: 95°C for 1 min, followed by 35 cycles of 95°C for 40 s, 54-64°C for 40 s, and 72°C for 1 min. The final extension was at 72°C for 3 min.

Table 1.The sequences of PCR primers

Exon	Forward primer	Reverse primer
1	CAggCAAgACCAggACTCgg	gTCgTggAgTTggCTgggTT
2	ACCATCCCAAgTCCCAgTgA	TgTTTCTgCAAAgACCTggC
3,4	ggCCAgAAgAgATCCTgAgT	CTgACCTgTCACTCCTgCTC
5	AgCAggAgTgACAggTCAgC	gggTCAggAgCACATAggAT
6	gTgTACCCTgACCTAgACCC	gAggTCACTTTATCTTgCCC
7	TCAggAgTgATAggTggTggC	CAgggATTCATggAgTCAgA
8	CAATTCTgCCAgCCTCTgAC	gCCTTgCAgACTCAggACTC
9	gTgAgAgATgTgggCTgAggggA	gCACATgggATgTCAgTggC
10	AggCTgggCACTTTCTTCAg	gTCAgACCAgCAgAggCCAT
11	gAAgggATggACAggCAAgg	AgCACAgCATAgAggCAgCC
12	CAgTgAgTgggggAggTgTC	gAAggAgAgggCTggAggTA
13	CCTTCTCACTCTgCgTCCCT	AACCAggACCAgAgTgAggC
14	TgAgTACTgCAggAggCTTg	TgAggTCAgAgggAAATgC
15	AATgAgggTATgggTgCCAg	ggAggAgggAgTgggATTCT
16	AgACTCCCATCATCTTCCCC	CACCTggACCCCCAATAAAC
17	ACAgAgTTTgCTAgCCCTgg	CTgggCAATCAggAACACAC
18	gCTgCCTAAAgTgACCTgTC	gCATACAgCAATggTTAggg
19	CCCTAACCATTgCTgTATGC	CCAAAggCTCACTACCAATC
20	CAgggTCTgAgAggAgggAg *new: CTggATTggAgAAAggACCA	CCATCAgTgTCCTCgCCTACC
21	AACCCAgTTAACAgAgCCAg	ggAggAgTCACTCAgAgTCg
22	ACCCAggATCTCAgATCTCT	TgCAggAgACAgAACTTgAT
23	AgTTggggCTCTgTggAgAC	CAAgTTACTgAAgCgggCAg
24	ATAgTgggCgTAgTgggAAg	TgTgAgAgAgCTgggAgAAT
25	CACCCTgATgTgTTTCTCCA *new: TCCTCCTCAggCTCTgTgTT	ggAAggACATgTCAgAACCC

26	gCATggACTCCTggggCTAT	TAAggTggggTCCAgTggCT
27	gTAAggAgTAggCTgATggg	AgggTCTCTTTgAggTTgAA
28~31	gggACTgggTggTAgAATAT	gAgACAgCTTTgAggAgTgC
28~29	TATAAggggTCTgggggTTC	ggCCCTAggAAgggTAATCA
30~31	ggAACCTggAgAgATggTgA	AACCTCgATggTCTCCACAC
32,33	TCTgCCTCACTgTTCCACCC	gCTCAggCgAATgTCAACgT
34,35	TgCTCTCTAAgTgTCTTCCC	CCCACTACACATCACTTgCC
36	ggTATgTggAggCAAgTgAT	CAAggATTTTgggAgAACTg
37,38	CTCCCAAAATCCTTgAAATC	AgAACTATgAAgCCCAgCAC
39,40	AgTggTTgggTgCTgggCTT	gCCAATAgCTCCAggAggTC
41,42	TTTCTCCTTCAgggTgACTC	CACgTTCgCCCTgATggAAA
42,43	AggTCAgAggTCgTggTTTT	ggTTAgAgCCTgTATCAgCA
44~46	TCTAgCCCTgTCTgTCCATA	TATAggAgggTCACTgCTCA
47,48	gACTTCCAATTCCATgTgAC	CTgTggATggAAggATAAgA
49,50	gggCAgTTggTgAAggTTgT	AAgAgggAggTgATgCAgg
51~53	CCTTgAgAACTgCTTgCTTC	TTTCCTATCACCTTCATgCC
54	TgATgggAACCTCTgATgTg	gAAgATTgggAgggTTTAgC
55	CAACCTTAACCCTCCAACCA	CTCAACTCTgCCCCCAAgT
55,56	ACACACgCATCTgAAggCTA	AggTTTCAgAgggACAgTgg
57~60	CCTCACAgACCCTgTATCCC	ggATCTgATAACCCAggCTC
61,62	ATgAgCCTgggTTATCAgAT	TCTCTCggATgCTgTgACTA
63,64	gCCCCAAgggATATCTCAgAg	TCTTggCTgTgTAggTgTgC
65~68	gTAgTgTCTTgCAgCCAgA	AACAAgAATgACCAggTggg
69,70	TgAgTgCggATgTTgggTAg	gCCCAAgTTCCCTTgAgTgT
71	gCAggAgCTTCTCTgTCATg	ACAgCAAgAggTCAgAggAg
72	TCAAggTgggTTgTTTAggg	ggAAgAgAgAATgCTggTgg
73	gggTgTAgCTgTACAgCCAC	CCCTCTTCCCTCACTCTCCT
74,75	CCAggAgAgTgAgggAAgAg	TAgggTCAgAAATTCCAggg

	*new: gAgCCTggAAAgCCTggT	*new: ACCAAgCTAAgggTggCTTC
76	TgACTAgTgACCAggAAgCC	TCAAgTCAgTCCCTAgTgCC
77,78	gCTAAggTCAgTgTgTggAA	CCCTgCACACAggACAATAC
79	gTAAgTCCTTgCCCAACAgCC	CAgAgAggCACACAgACACAg
80~82	CAAgTgAggCCCAgATTgA	ggCATggCACAgCTTgAA
83~85	TAgTgTgCgCCAACCTCCTg	CTgCCTgTCgACCCTTgACC
86,87	gTCAAgggTTgggCTCCAgg	TggAAACAggCTTgTgggTg
88,89	CACAAgCCTgTTTCCAAATg	gggTgggTAAACTATgggTC
90,91	CgCATATTTAAgCTCTggCC	CTTATgCCCgCCATCACACT
92,93	AgCCCgTgTCTgAACTCTgT	ACTCCCTCTTCCTCCTgTgg
94,95	TgATgAgAgTCCTgggAggg	CCCATCCTAAgTCCTCACgA
96,97	TCgTgAggACTTAggATggg	gAggTTggAAATCAgAggCA
98,99	CTCTTgCCTCTgATTTCCAA	CCCgCACCTgAATTCTAATA
100~102	gAAggTCCTggCATgAgTgg	TgCCCTCACAgTAgCTgTgg
103,104	CgggCTCgTTgTATTCTAAg	CAAAAgCTACCACACTggTg
105,106	CCACTATCCAgggCgATTCT	gCAgTggggTgAgCCTTAgg
107,108	gTACAgAggggATgggggCT	CTACACCCCCATgACCCgAC
109,110	gAgTTCAgggAggTTCCAgA	TggTTATgAggTTggAAggg
111,112	AgCTCTgACTCCTgATCCCT	gggACTATggTgAgACTgCA
113	TCCATgCAgTCTCACCATAg	CTTgACTgCTTgCCCTgTAA
114,115	CCCTCTgCCTgTgTgTCTCT	CTgCATTCATggACACCCAT
116	ACAgTggAAATCAgTgCTgC	AgggTTTgTgggAATCAgAg
117	CCCTgACCTTTCAACCCTCT	AAggACTCCTCCCCCAgAAC
118	TCTCCggggAAggTCAgATg	CATCACAggCTTgggTCAAg

* 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 from 50 normal, healthy Koreans was used as a control.

D. RNA extraction and Reverse Transcriptase (RT)-PCR analysis

Following informed consent, extraction of total RNA from skin biopsies of the patient and control was performed using Qiagen RNeasy Mini Kits (Qiagen, Hilden, Germany). Reverse transcription was performed with TaKaRa RNA PCK Kit (Takara, Shiga, Japan) with 1μg of total RNA, using oligo dT primers. The primers and the annealing temperature were summarized in Table 2.

Table 2.The summary of the primers and annealing temperature of RT-PCR

Target mutation	Forward primer	Reverse primer	Product size	Annealing temperature
2392G>A	ggatggactggagccagata	gctgactccaccttcgagac	298 bp	62°C
	ggatggactggagccagata	tcatggggccgcccaggtgat	181 bp	59°C
1094-1G>C	cggaactgaccatccagaat	aactggtagcgggtcacatc	421/274 bp	60°C

3. Computational analysis

To assess the potential pathogenicity of the mutations found in this study, information theory splice site analysis[10] was carried out via the world wide web interface at http://splice.cmh.edu/.

III. RESULTS

1. Clinical findings

Eighteen Korean unrelated DEB patients were studied (Table 3). In four DEB patients, the pedigree pattern was autosomal dominant; the other 14 patients showed autosomal recessive inheritance pattern. The RDEB cases were subclassified, based on clinical features, as follows: (a) HS-RDEB was characterized by severe mutilating phenotype with extensive erosions and blistering since birth, pseudosyndactyly and joint contractures; (b) moderate severe RDEB was characterized by milder and more localized involvement and lack of mutilating pseudosyndactyly; (c) mild or mitis RDEB was characterized by the mildest blisters and scarring limited to trauma-exposed sites; (d) a case of TBDN. Among the 14 RDEB cases, four patients were HS-RDEB, and seven patients were moderate severe RDEB, and two patients had mitis type RDEB, and one patient had a phenotype of TBDN.

Table 3. Phenotypes and genotypes of Korean patients with dystrophic epidermolysis bullosa

	phenotype	**mutation**		**exon**	
1	dominant	G2043R (6127G>A)		73	
2	dominant	Q2300R (6899A>G)		87	
3	dominant	G2043R (6127G>A)		73	
4	dominant	G2034R(6100G>A)		73	
5	TBDN	G798R (2392G>A)	6246del27	18	75
6	RDEB, mitis	G1694C (5080G>T)	682+1G>A	55	intron 5
7	RDEB, mitis[¶,13]	G114V (341G>T)	R1933X (5797C>T)	3	70
8	moderate	Q1211X (3631C>T)	not determined	27	-

9	moderate	242delCA	not determined	2	-
10	moderate	R1730X (5188C>T)	not determined	58	-
11	moderate	G2204S(6610G>A)	R669X (2005C>T)	82	15
12	moderate	R669X (2005C>T)	E2857X (8569G>T)	15	116
13	moderate	2645del4	E2857X (8569G>T)	20	116
14	moderate[¶, 14]	G2204S (6610G>A)	S1689X (5066C>G)	82	55
15	HS-RDEB	G2034E (6101G>A)	E2857X (8569G>T)	73	116
16	HS-RDEB	G798R (2392G>A) 2621ins5 (GCTTC)	Q1286X (3856C>T)	18 20	31
17	HS-RDEB	R578X (1732C>T)	2063delC	13	16
18	HS-RDEB	8291delGA	1094-1G>C	111	intron 8

¶ : previously reported patient

2. Mutational analysis

A. *COL7A1* mutation survey

We performed mutational analysis of 18 distinct Korean DEB families (4 dominant and 14 recessive) and the results are summarized in the Table 2 and the figure 1. The result demonstrated 30 pathogenic *COL7A1* mutations among total 33 alleles (It included 4 dominant alleles, 13 recessive alleles and the 3 mutations in 1 family.) with an overall mutation detection sensitivity of 90.9%. It included total 24 kinds of *COL7A1* mutation: 8 PTC, 6 insertion/deletion frameshift mutations, 5 GS, 5 alternative splicings. No such mutations were found in the 50 unrelated controls. We found out 10 novel mutations: 242delCA, 1094-1G>C, 2063delC, 2392G>A (G798R), 2621insGCTTC, 2645del4, Q1286X (3856C>T), G1694C (5080G>T), 6246del27, 8291delGA.

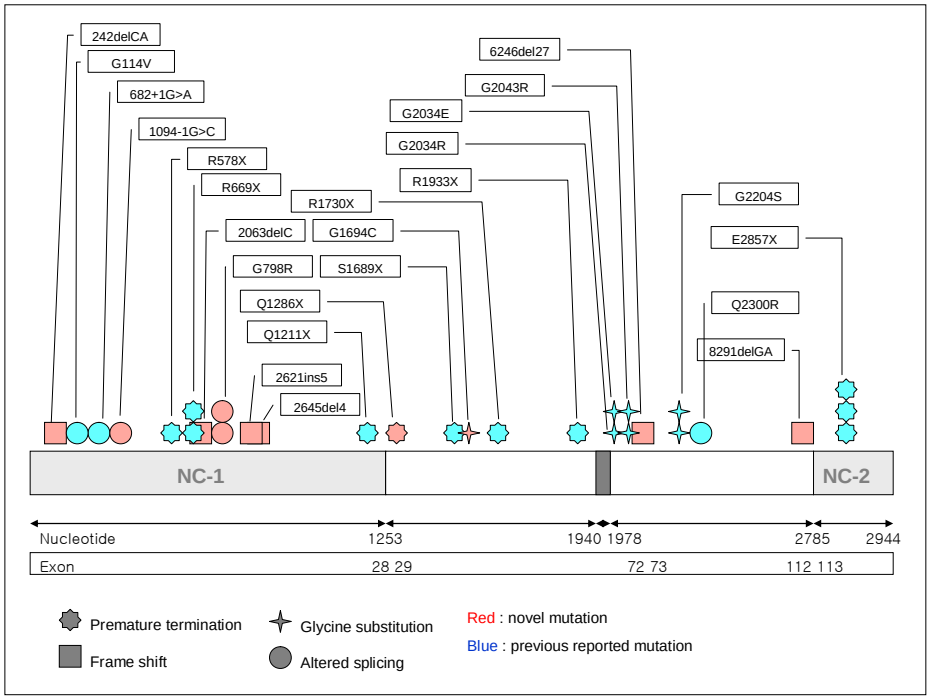

Figure 1. Schematic representation of type VII collagen domain organization, and position of *COL7A1* mutations disclosed in families with Korean DEB

B. RT-PCR analysis

We performed RT-PCR to access the possibility of cryptic splicing in the region of the mutation G798R (2392G>A) and 1094-1G>A. In the case of 2392G>A, we designed two sets of primers because the mutation 2392G>A results in downstream PTC (Table 2, Figure 3). The forward primer is same, but the reverse primer of former one is designed for the sequence of normal splicing product, which is located in exon 19. The reverse primer of the latter one is designed for that of cryptic splicing product, which is located just upsteam of the PTC in exon 19. Agarose gel electrophoresis of the amplified PCR products showed two bands in the proband's and mother's

samples compared with a single band in the control (Figure 2A). Direct sequencing of the smaller band (proband and mother) confirmed the anticipated cDNA sequence.

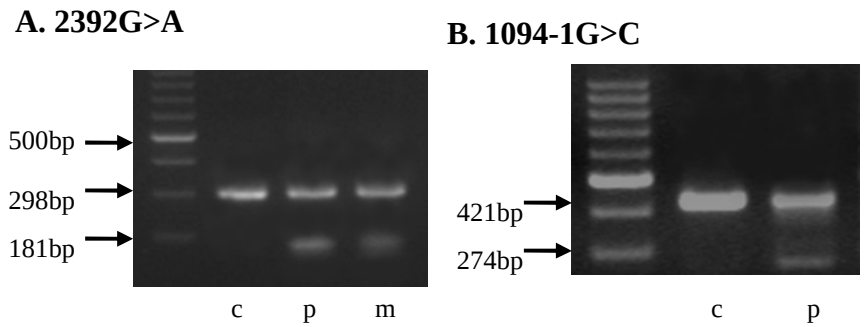

Figure 2. 2% agar gel electrophoresis of the cDNA samples of the mutation 2392G>A (A) and 1094-1G>C (B). (A) The RT-PCR amplification reveals one abnormal transcript of 181bp in the sample of patient (p) and the mother (m), in addition to the 298 bp band present in the control sample (c, p, m). **(B)** The mutation 1094-1G>C results in two transcripts of normal 421bp and abnormal 274bp (p), whereas the transcript of control reveals only normal 421bp transcript (c).

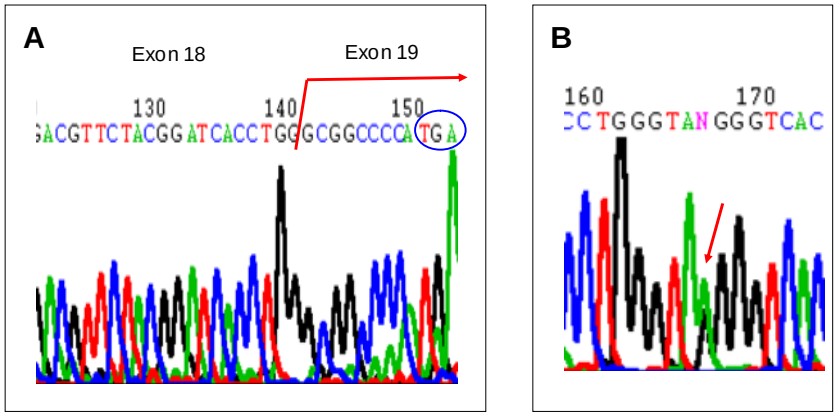

Figure 3. Direct sequencing of the RT-PCR product from the patient

with the mutation 2392G>A. (A) Direct sequencing of 181bp lower band reveals the expected cryptic splicing in exon 18, which results in PTC in exon 19. **(B)** On the other hand, direct sequencing of 298bp upper band reveals normal splicing sequences that include unaffected normal cDNA sequence and mutated sequences with normal splicing (arrow).

3. Computational analysis

A computer analysis was carried out to assess the potential for each mutation to affect the splicing of the COL7A1 mRNA. It predicted information contents score (Ri) changes in 10 distinct mutations (Table 3). Among the 10 mutations, 1094-G>C, 2392G>A (G798R), 6899A>G (Q2300R), 341G>T (G114V), 682+1G>A show significant Ri changes.

Table 3. Ri changes of each mutations

mutation		ΔRi of cryptic splicing site	ΔRi of natural splicing site	Predicted result
242delCA		no change	no change	frameshift
341G>T	G114V	0.0→8.2 (86 upstream)	6.6 (no change)	alternative splicing
682+1G>A		-	5.6→-7.2	alternative splicing
1094-1G>A		-	9.5 →1.9(acceptor)	alternative splicing
1732C>T	R578X	4.2→2.6 (46 downstream)	8.88 (no change)	PTC
2063delC		no change	no change	frameshift
2005C>T	R669X	-2.1→5.4 (47 upstream)	8.88 (no change)	PTC
2392G>A	G798R	5.5→8.0 (52 upstream)	8.04 (no change)	alternative splicing
2621insGCTTC		no change	no change	frameshift
2645del4		no change	no change	frameshift
3631C>T	Q1211X	no change	no change	PTC
386C>T	Q1286X	no change	no change	PTC

5066C>G	S1689X	no change	no change	PTC
5080G>T	G1694C	4.9→1.6 (17 upstream) -3.8→4.4 (19 upstream)	10.31 (no change)	GS
5188C>T	R1730X	no change	no change	PTC
5797C>T	R1933X	-1.7→5.8 (25 upstream)	8.7 (no change)	PTC
6100G>A	G2034R	no change	no change	GS
6101G>A	G2034E	no change	no change	GS
6127G>A	G2043R	no change	no change	GS
6246del27		6.6 → -6.6 (5 upstream)	6.84 (no change)	frameshift
6610G>A	G2204S	no change	no change	GS
6899A>G	Q2300R	3.2 (no change, 59 upstream) 6.5 (no change, 87 downstream)	6.5→4.2	alternative splicing
8291delGA		no change	no change	frameshift
8569G>T	E2857X	no change	no change	PTC

IV. DISCUSSION

Mutation analysis of *COL7A1* is urgently needed for precise diagnosis, prognostication, genetic counseling and reliable prenatal diagnosis, and identification of suitable candidates for future gene therapy trials. So far, there are some ethnic reports of mutational analysis of *COL7A1*, which is including British[15], Mexican[16], Italian[17], Japanese[18] and central European population[19,20]. However, there is a few sporadic case report of DEB in Korea, and this is the first report of ethnic mutational report in Korean DEB patients.

Among the 29 pathogenic mutations, we found 8 PTC, which appear to be fairly evenly distributed throughout the COL7A1 gene. Among the Six insertion/deletion frameshift mutations, 5 frameshift mutations are out-of-frameshift mutations and result in downstream PTC. However, 6246del27 is in-frameshift mutation, which results in the deletion of 9 amino acid sequences. We found 5 GS and 5 alternative splicings. All GS are located in collagenous domain. In spite of complete sequencing of the all exonic sequences including exon-intronic border, we could not detect pathogenic mutation in 3 RDEB patients. In these cases, the mutations could reside in the intron or outside the coding regions, such as the promoter region which was not analyzed in this study.

We investigated the genotype-phenotype correlation in each type of DEB. At first, we found 4 distinct mutations in 4 DDEB patients. Three of them are GS in the exon 73 (G2043R, G2034R), and the other one is Q2300R. G2043R is known to be a recurrent mutation in the DDEB[21], and is also detected recurrently in our case. Another GS, G2034R, is also reported previously in DDEB[22-25]. Interestingly, the substitution of glycine with glutamic acid[19] or typtophan[26] at the same amino acid position was reported in DDEB case. Furthermore, a mutation at the same codon, G2034E, was found in RDEB patient in this study, which does not affect any phenotype in the parents. Tryptophan is smaller than arginine or glutamic acid, and seems to be less

destabilizing effect to type VII collagen assembly, which may account for the silent mutation of G2034E. Similar examples were reported at the amino acid position 2028 and 2623. The mutation G2028R has been detected in a patient with DDEB pruriginosa and a family with toenail dystrophy without skin fragility, while the mutation G2028A has been implicated in a family with classic DDEB[27,28]. Similar reports showed the mutation G2623S[29] and G2623C[30] in families with RDEB and pretibial form of DDEB, respectively. The last mutation in DDEB, Q2300R, is non-GS within the collagenous region. Patients with DDEB usually harbour GS mutations within the collagenous region of collagen VII. So far, only 7 mutations other than GS have been reported in the literature. Of these, 4084-1G>C[31], 6899A>G (Q2300R)[32] and 8045A>G[33], were missense mutation, which all result in the cryptic splicing. The others were gross deletion mutation, 6847del27[34], 6863del16[35], 6081del28[34] and 8068del17inGA[36].

There were two patients with mitis type RDEB, which showed G1964C/682+1G>A and R1933X/341G>T (G114V) combination mutations. The last mutation, G114V, is previously reported mutation as a splicing mutation that results in deletion of 87bp in the non-collagenous domain[13]. It has been known that the splicing mutation results in milder phenotype. It is because the spicing mutation allows the synthesis of a certain quantity of normal chains and the consequent assembly of partially functional type VII collagen[37,38].

Among 14 RDEB patients, seven patients were classified as moderate severe RDEB, and 4 patients as HS-RDEB (Table 4). In the moderate severe RDEB mutations, 81.8% were PTC-causing mutations, *i.e.,* nonsense mutations or out-of-frame mutations. In the HS-RDEB mutations, 77.8% were PTC-causing mutations, which was similar proportion with the moderate severe RDEB. However, the position of PTC-causing mutation did not show any correlation between genotype and phenotype. Furthermore, we cannot find any genotypic differences between the two types of RDEB to explain the

difference of their phenotype. Among the non-PTC-causing mutations, glycine substitution mutations were found in 3 patients, and splicing site mutation was found in one patient. The glycine substitution mutations of moderate severe RDEB or HS-RDEB differ from that of mitis type RDEB in that their location is relatively adjacent to the hinge domain, exon 73 and exon 82. The splicing site mutation in HS-RDEB, 1094-1G>A, results in in-frame skipping of exon 9. The splicing site mutations in mitis type RDEB were 682+1G>A and 341G>T, which result in downstream PTC and in-frame deletion of 87 bp, respectively. However, 682+1G>A had a glycine substitution mutation on the other allele, which may explain its milder phenotype. The latter mutation, 341G>T, also results in in-frame deletion as 1094-1G>A does. However, The smaller size of deletion part, 87 bp rather than 147 bp in the case of HS-RDEB, may explain its milder phenotype.

Table 4. Summary of genotype and phenotype of the patients with moderate severe RDEB and HS-RDEB

	phenotype	***mutation***		***Effect***	
8	moderate	Q1211X	not detected	PTC	-
9	severe RDEB	242delCA	not detected	PTC	-
10		R1730X	not detected	PTC	-
11		G2204S	R669X	GS	PTC
12		R669X	E2857X	PTC	PTC
13		2645del4	E2857X	PTC	PTC
14		G2204S	S1689X	GS	PTC
15	HS-RDEB	G2034E	E2857X	GS	PTC
16		G798R	Q1286X	PTC	PTC
		2621insGCTTC		PTC	
17		R578X	2063delC	PTC	PTC
18		8291delGA	1094-1G>C	PTC	exon skipping

This study included one patient (patient 5) with rare variant of DEB, TBDN, characterized by transient blistering which manifests at birth or soon after, usually with marked improvement or even resolution in the first few months to years of life. So far, there are 3 reports of identification of pathogenic mutations. One case involves a patient with compound heterozygosity for two glycine substitution mutations, G1519D and G2251E[39]. Christiano et al. identified a dominant acceptor splice site mutation in intron 35, 4120-G>C, which results in in-frame skipping of exon 36[40]. The last report describes a dominant heterozygous glycine substitution mutation, G1522E[41]. The TBDN patient here described was a compound heterozygote for a 2392G>A (G798R) splicing site mutation and 6246del27 in-frame shift deletion mutation. We could not find any common feature of the genotype in the patients with TBDN, although the splicing site mutation of this case could ameliorate the phenotype. Hammami-Hauasli also described that transitory blistering in TBDN has possibly more to do with the quantity rather than the quality of the collagen[40].

We found out 5 different recurrent mutations, R669X, 2392G>A (G798R), G2043R, G2204S and E2857X, which account for approximately 30% of DEB alleles in Korean patients. Especially, E2857X is reported in Japanese study in DEB patient as an ethnic-specific recurrent mutation[18,42]. Another recurrent mutation, R578X, has been reported to be an ethnic-specific recurrent mutation, exclusive to British patients[15]. Murata et al.[42] also demonstrated the absence of the recurrent mutation R578X in 42 non-British patients, mainly Asian. However, the mutation study of our patient demonstrated that the R578X mutation is not exclusive to British patients. Among the 5 recurrent mutations in our study, G798R is a novel mutation, which seems to be an ethnic specific mutation in Korean DEB patients. However, it has limitation to interpret due to the small number of patients.

In computational analysis, we found the Ri changes in 10 distinct mutations (Table 3). We reevaluated them to choose the mutations that have changed the Ri enough to make a cryptic splicing site or abolish the original splicing site.

Among the 10 mutations, 1094-G>C, 2392G>A (G798R), 6899A>G (Q2300R), 341G>T (G114V), 682+1G>A show significant Ri changes. The mutations, 341G>T (G114V), 682+1G>A, 6899A>G, were previously reported as a splicing site mutation. The mutations, 1094-1G>A and 2392G>A, are the novel splicing site mutation. In the case of 1094-1G>A, the mutation is predicted to abolish natural acceptor site of exon 9 (Ri decreases from 9.5 to 1.9 bits) resulting in skipping of exon 9, which was proven in the cDNA level (Figure 2B, Figure 4). The mutation, 2392G>A, also induces cryptic splicing. Ri of natural donor site of exon 18 (8.04) is not changed, whereas Ri of cryptic donor site located 52 upstream of natural site increases 5.5 to 8.0 bits. As a consequence, 52 bp nucleotides were deleted from exon 18 in the out-of-frame pattern, which resulted in PTC in exon 19 (Figure 5). The mutation, 2392G>A, was detected in two opposite phenotype, transient bullous dermolysis of newborn (patient 5) and HS-RDEB (patient 16). As mentioned above, the spicing mutation allows the synthesis of a certain quantity of normal chains and the consequent assembly of partially functional type VII collagen. In the patient 5, we could also detect both normal splicing sequence and cryptic splicing one, which was confirmed by direct sequencing of RT-PCR product (Figure 3A, B). This may account for the spontaneous healing or mild phenotype in the patient with transient bullous dermolysis of newborn. By contrast, we detected 3 mutations in the patient 16 with HS-RDEB, two maternal mutations (2392G>A, 2621insGCTTC) and one paternal mutation (Q1286X). Two maternal mutations, 2392G>A and 2621insGCTTC, induce downstream PTCs in exon 19 and 20, respectively. In the case of cryptic splicing at the site of 2392G>A, PTC appears in exon 19. By the way, PTC appears in exon 20 with normal splicing due to the mutation 2621insGCTTC. Therefore, even if the COL7A1 gene escape cryptic splicing at the site of 2392G>A, it should meet the mutation, 2621insGCTTC, which results in downstream PTC (Figure 4). Consequently, 3 mutations in patient 16 result in severe phenotype. After all, we found 5 splicing site mutations, which involve

20% (6 alleles) of total 30 pathogenic mutations. This result is similar with previous reports in human disease[7] (15%) and in *COL7A1*[8] (17%). In this study, we performed computational tools, which can accurately predict cryptic splicing as previously reported[13].

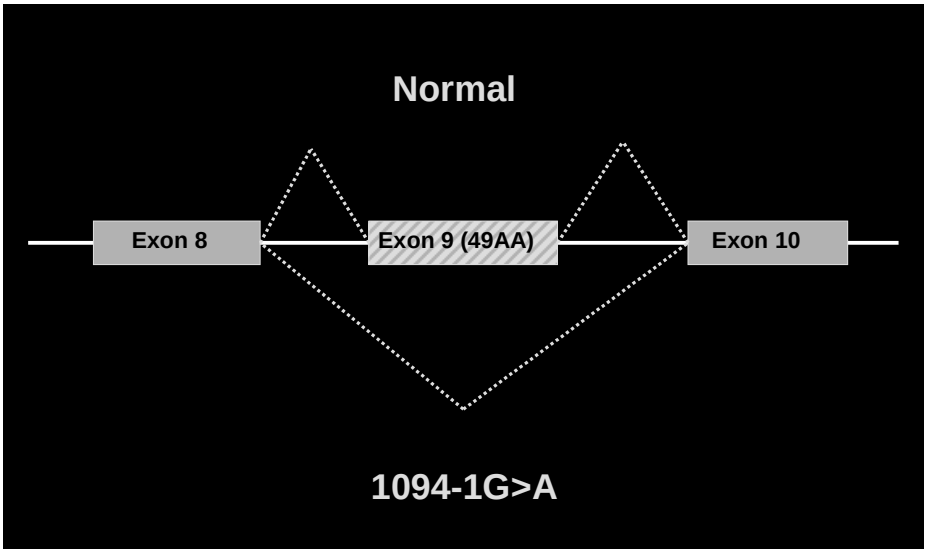

Figure 4. Schematic illustration of alternative splicing due to the mutation 1094-1G>A The mutation 1094-1G>A abolishes natural acceptor site of exon 9 (Ri decreases from 9.5 to 1.9 bits) resulting in skipping of exon 9 (49 amino acid sequences).

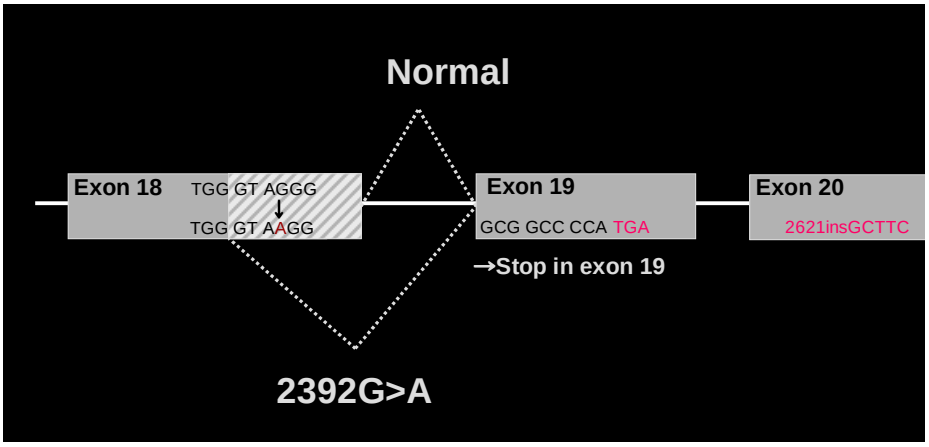

Figure 5. Schematic illustration of alternative splicing due to the mutation 2392G>A The mutation 2392G>A (arrow) induces cryptic splicing at the site of 52bp upstream from normal splicing site, which results in out-of-frame shift and PTC in exon 19, consequently. Even if it undergo normal splicing, there is another mutation, 2621inGCTTC in patient 16.

Comparing with other ethnic report of DEB, Korean patients with DEB seem to have relatively small number of patients with HS-RDEB. There are only 4 patients with HS-RDEB among 14 RDEB (28.6%). By contrast, the patients with HS-RDEB were reported with higher proportion in the Italian population[17] (55.1%), the report of Hovnanian et al[43] (86.7%) and the report of Kern et al[19] (83.8%). It seems that the Korean culture of uncommon consanguineous marriage in Korea may be one cause of small number of the patients with HS-RDEB. Actually, there is no homozygous mutation in this study, which may be due to non-consanguineous marriage. In Japanese study, we could find similar phenomenon. There was no homozygosity, and there were only 6 patients with HS-RDEB among 16 patients with RDEB (37.5%)[18]. The studies with Caucasian population, by contrast, show much higher proportion of homozygosity: 22.4% in Italian study[17]; 40% in the report of Hovnanian et al[43]; 37.8% in the report of Kern et al[19]. Thus, less frequent HS-RDEB can be a characteristic of Asian DEB. We could not find any other difference in the pattern of genotype in the patients with Korean DEB from other ethnic group.

In conclusion, we performed mutational analysis of 18 distinct Korean DEB families, and demonstrated 30 pathogenic *COL7A1* mutations among total 33 alleles. This study included the computational analysis that predicts cryptic splicing successfully. This is the first large mutational analysis of patients with Korean DEB. However, one of the limitations of this study is the small number of patients. Since Christiano et al[44]. had demonstrated a missense mutation in *COL7A1* in two affected siblings with RDEB, more than

300 mutations have been reported now. From the identification of an increasing number of mutations, some general genotype-phenotype correlation has been drawn. Moreover, elucidation of *COL7A1* mutations is of immediate clinical benefit in assisting clinical diagnosis and in improving genetic counseling, and also provides new insights into type VII collagen biology and helps determine potential strategies for newer forms of treatment, including gene therapy.

V. CONCLUSION

We performed mutational analysis of 18 distinct Korean DEB families. We sequenced *COL7A1* segments including all 118 exons and all exon-intron borders, and demonstrated 30 pathogenic *COL7A1* mutations among total 33 alleles. We also performed the computational analysis to predict cryptic splicing, and confirmed the result by RT-PCR. The summary of the results are described below.

1. The result demonstrated 30 pathogenic *COL7A1* mutations among total 33 alleles with an overall mutation detection sensitivity of 90.9%. We found out total 24 kinds of COL7A1 mutation including 10 novel mutations: 242delCA, 1094-1G>C, 2063delC, 2392G>A (G798R), 2621insGCTTC, 2645del4, Q1286X (3856C>T), G1694C (5080G>T), 6246del27, 8291delGA.
2. The mutations of DDEB families were 3 GS (all in the collagenous domain, exon 73) and 1 alternative splicing, and those of mild RDEB result from alternative splicing/GS, alternative splicing/PTC. Most of mutations observed in moderate severe RDEB and HS-RDEB are PTC-causing mutations, which did not show any difference between two groups. This study also included 1 patient with rare variant of DEB,

transient bullous dermolysis of newborn, which was compound heterozygote for G798R and 6246del27.

3. In the computational analysis, 1094-G>C, 2392G>A (G798R), 6899A>G (Q2300R), 341G>T (G114V) and 682+1G>A show significant Ri changes, which were confirmed in previous reports or by RT-PCR in this study.
4. We found out 5 different recurrent mutations, R669X, 2392G>A (G798R), G2043R, G2204S and E2857X.
5. Comparing with other ethnic report of DEB, Korean patients with DEB seem to have relatively small number of patients with HS-RDEB and homozygosity.

In conclusion, this is the first large mutational analysis of patients with Korean DEB. Furthermore, we performed the computational analysis to predict cryptic splicing successfully. This study will provide clinical benefit in assisting clinical diagnosis and in improving genetic counseling, as well as provide new insights into the genotype-phenotype correlation in DEB patients.

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Abstract (In Korean)

한국인 이영양형 수포성 표피박리증 환자에서의 제 VII형 콜라겐 유전자 돌연변이 분석

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이영양형 수포성 표피박리증(dystrophic epidermolysis bullosa, 이하 DEB)은 제 VII형 콜라겐의 유전자인 *COL7A1*의 돌연변이에 의해 고정 원섬유(anchoring fibril)에 결함이 생겨 약한 자극에도 쉽게 수포가 형성되는 질환이다. *COL7A1*은 32kb의 크기로 118개의 엑손(exon)으로 구성되어 있으며 인간의 단일 유전자로서는 가장 큰 유전자이다. 제 VII형 콜라겐은 중앙의 collagenous domain과 양쪽 아미노기(amino) 및 카르복시기(carboxy) 말단을 이루는 non-collagenous domain(각각 NC-1, NC-2)으로 이루어져 있다. DEB는 상염색체 우성 또는 열성으로 유전되며, 일반적으로 열성형인 경우 우성형보다 임상적으로 더 심한 임상양상을 나타내지만 같은 유전방식을 갖는 경우에서도 돌연변이의 위치 및 종류에 따라 다양한 임상양상을 나타낼 수 있다. 일례로 열성형 DEB 중 심한 임상양상을 보이는 경우 두 대립유전자 모두에서 조기 중단 코돈(premature termination codon, PTC)이 있는 반면, 우성형에서는 collagenous domain의 glycine 치환 돌연변이(glycine substitution, GS)를 보이는 경우가 많다. 현재까지 300개 이상의 *COL7A1* 돌연변이가 보고되었으나 아직 정확한 유전

형-표현형의 관계는 알려져 있지 않다. 더욱이 국내의 DEB에 대한 연구는 간헐적인 증례보고 외에는 없는 실정이다.

본 연구는 18가계(우성형 4가계, 열성형 14가계)를 대상으로 하였고, 총 33개의 대립유전자(한 가계에서 3개의 돌연변이 발견된 것 포함)에서 30개의 돌연변이를 발견하여 90.9%의 발견율을 보였다. 총 24가지의 돌연변이 중 기존에 보고되지 않은 새로운 돌연변이는 10가지 였으며, PTC 8개, insertion/deletion 6개, GS 5개, alternative splicing 5개였다. 반복적으로 나타나는 돌연변이(recurrent mutation)는 R669X, G798R, G2043R, G2204S, E2857X였으며 전체 돌연변이의 30%를 차지하였다. 발견된 각 돌연변이에 대해서 *COL7A1* mRNA의 splicing에 대한 영향을 알아보기 위해 in silico study를 시행하였으며 donor 혹은 acceptor site의 Ri 값의 변화가 10개에서 관찰되었다. 10개의 돌연변이에서 Ri 값의 유의한 변화는 1094-G>C, 2392G>A (G798R), 6899A>G (Q2300R), 341G>T (G114V), 682+1G>A의 5개에서 나타났다. 임상양상에 따른 돌연변이의 양상은 다음과 같았다. 4명의 우성형에서 3개의 GS와 1개의 alternative splicing을 발견하였다. 열성형의 경우 임상양상을 경증(mitis or mild), 중등증(moderate severe), 중증(Hallopeau-Siemens or severe)으로 나누었을 때 경증에서는 alternative splicing/GS, alternative splicing/PTC의 두 예가 있었고, 중등증 및 중증에서는 PTC가 가장 많이 관찰되었다. 1예에서 Transient bullous dermolysis of newborn의 임상양상을 보였으며 돌연변이는 열성형으로 G798R/6246del27의 조합이었다.

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