Acta Med. Okayama, 2018
Vol. 72, No. 3, pp. 275–282

Acta Medica Okayama
http://escholarship.lib.okayama-u.ac.jp/amo/

Original Article

Analysis of All 34 Exons of the *SPINK5* Gene in Japanese Atopic Dermatitis Patients

Shin Morizane[a]*, Mamoru Ouchida[b], Ko Sunagawa[a], Saeko Sugimoto[a], Mina Kobashi[a], Satoru Sugihara[a], Hayato Nomura[a], Kazuhide Tsuji[c], Atsushi Sato[d], Yoshihiro Miura[e], Hiroaki Hattori[f], Kotaro Tada[g], Wook-Kang Huh[h], Akemi Seno[i], and Keiji Iwatsuki[a]

Departments of [a]Dermatology and [b]Molecular Oncology, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama 700-8558, Japan, [c]Nishigawara Dermatology Clinic, Okayama 703-8258, Japan, [d]Sato Dermatology Clinic, Kurashiki, Okayama 710-0023, Japan, [e]Miura Dermatology Clinic, Kurashiki, Okayama 710-0055, Japan, [f]Hattori Dermatology and Allergy Clinic, Okayama 700-0027, Japan, [g]Tada Dermatology Clinic, Kurashiki, Okayama 710-0038, Japan, [h]Dr. Huh's Dermatology Clinic, Kurashiki, Okayama 710-0061, Japan, [i]Department of Dermatology, Mitoyo General Hospital, Kanonji, Kagawa 769-1695, Japan

Lympho-epithelial Kazal-type-related inhibitor (LEKTI) is a large multidomain serine protease inhibitor that is expressed in epidermal keratinocytes. Nonsense mutations of the *SPINK5* gene, which codes for LEKTI, cause Netherton syndrome, which is characterized by hair abnormality, ichthyosis, and atopy. A single nucleotide polymorphism (SNP) of *SPINK5*, p.K420E, is reported to be associated with the pathogenesis of atopic dermatitis (AD). We studied all 34 exons of the *SPINK5* gene in Japanese 57 AD patients and 50 normal healthy controls. We detected nine nonsynonymous variants, including p.K420E; these variants had already been registered in the SNP database. Among them, p.R654H (n = 1) was found as a heterozygous mutation in the AD patients, but not in the control. No new mutation was detected. We next compared the data of the AD patients with data from the Human Genetic Variation Database provided by Kyoto University; a significant difference was found in the frequency of the p.S368N genotype distribution. PolyPhen-2 and SIFT, two algorithms for predicting the functional effects of amino acid substitutions, showed significant scores for p.R654H. Therefore, R654H might be a risk factor for epidermal barrier dysfunction in some Japanese AD patients.

Key words: atopic dermatitis, SPINK5, LEKTI, serine protease inhibitor, epidermal barrier dysfunction

Atopic dermatitis (AD) is a chronic pruritic inflammatory skin disorder that is thought to be a multifactorial disease caused by both genetic and environmental factors [1]. AD is characterized by allergic inflammation and epidermal barrier dysfunction [1]. Since a loss-of-function mutation of the filaggrin (*FLG*) gene has been reported to be a major predisposing factor for AD [2], primary epidermal barrier dysfunction is thought to be an initial event of the pathogenesis of AD. However, there are quite a few AD patients who do not carry any *FLG* mutations, and some individuals with a *FLG* loss-of-function mutation do not develop AD [3]. Therefore, other genetic factors are also likely to be associated with the pathogenesis of AD.

Excessive protease activity is a characteristic abnor-

Received October 3, 2017; accepted December 13, 2017.
*Corresponding author. Phone : +81-86-235-7282; Fax : +81-86-235-7283
E-mail : zanemori@cc.okayama-u.ac.jp (S. Morizane)

Conflict of Interest Disclosures: No potential conflict of interest relevant to this article was reported.

mality that affects the epidermal barrier in AD patients [1, 4]. In humans, the kallikrein-related peptidases (KLKs) are 15 trypsin-like or chymotrypsin-like secreted serine proteases, and in the skin, mainly KLK5 and KLK7 are involved in the degradation of corneodesmosome proteins, such as desmoglein 1, desmocollin 1, and corneodesmosin, leading to the desquamation of the corneal layers [5, 6]. The expression and activity of several KLKs are increased in AD lesions, and they trigger or enhance epidermal barrier dysfunction [4, 7].

In particular, KLK7 has been shown to be induced by Th2 cytokines, interleukin (IL)-4, and IL-13 in epidermal keratinocytes, which might explain how the Th2 environment exacerbates epidermal barrier dysfunction [8]. In addition, KLK5 activates protease-activated receptor-2, and it induces inflammatory cytokines and chemokines related to the Th2 environment in cells [9].

The KLK inhibitor lympho-epithelial Kazal-type-related inhibitor (LEKTI) is a large multidomain serine protease inhibitor that is expressed in epidermal keratinocytes, and it tightly regulates KLK activities [6]. The *SPINK5* gene encodes LEKTI. Notably, nonsense mutations of the *SPINK5* gene cause Netherton syndrome, which is characterized by hair abnormalities, ichthyosis, and atopy [10, 11]. A single nucleotide polymorphism (SNP) in *SPINK5*, p.K420E (rs2303067), has been reported to be associated with AD [12-18]. Fortugno *et al.* showed that this variant has altered serine protease inhibitor function that results in protease deregulation, which might be involved in the pathogenesis of AD [19]. However, several other groups have reported that there is no association between this variant and AD [20-24].

The effects of nonsynonymous SNPs or mutations in *SPINK5* on the epidermal barrier seem to be very serious, which suggests that other variants of the gene might also be involved in the pathogenesis of AD. Walley *et al.* analyzed 33 exons of the *SPINK5* gene in AD patients [16]. Many other groups have also focused on the analysis of exons 13, 14, and 26 [12-15, 17, 18, 20-24]. However, it has been reported that there are 34 exons in the *SPINK5* gene and three splicing isoforms in humans [25]. Fig. 1 shows the genomic structure of the human *SPINK5* gene and the splicing pattern of the isoforms (Fig. 1). As far as we know, no comprehensive analysis of all 34 exons of the *SPINK5* gene has been reported in AD patients. Here we studied all of the exons of the *SPINK5* gene in Japanese AD patients.

Materials and Methods

Patient and control groups. This study was approved by the Ethics Committee of Okayama University (No.1605-027), and was performed in accordance with the Helsinki Declaration. The diagnosis of AD was based on the diagnostic criteria of Hanifin and Rajka, and was made by dermatologists. Blood samples for genetic analyses were collected with written informed consent from AD patients (n = 57; ages 37.1 ± 10.6 years) and normal healthy controls (n = 50; ages 35.5 ± 11.1 years) at Okayama University Hospital and its affiliated hospitals or clinics. We also compared the data of our AD patients with data from the Human Genetic Variation Database provided by Kyoto University (n = 1,029-1,205) < http://www.hgvd.genome.med.kyoto-u.ac.jp/ > (accessed: August 2017).

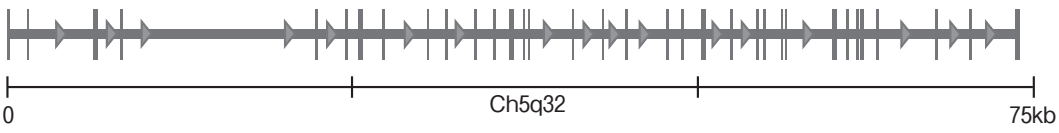
Analysis of the SPINK5 gene. Genomic DNA was extracted from whole blood using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA). All 34 exons were amplified with specific primers (Table 1), and the polymerase change reaction (PCR) products were purified with exonuclease I and shrimp alkaline phosphatase (USB Corporation, Cleveland, OH, USA), reacted with Big Dye Terminator (Applied Biosystems, Foster City, CA, USA), and analyzed with an ABI3100 DNA sequencer (Applied Biosystems) at the Central Research Laboratory, Okayama University Medical School.

Polyphen-2. An algorithm for predicting the functional effects of amino acid substitutions, PolyPhen-2 < http://genetics.bwh.harvard.edu/pph2/ >, accessed August, 2017 was used. Variants were predicted as 'benign (score 0.0-0.15)', 'possibly damaging (0.15-1.0)', or 'probably damaging (0.85-1.0)'.

SIFT. We used the algorithm SIFT < http://sift.jcvi.org/ > to predict whether an amino acid substitution affects protein function. SIFT scores range from 0 to 1. The amino acid substitution is predicted to be damaging if the score is ≤ 0.05, and tolerated if the score is > 0.05.

Statistical analyses. Staistical analyses were performed using IBM SPSS Statistics 20 (IBM, Armonk, NY, USA). Odds ratios (ORs) were calculated with 95% confidence intervals (CIs). The correlation between genotypes and AD was examined by the *Chi-squared* test. Values of $p < 0.05$ were considered to be significant.

a



b

```
Isoform A    1 MKIATVSVLLPLALCLIQDAASKNEDQEMCHEFQAFMKNGKLFCPQDKKFFQSLDGIMFI
Isoform B    1 MKIATVSVLLPLALCLIQDAASKNEDQEMCHEFQAFMKNGKLFCPQDKKFFQSLDGIMFI
Isoform C    1 MKIATVSVLLPLALCLIQDAASKNEDQEMCHEFQAFMKNGKLFCPQDKKFFQSLDGIMFI

Isoform A  121 TDGKTYDNRCALCAENAKTGSQIGVKSEGECKSSNPEQDVCSAFRPFVRDGRLGCTREND
Isoform B  121 TDGKTYDNRCALCAENAKTGSQIGVKSEGECKSSNPEQDVCSAFRPFVRDGRLGCTREND
Isoform C  121 TDGKTYDNRCALCAENAKTGSQIGVKSEGECKSSNPEQDVCSAFRPFVRDGRLGCTREND

Isoform A  241 RESDPVRGPDGRMHGNKCALCAEIFKQRFSEENSKTDQNLGKAEEKTKVKREIVKLCSQY
Isoform B  241 RESDPVRGPDGRMHGNKCALCAEIFKQRFSEENSKTDQNLGKAEEKTKVKREIVKLCSQY
Isoform C  241 RESDPVRGPDGRMHGNKCALCAEIFKQRFSEENSKTDQNLGKAEEKTKVKREIVKLCSQY

Isoform A  361 TSYAELCSEYRKLVRNGKLACTRENDPIQGPDGKVHGNTCSMCEVFFQAEEEEKKKKEGK
Isoform B  361 TSYAELCSEYRKLVRNGKLACTRENDPIQGPDGKVHGNTCSMCEVFFQAEEEEKKKKEGK
Isoform C  361 TSYAELCSEYRKLVRNGKLACTRENDPIQGPDGKVHGNTCSMCEVFFQAEEEEKKKKEGK

Isoform A  481 ERARAKAKREAAKEICSEFRDQVRNGTLICTREHNPVRGPDGKMHGNKCAMCASVFKLEE
Isoform B  481 ERARAKAKREAAKEICSEFRDQVRNGTLICTREHNPVRGPDGKMHGNKCAMCASVFKLEE
Isoform C  481 ERARAKAKREAAKEICSEFRDQVRNGTLICTREHNPVRGPDGKMHGNKCAMCASVFKLEE

Isoform A  601 SMCEAFFQQEAKEKERAEPRAKVKREAEKETCDEFRRLLQNGKLFCTRENDPVRGPDGKT
Isoform B  601 SMCEAFFQQEAKEKERAEPRAKVKREAEKETCDEFRRLLQNGKLFCTRENDPVRGPDGKT
Isoform C  601 SMCEAFFQQEAKEKERAEPRAKVKREAEKETCDEFRRLLQNGKLFCTRENDPVRGPDGKT

Isoform A  721 CTRESDPVRDADGKSYNNQCTMCKAKLEREAERKNEYSRSRSNGTGSESGKDTCDEFRSQ
Isoform B  721 CTRESDPVRDADGKSYNNQCTMCKAKLEREAERKNEYSRSRSNGTGSESGKDTCDEFRSQ
Isoform C  721 CTRESDPVRDADGKSYNNQCTMCKAKLEREAERKNEYSRSRSNGTGSESGKDTCDEFRSQ

Isoform A  841 RSNDKEDLCREFRSMQRNGKLICTRENNPVRGPYGKMHINKCAMCQSIFDREANERKKKD
Isoform B  841 RSNDKEDLCREFRSMQRNGKLICTRENNPVRGPYGKMHINKCAMCQSIFDREANERKKKD
Isoform C  841 RSNDKEDLCREFRSMQRNGKLICTRENNPVRGPYGKMHINKCAMCQSIFDREANERKKKD

Isoform A  961 PRENDPVHGADGKFYTNKCYMCRAVFLTEALERAKLQEKPSHVRASQEEDSPDSFSSLDS
Isoform B  931 PRENDPVHGADGKFYTNKCYMCRAVFLTEALERAKLQEKPSHVRASQEEDSPDSFSSLDS
Isoform C  917 ----------------------------------------------------------

Isoform A 1081 TPGTTAASMPPSDE
Isoform B 1051 TPGTTAASMPPSDE
Isoform C  917 --------------

  61 NKCATCKMILEKEAKSQKRARHLARAPKATAPTELNCDDFKKGERDGDFICPDYYEAVCG
  61 NKCATCKMILEKEAKSQKRARHLARAPKATAPTELNCDDFKKGERDGDFICPDYYEAVCG
  61 NKCATCKMILEKEAKSQKRARHLARAPKATAPTELNCDDFKKGERDGDFICPDYYEAVCG

 181 PVLGPDGKTHGNKCAMCAELFLKEAENAKREGETRIRRNAEKDFCKEYEKQVRNGRLFCT
 181 PVLGPDGKTHGNKCAMCAELFLKEAENAKREGETRIRRNAEKDFCKEYEKQVRNGRLFCT
 181 PVLGPDGKTHGNKCAMCAELFLKEAENAKREGETRIRRNAEKDFCKEYEKQVRNGRLFCT

 301 QNQAKNGILFCTRENDPIRGPDGKMHGNLCSMCQAYFQAENEEKKKAEARARNKRESGKA
 301 QNQAKNGILFCTRENDPIRGPDGKMHGNLCSMCQAYFQAENEEKKKAEARARNKRESGKA
 301 QNQAKNGILFCTRENDPIRGPDGKMHGNLCSMCQAYFQAENEEKKKAEARARNKRESGKA

 421 SRNKRQSKSTASFEELCSEYRKSRKNGRLFCTRENDPIQGPDGKMHGNTCSMCEAFFQQE
 421 SRNKRQSKSTASFEELCSEYRKSRKNGRLFCTRENDPIQGPDGKMHGNTCSMCEAFFQQE
 421 SRNKRQSKSTASFEELCSEYRKSRKNGRLFCTRENDPIQGPDGKMHGNTCSMCEAFFQQE

 541 EEKKNDKEEKGKVEAEKVKREAVQELCSEYRHYVRNGRLPCTRENDPIEGLDGKIHGNTC
 541 EEKKNDKEEKGKVEAEKVKREAVQELCSEYRHYVRNGRLPCTRENDPIEGLDGKIHGNTC
 541 EEKKNDKEEKGKVEAEKVKREAVQELCSEYRHYVRNGRLPCTRENDPIEGLDGKIHGNTC

 661 HGNKCAMCKAVFQKENEERKRKEEEDQRNAAGHGSSGGGGGNTQDECAEYREQMKNGRLS
 661 HGNKCAMCKAVFQKENEERKRKEEEDQRNAAGHGSSGGGGGNTQDECAEYREQMKNGRLS
 661 HGNKCAMCKAVFQKENEERKRKEEEDQRNAAGHGSSGGGGGNTQDECAEYREQMKNGRLS

 781 MKNGKLICTRESDPVRGPDGKTHGNKCTMCKEKLEREAAEKKKKEDEDRSNTGERSNTGE
 781 MKNGKLICTRESDPVRGPDGKTHGNKCTMCKEKLEREAAEKKKKEDEDRSNTGERSNTGE
 781 MKNGKLICTRESDPVRGPDGKTHGNKCTMCKEKLEREAAEKKKKEDEDRSNTGERSNTGE

 901 EEKSSSKPSNNAKDQCRQVQNEAEDAKFRQPGRSLASVARMSTDECSEFRNYIRNNELIC
 901 EEKSSSKPSNNAK----------DECSEFRNYIRNNELIC
 901 EEKSSSKPSNNAKVIY----------

1021 EMCKDYRVLPRIGYLCPKDLKPVCGDDGQTYNNPCMLCHENLIRQTNTHIRSTGKCEESS
 991 EMCKDYRVLPRIGYLCPKDLKPVCGDDGQTYNNPCMLCHENLIRQTNTHIRSTGKCEESS
 917 ----------
```

c

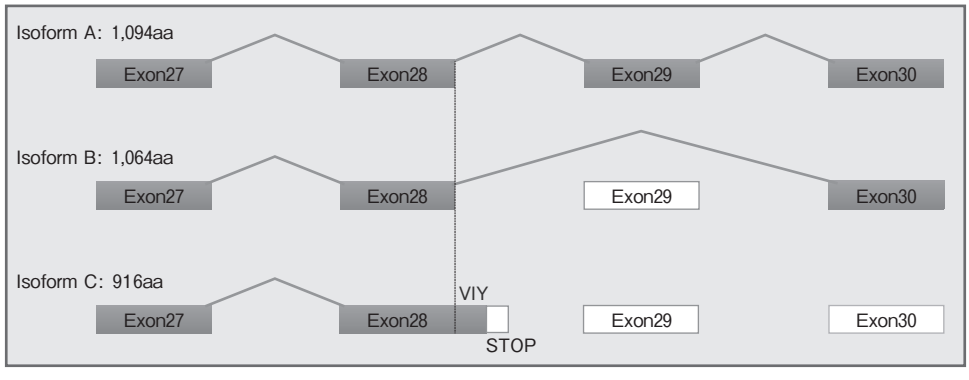


Fig. 1 *SPINK5* gene structure. **a**, Genomic structure of the *SPINK5* gene including 34 exons; **b**, Amino acid alignment of splicing isoforms A-C; **c**, Shames of exons 27-30 splicing pattern of isoforms A-C.

Table 1 Primer for exons 1-34 of *SPINK5*

Exon No.	Primer name	Primer (Forward 5′ - -3′)	Coding exon length (bp)	Products length (bp)
1	SPINK5-E1-FW	CCCATGACCTTGAACAACTGG	55	240
	SPINK5-E1-RV	TGAGATGTGTGGCAAGAGGCT		
2	SPINK5-E2-FW	CTGGTCCAGTGCCCTTCTTTT	26	277
	SPINK5-E2-RV	AGGTGTTGGCCACAAACCACT		
3	SPINK5-E3-FW	AGGCATTCTTTGAGGGCTTTAC	128	430
	SPINK5-E3-RV	AATGTCAGAGTGATCTGCTCAG		
4	SPINK5-E4-FW	TTTGACATGCCAGGCTAGGCT	73	311
	SPINK5-E4-RV	CCCTCCTAAGTTGTTGGCTTC		
5	SPINK5-E5-FW	ATTAGCTCAATGTAGCCTTCATGA	128	392
	SPINK5-E5-RV	ATTTAGGAATGGCTAACTCCCAC		
6	SPINK5-E6-FW	TCGGGAGTAAAGGAGAATGACAA	64	264
	SPINK5-E6-RV	TTCCAAGGCTCCCACCTAAGAA		
7	SPINK5-E7-FW	CACAGCTGGTACTGCTCTAAGT	128	393
	SPINK5-E7-RV	GTGAGAAAGGCTATTTCACAGTG		
8	SPINK5-E8-FW	TTGAAGACGGAAATGCTTGTCTC	64	281
	SPINK5-E8-RV	CTCAAAGGTTTAGGCATACATTTG		
9	SPINK5-E9-FW	TCTCTGAGCCTAGAGGTTAGAG	128	400
	SPINK5-E9-RV	GGGCATAAAACTGCTCAGATGG		
10	SPINK5-E10-FW	GGTCTTTTTGGGGATTTGAGGTG	88	276
	SPINK5-E10-RV	CACACACACACAAGTTGTAACT		
11	SPINK5-E11-FW	CATAGAAAACAGAACTATATCTCAAC	128	327
	SPINK5-E11-RV	CTCCCAGCTCTATCACTGATATT		
12	SPINK5-E12-FW	TAGCACCATACTATCCTGGAGG	82	336
	SPINK5-E12-RV	GGGCAAAATCTCACCCTTTAGG		
13	SPINK5-E13-FW	TTCCTATCTCTTGGCATATGATGT	128	453
	SPINK5-E13-RV	TGTCTCCAATCAGACAGTTTCTC		
14	SPINK5-E14-FW	CAGGGTTAGGCACATCACATTC	82	294
	SPINK5-E14-RV	TAAGGAATGCACGTGTTCCCTG		
15	SPINK5-E15-FW	TTTCTTGGTTGTTGGGTTCATTGT	128	434
	SPINK5-E15-RV	TATGACACCCAAGACTGAATGCT		
16	SPINK5-E16-FW	GCTATGAAAGTTAAGGATCTAGTC	49	596
	SPINK5-E16-RV	GGACACTTCTTATCAGGACCC		
17	SPINK5-E17-FW	CAATTCTGATTGATGACGGAAGC	128	391
	SPINK5-E17-RV	AACTCACGGTCTATACTCTACAC		
18	SPINK5-E18-FW	GCAAAAGCACCTCTCAGACTAG	85	441
	SPINK5-E18-RV	ATTGATCTCTCTCCTGCACGAG		
19	SPINK5-E19-FW	CTTTCACAGTGCCTAGTGCAG	128	451
	SPINK5-E19-RV	CTTCCAATGTCACCCCTCTTG		
20	SPINK5-E20-FW	GATGTGTGTTTACCTTTCCACTC	67	336
	SPINK5-E20-RV	AACCCCTGGACTAAATGAACCC		
21	SPINK5-E21-FW	TACAGCCACCTTCTTAAGTCCTT	128	482
	SPINK5-E21-RV	CTAGAACTGTGAATCTTGGCCAT		
22	SPINK5-E22-FW	TAGAGAAGGCCTGCAGCATAGT	97	417
	SPINK5-E22-RV	ACCCCAGAGCAGTGAAAATGTG		
23	SPINK5-E23-FW	ACCGTACCCGGCAATGTTATGT	128	432
	SPINK5-E23-RV	CCTTGCTAAGGCTCATTGTTCC		
24	SPINK5-E24-FW	CGGCCATTTGGAATCATTGACC	73	331
	SPINK5-E24-RV	GGTAACTCCTGACCTTCTAAGG		
25	SPINK5-E25-FW	TCACAGCAAGGTTACATGGCTG	128	467
	SPINK5-E25-RV	GCTCCTTTCTCCTGTATTGCTC		
26	SPINK5-E26-FW	CATCTGCACTCGAGAAAGTGAC	97	435
	SPINK5-E26-RV	TGGGACAGAGTCAGCATTTCAC		
27	SPINK5-E27-FW	CCTGTGTTATGAGTTTATATCTAATCGG	128	323
	SPINK5-E27-RV	ATGTGTGATGCCAAGTATCTTAGG		
28	SPINK5-E28-FW	GGTATAAGAAAACAGTGTTAGATTTGC	73	448
	SPINK5-E28-RV	CATATTTCTCCCAAGGAGGAAAGAC		
29+30	SPINK5-E29-FW	GTTAGATCCCTGAGCAATTGTCC	90+128	611
	SPINK5-E30-RV	CAGGTGTTCTCAATTTGAGGGC		
31	SPINK5-E31-FW	GGTTACAGGCGTGAACTACCAT	97	341
	SPINK5-E31-RV	ACTTCAGGCTGCACTGAATCAG		
32	SPINK5-E32-FW	TCCACCTCATATGGTGCAAAATC	131	365
	SPINK5-E32-RV	TATGGGTTAACACTCTAGCTGAG		
33	SPINK5-E33-FW	TGAATGCAGATCCCAGATCCTC	91	364
	SPINK5-E33-RV	TGACCAATTCTGGGTCACCTGA		
34	SPINK5-E34-FW	TGCTACTTCTTTGCAGAATGCAG	9	289
	SPINK5-E34-RV	TGAAAATCAGTGAGTCCATTCCC		

The primers were designed to amplify and sequence 34 exons. Exons 29 and 30 were amplified together.

Results

Nine nonsynonymous variants, including p.K420E, were detected (Table 2); these variants had already been registered in the SNP database (dbSNP). We also found 10 synonymous variants (data not shown). No new mutation was detected in this study. We found no significant difference in the frequency of p.K420E between the AD patient group and control group. Similarly, we found no significant difference in the frequencies of p.D106N (rs17860502), p.Q267R (rs6892205), p.A335V (rs34482796), p.S368N (rs2303063), p.D386N (rs2303064), p.R711Q (rs3777134), or p.E825D (rs2303070) between the two groups. However, among the nine variants, p.R654H (n = 1) was found as a heterozygous variant in the AD patient group, but not in the control group. Although p.R654H has been reported as rs182767534 in dbSNP <https://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?rs=182767534> (accessed August 2017), the frequency of heterozygosity was < 0.001.

We next compared the data of our AD patients with data from the Human Genetic Variation Database provided by Kyoto University (Table 3), because the number of controls in our cohort was small. The frequency of p.R654H was 4 heterozygotes per 1,200 people in the Human Genetic Variation Database, indicating that the frequency of p.R654H did not differ significantly. Only the frequency of S368N differed significantly between our AD patients and the data of the Human Genetic Variation Database ($p = 0.048$). The Polyphen-2 algorithm for predicting the functional effects of amino acid substitutions showed that the scores of p.R654H and p.S368N were 1.000 (probably damaging) and 0.002 (benign), respectively. The other algorithm, SIFT, indicated a significant score (0.05, *i.e.*, damaging) for p.R654H.

Discussion

LEKTI consists of 15 domains (D1 to D15), and each domain has a Kazal-type structure that is thought to function as a serine protease inhibitor [10]. The fragments of LEKTI processing, including D6D9, D7D9, D8D9, D10D15, and D10D13, are also bioactive [26]. p.K420E has been shown to change the cleavage pattern of D6D9, and to change the function of serine protease inhibitors [19], indicating that the substitution of even a single amino acid is capable of significantly affecting the function of the LEKTI protein. In our study, there was no difference in the frequency of p.K420E between the AD patient group and the control group. However, some other Japanese groups have reported an association between this variant and AD [13-15], suggesting that there might be regional differences even in Japan.

p.R654H is located within D10, and both the PolyPhen-2 and SIFT algorithms showed significant scores for this variant. Similar to p.K420E, p.R654H might also affect the functions of LEKTI fragments, including D10, D10D13, and D10D15.

The frequency of the p.S368N genotype differed significantly between our AD patients and the data from the Human Genetic Variation Database ($p = 0.048$). As shown in Table 3, the OR of the AA genotype (Asn) of p.S368N was 0.41 (*i.e.*, < 1), and the 95% CI was 0.17 to 0.991 (< 1), suggesting that this genotype might be a protective SNP that protects against the occurrence of AD in Japanese subpopulations. Although the frequency of the genotype did not differ significantly between the AD patients and the control in the present study, a large-scale cohort study would be necessary to confirm and clarify this result in future analyses.

In conclusion, we performed a sequencing analysis of all 34 exons of the *SPINK5* gene; no such comprehensive analysis of all 34 exons had been reported, to our knowledge. Nine nonsynonymous variants were detected, although no new mutation was found. We detected p.R654H only in the AD patients, not the control, and p.R654H might be a risk factor for epidermal barrier dysfunction in some Japanese AD patients. Further investigations, such as a large-scale cohort analysis and a functional analysis using mutant proteins, are required to clarify the clinical significance of the p.R654H variant in AD. The frequency of this variant should also be evaluated in other ethnicities.

Acknowledgments. This work was financially supported by Daiichi Sanko Co., Ltd., Mitsubishi Tanabe Pharma Corp., and Kyowa Hakko Kirin Co., Ltd.

Table 2 Nonsynonymous variants of *SPINK5* in Japanese AD patients

Amino acid	Genotype	AD (n=57)	Control (n=50)	*P* value	OR	95% Conf.	Interval
D106N	GG	36	30				
	GA	18	18	0.66	0.833	0.372	1.866
	AA	3	2	0.813	1.25	0.232	6.667
	GA+AA	21	20	0.737	0.875	0.403	1.899
(Allele frequencies)	G	90	78				
	A	24	22	0.866	0.945	0.495	1.806
Q267R	AA	22	19				
	GA	29	22	0.759	1.138	0.501	2.587
	GG	6	9	0.365	0.576	0.179	1.861
	GA+GG	35	31	0.95	0.975	0.449	2.118
(Allele frequencies)	A	73	60				
	G	41	40	0.544	0.842	0.485	1.462
A335V	CC	22	19				
	CT	29	22	0.759	1.138	0.501	2.587
	TT	6	9	0.365	0.576	0.179	1.861
	CT+TT	35	31	0.95	0.975	0.449	2.118
(Allele frequencies)	C	73	60				
	T	41	40	0.544	0.842	0.485	1.462
S368N	GG	24	19				
	GA	27	22	0.945	0.972	0.429	2.202
	AA	6	9	0.291	0.528	0.165	1.695
	GA+AA	33	31	0.666	0.847	0.39	1.822
(Allele frequencies)	G	75	60				
	A	39	40	0.381	0.78	0.448	1.358
D386N	GG	13	17				
	GA	26	21	0.305	1.619	0.649	4.037
	AA	18	12	0.196	1.962	0.71	5.419
	GA+AA	44	33	0.198	1.744	0.751	4.043
(Allele frequencies)	G	52	55				
	A	62	45	0.171	1.457	0.851	2.495
K420E	AA	22	19				
	GA	29	22	0.759	1.138	0.501	2.587
	GG	6	9	0.365	0.576	0.179	1.861
	GA+GG	35	31	0.95	0.975	0.449	2.118
(Allele frequencies)	A	73	60				
	G	41	40	0.544	0.842	0.485	1.462
R654H	GG	56	50				
	GA	1	0	0.347	Inf	0.227	Inf
	AA	0	0	1	NaN	NaN	NaN
	GA+AA	1	0	0.347	Inf	0.227	Inf
(Allele frequencies)	G	113	100				
	A	1	0	0.348	Inf	0.228	Inf
R711Q	GG	21	19				
	GA	28	21	0.661	1.206	0.524	2.776
	AA	8	10	0.57	0.724	0.242	2.169
	GA+AA	36	31	0.902	1.051	0.482	2.289
(Allele frequencies)	G	70	59				
	A	44	41	0.72	0.905	0.524	1.563
E825D	GG	28	33				
	GT	26	16	0.11	1.915	0.865	4.239
	TT	3	1	0.259	3.536	0.47	25.819
	GT+TT	29	17	0.079	2.011	0.924	4.371
(Allele frequencies)	G	82	82				
	T	32	18	0.082	1.778	0.93	3.396

All 34 exons of SPINK5 were amplified with specific primers and sequenced with an ABI3100 DNA sequencer. Nine nonsynonymous variants were found in this analysis. OR, odds ratio; Inf, infinity; NaN, Not a Number.

Table 3 Nonsynonymous variants of *SPINK5* in Japanese AD patients (The comparison with the Human Genetic Variation Database)

Amino acid	Genotype	AD (n = 57)	Kyoto Database	*P* value	OR	95% Conf. Interval	
D106N	GG	36	719				
	GA	18	271	0.341	1.327	0.746	2.36
	AA	3	39	0.487	1.536	0.483	4.914
	GA + AA	21	310	0.284	1.353	0.782	2.342
(Allele frequencies)	G	90	1709				
	A	24	349	0.259	1.306	0.824	2.071
Q267R	AA	22	368				
	GA	29	562	0.612	0.863	0.491	1.516
	GG	6	231	0.068	0.434	0.179	1.059
	GA + GG	35	793	0.276	0.738	0.429	1.269
(Allele frequencies)	A	73	1298				
	G	41	1024	0.087	0.712	0.483	1.05
A335V	CC	22	371				
	CT	29	601	0.477	0.814	0.464	1.428
	TT	6	233	0.067	0.434	0.179	1.058
	CT + TT	35	834	0.214	0.708	0.412	1.216
(Allele frequencies)	C	73	1343				
	T	41	1067	0.081	0.707	0.479	1.043
S368N	GG	24	377				
	GA	27	547	0.377	0.775	0.443	1.356
	AA	6	230	0.048	0.41	0.17	0.991
	GA + AA	33	777	0.139	0.667	0.391	1.138
(Allele frequencies)	G	75	1301				
	A	39	1007	0.047	0.672	0.453	0.996
D386N	GG	13	331				
	GA	26	585	0.721	1.132	0.58	2.207
	AA	18	254	0.11	1.804	0.88	3.7
	GA + AA	44	839	0.368	1.335	0.716	2.488
(Allele frequencies)	G	52	1247				
	A	62	1093	0.184	1.286	0.888	1.862
K420E	AA	22	355				
	GA	29	460	0.953	1.017	0.578	1.789
	GG	6	231	0.056	0.419	0.172	1.022
	GA + GG	35	691	0.47	0.817	0.475	1.406
(Allele frequencies)	A	73	1170				
	G	41	922	0.089	0.713	0.483	1.053
R654H	GG	56	1200				
	GA	1	4	0.095	5.357	0.795	36.477
	AA	0	0	1	NaN	NaN	NaN
	GA + AA	1	4	0.095	5.357	0.795	36.477
(Allele frequencies)	G	113	2404				
	A	1	4	0.095	5.319	0.795	35.79
R711Q	GG	21	382				
	GA	28	581	0.656	0.877	0.494	1.555
	AA	8	218	0.338	0.668	0.297	1.503
	GA + AA	36	799	0.479	0.82	0.475	1.415
(Allele frequencies)	G	70	1345				
	A	44	1017	0.347	0.831	0.566	1.22
E825D	GG	28	670				
	GT	26	436	0.201	1.427	0.83	2.452
	TT	3	85	0.784	0.845	0.268	2.673
	GT + TT	29	521	0.289	1.332	0.787	2.255
(Allele frequencies)	G	82	1776				
	T	32	606	0.53	1.144	0.754	1.734

Nine nonsynonymous variants found in this analysis were compared with the Human Genetic Variation Database. OR, odds ratio; NaN, Not a Number.

References

1. Cork MJ, Robinson DA, Vasilopoulos Y, Ferguson A, Moustafa M, MacGowan A, Duff GW, Ward SJ and Tazi-Ahnini R: New perspectives on epidermal barrier dysfunction in atopic dermatitis: gene-environment interactions. J Allergy Clin Immunol (2006) 118: 3–21; quiz 22–23.
2. Palmer CN, Irvine AD, Terron-Kwiatkowski A, Zhao Y, Liao H, Lee SP, Goudie DR, Sandilands A, Campbell LE, Smith FJ, O'Regan GM, Watson RM, Cecil JE, Bale SJ, Compton JG, DiGiovanna JJ, Fleckman P, Lewis-Jones S, Arseculeratne G, Sergeant A, Munro CS, El Houate B, McElreavey K, Halkjaer LB, Bisgaard H, Mukhopadhyay S and McLean WH: Common loss-of-function variants of the epidermal barrier protein filaggrin are a major predisposing factor for atopic dermatitis. Nat Genet (2006) 38: 441–446.
3. McAleer MA and Irvine AD: The multifunctional role of filaggrin in allergic skin disease. J Allergy Clin Immunol (2013) 131: 280–291.
4. Voegeli R, Rawlings AV, Breternitz M, Doppler S, Schreier T and Fluhr JW: Increased stratum corneum serine protease activity in acute eczematous atopic skin. Br J Dermatol (2009) 161: 70–77.
5. Caubet C, Jonca N, Brattsand M, Guerrin M, Bernard D, Schmidt R, Egelrud T, Simon M and Serre G: Degradation of corneodesmosome proteins by two serine proteases of the kallikrein family, SCTE/KLK5/hK5 and SCCE/KLK7/hK7. J Invest Dermatol (2004) 122: 1235–1244.
6. Sotiropoulou G, Pampalakis G and Diamandis EP: Functional roles of human kallikrein-related peptidases. J Biol Chem (2009) 284: 32989–32994.
7. Komatsu N, Saijoh K, Kuk C, Liu AC, Khan S, Shirasaki F, Takehara K and Diamandis EP: Human tissue kallikrein expression in the stratum corneum and serum of atopic dermatitis patients. Exp Dermatol (2007) 16: 513–519.
8. Morizane S, Yamasaki K, Kajita A, Ikeda K, Zhan M, Aoyama Y, Gallo RL and Iwatsuki K: TH2 cytokines increase kallikrein 7 expression and function in patients with atopic dermatitis. J Allergy Clin Immunol (2012) 130: 259–261 e251.
9. Briot A, Deraison C, Lacroix M, Bonnart C, Robin A, Besson C, Dubus P and Hovnanian A: Kallikrein 5 induces atopic dermatitis-like lesions through PAR2-mediated thymic stromal lymphopoietin expression in Netherton syndrome. J Exp Med (2009) 206: 1135–1147.
10. Hovnanian A: Netherton syndrome: skin inflammation and allergy by loss of protease inhibition. Cell Tissue Res (2013) 351: 289–300.
11. Chavanas S, Bodemer C, Rochat A, Hamel-Teillac D, Ali M, Irvine AD, Bonafe JL, Wilkinson J, Taieb A, Barrandon Y, Harper JI, de Prost Y and Hovnanian A: Mutations in SPINK5, encoding a serine protease inhibitor, cause Netherton syndrome. Nat Genet (2000) 25: 141–142.
12. Dezman K, Korosec P, Rupnik H and Rijavec M: SPINK5 is associated with early-onset and CHI3L1 with late-onset atopic dermatitis. Int J Immunogenet (2017) 44: 212–218.
13. Kato A, Fukai K, Oiso N, Hosomi N, Murakami T and Ishii M: Association of SPINK5 gene polymorphisms with atopic dermatitis in the Japanese population. Br J Dermatol (2003) 148: 665–669.
14. Kusunoki T, Okafuji I, Yoshioka T, Saito M, Nishikomori R, Heike T, Sugai M, Shimizu A and Nakahata T: SPINK5 polymorphism is associated with disease severity and food allergy in children with atopic dermatitis. J Allergy Clin Immunol (2005) 115: 636–638.
15. Nishio Y, Noguchi E, Shibasaki M, Kamioka M, Ichikawa E, Ichikawa K, Umebayashi Y, Otsuka F and Arinami T: Association between polymorphisms in the SPINK5 gene and atopic dermatitis in the Japanese. Genes Immun (2003) 4: 515–517.
16. Walley AJ, Chavanas S, Moffatt MF, Esnouf RM, Ubhi B, Lawrence R, Wong K, Abecasis GR, Jones EY, Harper JI, Hovnanian A and Cookson WO: Gene polymorphism in Netherton and common atopic disease. Nat Genet (2001) 29: 175–178.
17. Zhao LP, Di Z, Zhang L, Wang L, Ma L, Lv Y, Hong Y, Wei H, Chen HD and Gao XH: Association of SPINK5 gene polymorphisms with atopic dermatitis in Northeast China. J Eur Acad Dermatol Venereol (2012) 26: 572–577.
18. Hubiche T, Ged C, Benard A, Leaute-Labreze C, McElreavey K, de Verneuil H, Taieb A and Boralevi F: Analysis of SPINK5, KLK 7 and FLG genotypes in a French atopic dermatitis cohort. Acta Derm Venereol (2007) 87: 499–505.
19. Fortugno P, Furio L, Teson M, Berretti M, El Hachem M, Zambruno G, Hovnanian A and D'Alessio M: The 420K LEKTI variant alters LEKTI proteolytic activation and results in protease deregulation: implications for atopic dermatitis. Hum Mol Genet (2012) 21: 4187–4200.
20. Folster-Holst R, Stoll M, Koch WA, Hampe J, Christophers E and Schreiber S: Lack of association of SPINK5 polymorphisms with nonsyndromic atopic dermatitis in the population of Northern Germany. Br J Dermatol (2005) 152: 1365–1367.
21. Jongepier H, Koppelman GH, Nolte IM, Bruinenberg M, Bleecker ER, Meyers DA, te Meerman GJ and Postma DS: Polymorphisms in SPINK5 are not associated with asthma in a Dutch population. J Allergy Clin Immunol (2005) 115: 486–492.
22. Kabesch M, Carr D, Weiland SK and von Mutius E: Association between polymorphisms in serine protease inhibitor, kazal type 5 and asthma phenotypes in a large German population sample. Clin Exp Allergy (2004) 34: 340–345.
23. Liu Q, Xia Y, Zhang W, Li J, Wang P, Li H, Wei C and Gong Y: A functional polymorphism in the SPINK5 gene is associated with asthma in a Chinese Han Population. BMC Med Genet (2009) 10: 59.
24. Lan CC, Tu HP, Wu CS, Ko YC, Yu HS, Lu YW, Li WC, Chen YC and Chen GS: Distinct SPINK5 and IL-31 polymorphisms are associated with atopic eczema and non-atopic hand dermatitis in Taiwanese nursing population. Exp Dermatol (2011) 20: 975–979.
25. Tartaglia-Polcini A, Bonnart C, Micheloni A, Cianfarani F, Andre A, Zambruno G, Hovnanian A and D'Alessio M: SPINK5, the defective gene in netherton syndrome, encodes multiple LEKTI isoforms derived from alternative pre-mRNA processing. J Invest Dermatol (2006) 126: 315–324.
26. Fortugno P, Bresciani A, Paolini C, Pazzagli C, El Hachem M, D'Alessio M and Zambruno G: Proteolytic activation cascade of the Netherton syndrome-defective protein, LEKTI, in the epidermis: implications for skin homeostasis. J Invest Dermatol (2011) 131: 2223–2232.