Ann Dermatol Vol. 23, No. 2, 2011 DOI: 10.5021/ad.2011.23.2.170

ORIGINAL ARTICLE

Genetic Polymorphism of *FLG* in Korean Ichthyosis Vulgaris Patients

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Background: Filaggrin is a key protein that facilitates the formation of skin barrier by forming a stratum corneum. Mutations in the gene encoding filaggrin (*FLG*) have recently been reported in patients with ichthyosis vulgaris (IV). Interestingly, there are ethnic differences between *FLG* mutations identified in Asians and Europeans, and few *FLG* mutations are overlapping between Chinese and Japanese IV patients. **Objective:** The aim of this study was to investigative the genetic polymorphism of *FLG* in Korean IV patients. **Methods:** Genomic DNA was extracted from whole venous blood specimen of Korean patients with IV and a control group, and the full sequence of *FLG* was determined via overlapping long-range polymerase chain reaction method. **Results:** Analysis of base sequence previously unreported reveal new nonsense mutation p.Y1767X in a Korean IV patient, and additional new single nucleotide polymorphisms. **Conclusion:** On the basis of this study, it is anticipated that analysis of *FLG* gene sequence be extended to other dermatoses associated with *FLG*, such as atopic dermatitis. **(Ann Dermatol 23(2) 170～176, 2011)**

-Keywords-
Filaggrin, Genetics, Ichthyosis vulgaris, Mutation, Polymorphisms

Received February 9, 2010, Revised December 21, 2010, Accepted for publication December 21, 2010
*This study was supported by a grant of the Korean Health Technology R&D Project, Ministry of Health & Welfare, Republic of Korea (A091121).

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INTRODUCTION

Filaggrin is a key protein involved in the terminal differentiation of the epidermis and formation of skin barrier. The filaggrin gene (*FLG*) is located on human chromosome 1q21.3. It encodes the polyprotein profilaggrin, which is consisted of 10～12 tandemly repeated filaggrin subunits[1,2]. The aforementioned repeat number polymorphism is rarely found in other genes - a unique phenomenon in *FLG*. Of the 10 *FLG* repeat units, the eighth and tenth may possess one more repeat unit with similar base sequence, in contrast to the other repeat units, resulting 10, 11, or 12 repeat unit variants in *FLG*[3].

Skin diseases that are attributable to the functional defects of *FLG* include ichthyosis vulgaris (IV; Online Mendelian Inheritance in Man (OMIM) #146700). It has long been known that IV patients have significantly reduced granular layer according to hematoxylin-eosin (H&E) staining of the patient tissue specimens. Such decrease in granular layer is due to decrease in keratohyaline granule, a key component of the granular layer. At the same time, immunohistological stain revealed reduced expression of filaggrin[4].

Recently, efficient method to determine the base sequence of *FLG* has been developed, and some patients with IV were found to have loss-of-function mutation in *FLG*, indicating that *FLG* abnormality is involved in the pathogenesis of IV[5]. Based on the fact that *FLG* plays an important role as a skin-barrier, studies have been extended to patients with atopic dermatitis (AD), and *FLG* mutation was found in some cases of AD[6]. In addition, several studies based on European population suggested that *FLG* null mutations (R501X and 2282del4) predispose to other atopic disorders (asthma and allergic rhinitis). These mutations also predispose to atopic march[7], early-

onset AD that persists into adulthood as well as more severe asthma[2,8,9].

Interestingly, distinctive differences have been found in *FLG* mutation between ethnic groups. Studies on *FLG* mutation showed that of the 27 *FLG* mutations reported, only two are common to Europeans and Asians[10]. Genetic polymorphisms (including *FLG* mutation) in the Koreans could be different from that in the other nations; Japanese and Chinese have more differences than similarities between them, even though they are both Asians. A recent study by Akiyama[10] which examined the relationship between *FLG* mutation and population genetics, confirmed certain genetic variations to be unique to Europeans and Asians. *FLG* variations differed even between Asians. Specifically, *FLG* varied among Chinese[11], Japanese[12-14], and Taiwanese[15]. Only 3321delA was found to be common among Asians[10].

To date, a genetic study on Korean patient was conducted that included only one subject, and the patient was demonstrated to have 3321delA, a mutation that commonly occurs in Asians[16]. The aim of this study was to investigate genetic polymorphisms, including *FLG* mutation, in Korean IV patients.

MATERIALS AND METHODS

Study population

Blood samples were obtained from 7 patients with IV (<60 years of age) from independent Korean families. The diagnosis of IV was performed clinically by experienced dermatologists on the basis of clinical manifestation. Autosomal dominant pattern of inheritance was confirmed by careful history taking. The normal control group was consisted of 13 subjects who neither experienced IV nor AD. This study was approved by the Institutional Review Board of the Chung-Ang university hospital, and written informed consent was obtained from each participant prior to study entry.

Filaggrin genotyping

Polymerase chain reaction (PCR) and sequencing analysis was performed using a protocol described in a previous study[17] with some modifications. Briefly, genomic DNA from IV patients was extracted from peripheral blood samples using the G-DEX™ Genomic DNA Extraction Kit (iNtRON Biotechnology, Sungnam, South Korea). The purity of their DNA was determined using a Nanodrop ND-1000R spectrophotometer (Thermo Fisher Scientific, Wilminton, DE, USA). DNA with an optical-density ratio

Table 1. PCR primers used for *FLG* mutation analysis

FLG exon	Primer pairs	Annealing temperature	Size*
Exon 1	F:5′-CGTGAGGAAGCTGGGAAGTA-3′ R:5′-TTATGCCCTCATTTTCCTTCT-3′	60°C	381 bp
Exon 2	F:5′-CTACTAAGTCCAGCTGTAAGTG-3′ R:5′-GCTCTATCTTTGGTCTTGTCAG-3′	60°C	431 bp
Exon 3 repeats	Primer pairs	Annealing temperature	Size
1～3 (1)	F:5′-GCTGATAATGTGATTCTGTCTG-3′ R:5′-GACCCCGATGATTGTTCCTGT-3′	60.1°C	1,710 bp
1～3 (2)	F:5′-CACGGAAAGGCTGGGCTGA-3′ R:5′-GACCCCGATGATTGTTCCTGT-3′	67.2°C	2,642 bp
3～5	F:5′-GCAAGCAGACAAACTCGTAAG-3′ R:5′-ACATCAGACCTTTCCTGGGAC-3′	65°C 66°C	1,916 bp
4～7	F:5′-GACAAGATTCATCTGTAGTCG-3′ R:5′-CTGGCTAAAACTGGATCCCCA-3′	59.9°C 60.°C	2,609 bp
7～8	F:5′-CCACACGTGGCCGGTCAGCA-3′ R:5′-CTACCGAATGCTCGTGGTGGT-3′	65°C 66°C	1,224/2,196 bp
7～10	F:5′-CCCAGGACAAGCAGGAACT-3′ R:5′-GCTTCATGGTGATGCGACCA-3′	61.8°C	3,331/4,303 bp
9～10	F:5′-CCCAGGACAAGCAGGAACT-3′ R:5′-GCTTCATGGTGATGCGACCA-3′	65°C 66°C	1,367/2,342 bp
10	F:5′-GCCCATGGGCGGACCAGGA-3′ R:5′-CTGCACTACCATAGCTGCC-3′	65°C 66°C	1,753/2,728 bp
End	F:5′-CTAGTACCGCTAAGGAACATGG-3′ R:5′-TGGCTCCTTCGATATTTCTGA-3′	60°C	781 bp

*Additional repeat(s) after repeat 8 and 10, respectively repeat 8.2 and repeat 10.2, make two PCR products in exon 3 (7～8), (7～10), (9～10), (10).

260/280 of 1.8 or more was used.

PCR amplifications were performed in 40 μl reaction volumes containing 200 ng genomic DNA, 2.5 mmol/L $MgCl_2$, 200 μmol/L for each deoxynucleotide triphosphate, 10 pmol for each primer, 5xBD (Solgent Co. Ltd., Daejeon, Korea), and 2 U Taq DNA polymerase (TaKaRa Bio Inc, Otsu, Japan) using a GeneAmp PCR system 2700 (Applied Biosystems, Princeton, NJ, USA). Each genomic DNA was amplified using the primers listed in Table 1. The amplified PCR products were separated by electrophoresis in 0.8% agarose gel.

Sequencing

DNA sequencing was performed using Applied Biosystems 3100/3700 DNA sequencers Applied Biosystems, Princeton, NJ, USA) after conducting purification with QIAquick PCR purification kits (Qiagen, Valencia, CA, USA). Sequencing conditions were as follows: 96°C for 1 min followed by 25 cycles of 96°C for 10s, 50°C for 5 sec and 60°C for 4 min and a final extension at 10°C for 10 min. Several sets of forward and reverse sequencing primers were used depending on the size of the PCR product fragment, as follows (Table 2).

Table 2. Primers for sequencing reaction

Exon region	Primer pairs
Exon 1	1F:5′-CGTGAGGAAGCTGGGAAGTA-3′
	1R:5′-TTATGCCCTCATTTTCCTTCT-3′
Exon 2	1F:5′-CTACTAAGTCCAGCTGTAAGTG-3′
	1R:5′-GCTCTATCTTTGGTCTTGTCAG-3′
Exon 3 (1-3)-(1)	1F:5′-GCTGATAATGTGATTCTGTCTG-3′
	1R:5′-TCAGCCCAGCCTTTCCGTG-3′
	2F:5′-CACGGAAAGGCTGGGCTGA-3′
	2R:5′ACCTGAGTGTCCAGACCTATT-3′
Exon 3 (1-3)-(2)	1F:5′-CACGGAAAGGCTGGGCTGA-3′
	1R:5′-GCAAGCAGACAAACTCGTAAG-3′
	2F:5′-CCAGACAATCAGGAACTCC-3′
	2R:5′-GGAGTTCCTGATTGTCTGG-3′
Exon 3 (3-5)	1F:5′-GCA AGCAGACAAACTCGTAAG-3′
	1R:5′-ACATCAGACCTTTCCTGGGAC-3′
	2F:5′-GGATCCTACCACGAGCAATCA-3′
	2R:5′-GGACCTTGACCTTGCCTGTTC-3′
	3F:5′-CAG AAGTGCAAGCAGGCAAACAA-3′
	3R:5′-ACTGTGTGTCTGACTCTTCTGAG-3′
Exon 3 (4-7)	1F:5′-GACAAGATTCATCTGTAGTGC-3′
	1R:5′-CTGGCTAAAACTGGATCCCCA-3′
	2F:5′-AATGAGAAACAATCAGGAGACG-3′
	2R:5′-GCTGTCTTGTGCCTGATCATAA-3′
	3F:5′-CACAGTCAGTGTCAGCACAG-3′
	4F:5′-CAGAAGTGCAAGCAGAAAAACATA-3′
	5F:5′-AGAGGCGGTCTGGGTCTGCG-3′
Exon 3 (7-8)	1F:5′-CCACACGTGGCCGGTCAGCA-3′
	1R:5′-CTAGGCAATGCTCGTGGTGGT-3′
Exon 3 (7-10)	1F:5′-CCCAGGACAAGCAGGAACT-3′
	1R:5′-GCTTCATGGTGATGCGACCA-3′
Exon 3 (9-10)	1F:5′-GAA ACGTCTGGACATTCAGGA-3′
	1R:5′-GCT TCATGGTGATGCGACCA-3′
	2F:5′-AATGAGGAACAATCAGGAGACA-3′
	2R:5′-GAAGCTTCATGGTGACGTGACA-3′
	3F:5′-AGA AACCATCGTGGATCTGT-3′
	3R:5′-GTGCCTTGACTGCTCCTGAA-3′
	4F:5′-ACGGGCACTCTGCAGACC-3′
Exon 3 (10)	1F:5′-GCCCATGGGCGGACCAGGA-3′
	1R:5′-CTGCACTACCATAGCTGCC-3′
	2F:5′-ACCATGAAGCTTCCACTCAGGCG-3′
FLG end	1F:5′-CTAGTACCGCTAAGGAACATGG-3′
	1R:5′-TGGCTCCTTCGATATTTCTGA-3′

A statistical-analysis

χ^2 test was performed to investigate if there was any difference in the frequency of the genotype and allele between the IV patient group and the normal control group. A *p*-value of less than 0.05 ($p<0.05$) was considered statistically significant.

RESULTS

This study was performed to determine genetic diversity (including mutation) of *FLG* in Korean IV patients. For the exon 1 and 2 regions, the obtained sequences were compared to the reference sequence, but no meaningful result was found. The extremely large size of exon 3, in which most of the reported mutations were found, was challenging for PCR-based sequencing method. Thus the exon 3 region was divided into 9 overlapping fragments, and PCR and sequencing analysis were carried out. Of the IV patients participated in this study, one patients had a personal and family history of AD and allergic asthma. After the full sequencing, nonsense mutation p.Y1767X (=c.5301C>G), which had never been reported in literature for IV or AD patients, was found. Later, it was revealed that his father, who had concomitant AD and asthma, carried the same mutation, in contrast to his mother who did not experience IV or AD (Fig. 1).

In patients from the other families, additional mutations were not found. However, 81 single nucleotide polymorphisms (SNPs) were found, more than that in the reference sequence of *FLG* (NG_016190.RefSeqGene). Out of 81 SNPs, 30 SNPs were new and had never been reported (Table 3). The pattern of genetic polymorphism (including repeat number polymorphism) shown by the 7 patients with IV, and by the 13 subjects in the normal control group, were compared. The chi-square test of the alleles showed no significant difference (data not shown).

DISCUSSION

After the full sequencing of *FLGs* in Korean IV patients and normal control, a unique diversity of the *FLG* gene is

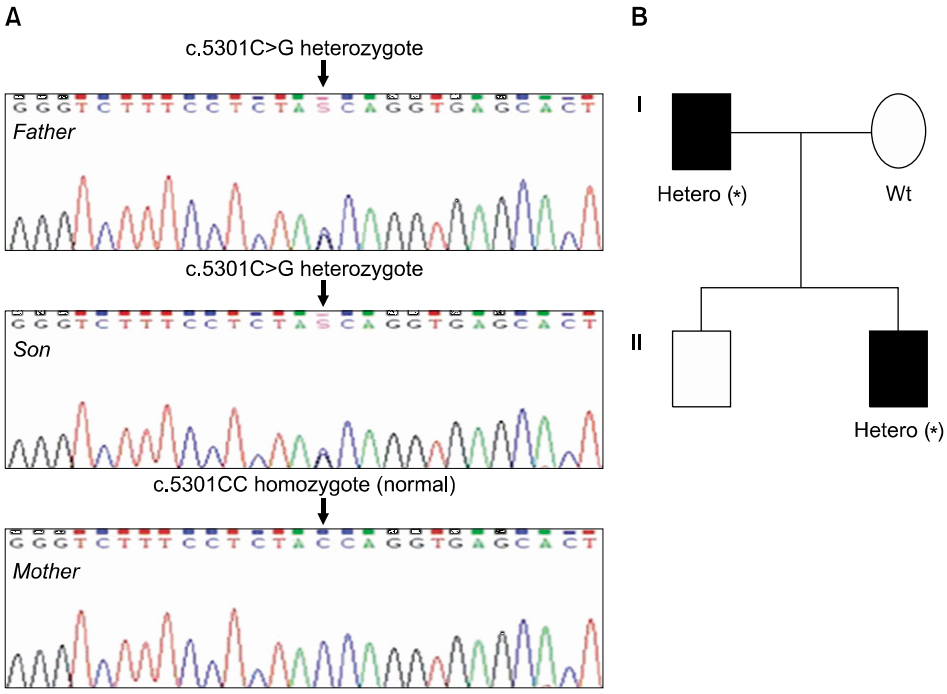

Fig. 1. Detection of filaggrin (*FLG*) mutation, Y1767X. (A) Direct DNA sequencing of specific *FLG* polymerase chain reaction products. Normal control sequence from filaggrin repeat 5 in exon3, corresponding to codons 5299-5301. Upper panels show identification of the novel Y1767X (=c.5301C>G) heterozygous mutation of the family. (B) A pedigree of IV family, showing semidominant inheritance pattern. Filled symbols refer to the IV presentation. Hetero (*): heterozygous for Y1767X, Wt: wild type for Y1767X.

Table 3. Detection of SNP in Korean ichthyosis vulgaris patients

Exon 3	Change_HUGO	Change	Amino acid_HUGO	SNP (NCBI)	Genotype frequency* (AA/Aa/aa)
1-3	995	GGC>GTC	p.G332V	rs41267154	2/7/11
1-3	1236	CGT>CGC	p.=(412R)	rs11582620	16/3/1
1-3	1330	GGG>AGG	p.G444R	rs11588170	2/7/11
1-3	1360	ACA>GCA	p.T454A	rs2011331	2/11/7
1-3	1432	CCT>TCT	p.P478S	rs11584340	2/7/11
1-3	1555	CAC>AAC	p.H519N	rs12036682	18/2/0
1-3	2181	CAC>CAA	p.H727Q	rs35904544	2/11/7
1-3	2263	GAA>AAA	p.E755K	rs74129461	2/7/11
1-3	2508	GAT>GAC	p.=(836D)	rs3120653	2/11/7
1-3	2938	CAT>GAT	p.H980D	rs12756586	2/7/11
1-3	3387	TCT>TCC	p.=(1129S)	rs9436067	2/7/11
1-3	3397	CGG>TGG	p.R1133W	New	19/1/0
1-3	3500	GCA>GGA	p.A1167G	rs7530018	2/7/11
3-5	4079	CGC>CAC	p.R1360H	rs11586631	16/4/0
3-5	4126	AGA>GGA	p.R1376G	rs11581433	2/7/11
3-5	4410	CAT>CAC	p.=(1470H)	rs12732920	16/4/0
3-5	4445	TCC>TAC	p.S1482Y	rs11204978	16/4/0
3-5	4452	GAC>GAG	p.D1484E	rs71626706	2/7/11
3-5	4568	ACA>ATA	p.T1523I	rs12750081	16/4/0
3-5	5051	CGC>CAC	p.R1684H	rs12407807	2/7/11
3-5	5095	CGC>TGC	p.R1699C	rs12405278	2/7/11
3-5	5301	TAC>TAG	p.Y1767X	Nonsense mutation	19/1/0
4-7	5414	GCG>GTG	p.A1805V	rs12405241	2/7/11
4-7	5672	GGG>TGG	p.R1891Q	rs12407748	2/11/7
4-7	5883	CAC>CAA	p.H1961Q	rs3126079	2/7/11
4-7	6045	GAC>GAA	p.D2015E	rs71626704	2/7/11
4-7	6498	TCT>TCC	p.=(2166S)	rs2184954	18/2/0
4-7	6574	AAA>CAA	p.K2192Q	New	19/1/0
4-7	6580	TAT>CAT	p.Y2194H	rs2184953	2/7/11
4-7	6990	CAC>CAT	p.=(2330H)	rs71626703	2/7/11
4-7	7015	GAC>AAC	p.D2339N	New	2/7/11
4-7	7097	AGT>ACT	p.S2366T	rs71625202	2/7/11
4-7	7192	GAG>CAG	p.E2398Q	rs71625201	2/7/11

Table 3. Continued

Exon 3	Change_HUGO	Change	Amino acid_HUGO	SNP (NCBI)	Genotype frequency* (AA/Aa/aa)
7-8	7330	AAG>GAG	p.K2444E	rs71625200	2/7/11
7-8	7398	CCG>CCA	p.=(2466P)	rs71625199	16/4/0
7-8	7442	TTG>TCG	p.L2481S	rs55650366	2/7/11
7-8	7521	CAC>CAG)	p.H2507Q	rs3126074	2/7/11
7-8	7633	GGA>AGA	p.G2545R	rs3126072	2/7/11
7-8	8360	CGT>CAT	p.R2787H	New	2/7/11
7-10	8570	CGT>CAT	p.R2857H	New	2/7/11
7-10	8673	GTG>GTT	p.=(2891V)	New	2/7/11
7-10	8807	GAC>GGC	p.D2936G	New	2/7/11
7-10	9313	TAC>GAC	p.Y3105D	rs2065958	2/7/11
7-10	9536	GTG>GGG	p.V3179G	Rs2065957	2/7/11
7-10	9540	TCA>TCG	p.=(3180S)	rs3126069	2/7/11
7-10	9645	GTG>GTT	p.=(3215V)	rs9436066	2/11/7
9-10	9966	CAA>CAG	p.=(3322Q)	rs6681433	2/7/11
9-10	10017	CAG>CAA	p.=(3339Q)	rs2065956	2/7/11
9-10	10194	TCT>TCC	p.=(3732S)	rs3091276	16/4/0
9-10	10307	GGA>GCA	p.G3436A	rs2065955	2/11/7
9-10	10473	AAT>AAC	p.=(3491N)	rs3126067	2/11/7
10	10491	GAT>GAC	p.=(3497D)	rs3126066	2/11/7
10	10559	CAG>CCG	p.Q3520P	New	19/1/0
10	10561	TCC>GCC	p.S3521A	New	18/2/0
10	10563	TCC>TCG	p.(3521S)	New	19/1/0
10	10590	AGG>AGT	p.R3530S	rs72697000	18/2/0
10	10691	CGT>CAT	p.R3564H	rs7518080	19/1/0
10	10699	GCT>TCT	p.A3567S	New	18/2/0
10	10702	CAG>GAG	p.Q3568E	rs7540123	2/11/7
10	10703	CAG>CGG	p.Q3568R	rs7532285	2/11/7
10	10734	CCC>CCT	p.=(3578P)	New	2/11/7
10	10735	ACG>GCG	p.T3579A	New	2/11/7
10	10736	ACG>AGG	p.T3579R	rs3126075	2/11/7
10	10746	CAC>CAA	p.H3582Q	New	2/11/7
10	10779	GAG>GAC	p.E3593D	rs12083389	19/1/0
10	10802	CAT>CGT	p.H3601R	New	2/11/7
10	10806	CAT>CAC	p.=(3602H)	New	2/11/7
10	10807	GCA>ACA	p.A3604T	New	2/11/7
10	10813	AAT>TAT	p.N3605Y	New	2/11/7
10	10814	AAT>ACT	p.N3605T	New	2/11/7
10	10822	GGT>CGT	p.G3608R	New	2/11/7
10	10836	GCA>GCG	p.=(3612A)	New	2/11/7
10	11116	AGA>GGA	p.R3706G	New	19/1/0
10	11271	GAC>GAT	p.=(3757D)	New	19/1/0
10	11419	CCC>TCC	p.P3807S	New	18/2/0
10	11806	CCT>GCT	p.P3936A	New	2/11/7
End	11902	GGT>CGT	3968	New	19/1/0
End	11909	TCA>TTA	3970	rs3814299	18/2/0
End	12018	GTT>GTC	4006	New	18/2/0
End	12090	ACG>ACA	4030	New	18/2/0
End	12884	TCA>TTA	p.S4295L	New	19/1/0

*Difference of genotype (or allele) frequency between IV and normal control group was not observed. Thus, combined genotype frequency of 20 subjects (7 IV and 13 normal control) was presented. SNP: single nucleotide polymorphism, AA: wild, Aa: hetero, aa: homo.

confirmed to exist in the Koreans. Nonsense mutation, p.Y1767X, which had never been reported, was found, and numerous SNPs that are not in the registry were observed. In some SNPs (e.g., 7th base (A>G), 310th base (C>T) of repeat 10.2, i.e. repeat after repeat 10), a homozygote of a minor allele was found in all of the subjects. This is considered to be attributable to the fact that the reference base sequence (NG_016190.1) of *FLG* was obtained from European populations. This justifies the necessity for the reference sequence for the Korean population.

FLG is a histidine-rich cation protein that is produced in

the proteolytic degradation of profilaggrin, a large protein mass with a significant molecular weight comprised of keratohyaline granule, which exists in keratinocyte of granular layer[15]. In IV patients, keratohyaline granule considerably decreases or disappears, and filaggrin also decreases[5,10]. Filaggrin, via degradation, changes into molecules that contain urocanic acid (UCA) and pyrrolidone carboxylic acid, which block ultraviolet rays and moisturize the keratin layer[15,17].

R501X and 2282del4 - mutations in the *FLG* genes - were first found in pediatric patients with IV who skin barrier showed abnormal functioning[18]. Among patients harbored either one of the two mutations, 44% of their family members have had AD, whereas among patients harbored both mutations, 76% of their family members have had AD. Among patients without any of the two mutations, no family member had AD[6]. A previous study showed that mutation in the *FLG* gene was correlated with AD of onset before the age of 2. Other studies indicate that mutations in the *FLG* gene that cause functional abnormality are markers of poor prognosis, and predictive that AD in an infant may manifest into adulthood[19]. In addition, it was also shown that the two mutations (R501X and 2282del4) that cause functional abnormalities are significantly correlated to AD ($p=0.0001$), asthma ($p=0.006$), and atopic allergy ($p=0.002$)[12,20]. Consistent with the above literature review, we could find nonsense mutation only in IV patients with concomitant personal history of AD and family history of atopic disease. This result is consistent with the previous finding for a Korean IV patient, who also experienced concomitant AD[11]. We found relatively lower frequency of *FLG* nonsense mutation in this study. One out of 7 IV patients, compared to European (ca. 50%) and other Asian populations (ca. 20%)[10]. We think that IV patients with personal or family history of AD should undergo investigations for *FLG* nonsense mutation.

The focus of previous studies was on the detection of *FLG* mutation in patients with IV or AD. Interestingly, however, in most of the AD patients and in a considerably large number of IV patients, loss-of-function *FLG* mutation did not exist. Meanwhile, reduced expression of *FLG* was observed in AD patients in whom such mutation was not observed[21]. Previous studies reported that mediators, such as sphigosylphosphorulcholine, which is involved in the inflammatory reaction of the skin, or Th2 cytokine, which is associated with pathophysiology of AD, reduced the expression of *FLG*[22]. This means that another mechanism other than *FLG* nonsense mutation is involved in the pathogenesis of IV or AD. Given the study finding that repeat number polymorphism can be associated with xeroderma[23], we conjectured *FLG* length polymorphism could be an alternative explanation for most cases of IV that cannot be explalind by mutation. However, after we compared the frequency of repeat number variants between IV patients and the control group, no significant difference can not be found in the repeat number polymorphism. As for other SNPs, we could not find significant difference between IV patients and the control group. We think the so called "filaggrin-processing" (proteolytic degradation of progilaggrin to filaggrin finally leading to NMF or UCA) is another candidate to be investigated for the large portion of IV or AD patients not attributable to *FLG* mutations.

In summary, we established *FLG* full sequencing protocols applicable to the Korean population and established the Korean reference sequence for *FLG*. Applying these methods, we revealed previously unknown mutation in Korean IV patients and their family members. Although we could not find additional mutation or SNPs specific for Korean IV patients, we confirmed the fact that *FLG* nonsense mutation is likely to be found in patients with personal or family history of IV, AD, or other respiratory atopy. In the future, we hope to extend this study to Korean patients with AD, and compare the genetic diversity of *FLG* with other ethnic groups.

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