ORIGINAL ARTICLE

Correlation between *SPINK5* Gene Mutations and Clinical Manifestations in Netherton Syndrome Patients

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Netherton syndrome (NS) is a congenital ichthyosiform dermatosis caused by serine protease inhibitor Kazal-type 5 (*SPINK5*) mutations. Tissue kallikreins (KLKs) and lymphoepithelial Kazal-type-related inhibitor (LEKTI) (*SPINK5* product) may contribute to the balance of serine proteases/inhibitors in skin and influence skin barrier function and desquamation. *SPINK5* mutations, causing NS, lead to truncated LEKTI; each NS patient possesses LEKTI of a different length, depending on the location of mutations. This study aims to elucidate genotype/phenotype correlations in Japanese NS patients and to characterize the functions of each LEKTI domain. Since we were unable to demonstrate truncated proteins in tissue from patients with NS, we used recombinant protein to test the hypothesis that the length of LEKTI correlated with protease inhibitory activity. Genotype/phenotype correlations were observed with cutaneous severity, growth retardation, skin infection, stratum corneum (SC) protease activities, and KLK levels in the SC. Predominant inhibition by LEKTI domains against overall SC protease activities was trypsin-like (Phe-Ser-Arg-) activity by LEKTI domains 6–12, plasmin- and trypsin-like (Pro-Phe-Arg-) activities by domains 12–15, chymotrypsin-like activity by all domains, and furin-like activity by none. KLK levels were significantly elevated in the SC and serum of NS patients. These data link LEKTI domain deficiency and clinical manifestations in NS patients and pinpoints to possibilities for targeted therapeutic interventions.

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INTRODUCTION

Netherton syndrome (NS) (OMIM 256500) is an autosomal recessive disorder caused by mutations in the serine protease inhibitor Kazal-type5 (*SPINK5*) gene (Chavanas *et al.*, 2000).

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Abbreviations: Ab, antibody; AMC, 7-amino-4-methyl-coumarin; hGH, human growth hormone; KLK, kallikrein; LEKTI, lymphoepithelial Kazal-type-related inhibitor; NS, Netherton syndrome; pNA, para-nitroanilide; SC, stratum corneum; SD, standard deviation; SPINK5, serine protease inhibitor Kazal-type 5

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NS presents as an ichthyosiform dermatosis with variable erythroderma, hair-shaft defects (bamboo hair), atopic features, and growth retardation (Griffiths *et al.*, 1998).

Lymphoepithelial Kazal-type-related inhibitor (LEKTI) (Magert *et al.*, 1999), the product of *SPINK5* (Chavanas *et al.*, 2000), includes in its primary structure 15 different serine protease inhibitory domains (Magert *et al.*, 1999). The inhibitory functions of LEKTI are highly diverse, that is, toward trypsin, plasmin, subtilisin A, cathepsin G, and human neutrophil elastase (Mitsudo *et al.*, 2003). LEKTI is expressed in the normal stratum corneum (SC), stratum granulosum, and skin appendages (Bitoun *et al.*, 2003; Raghunath *et al.*, 2004). Partial LEKTI domains, for example, 1–6, 6–9′, 9–12, 12–15, 6, or 15, display distinct inhibitory profiles (Jayakumar *et al.*, 2004; Egelrud *et al.*, 2005; Schechter *et al.*, 2005; Borgono *et al.*, 2007), implying that each LEKTI domain may possess a selective/specific inhibitory function.

Human tissue kallikreins (KLKs) are a family of 15 trypsin or chymotrypsin-like secreted serine proteases (KLK1–KLK15) (Yousef and Diamandis, 2001). At least eight KLKs are expressed in normal skin (Komatsu *et al.*, 2005b, 2006a). KLKs are colocalized with LEKTI in skin (Ekholm *et al.*, 2000; Bitoun *et al.*, 2003; Komatsu *et al.*, 2005a). In addition, KLKs and LEKTI are secreted together in lamellar bodies to the

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intercellular space, in the uppermost stratum granulosum (Sondell *et al*., 1995; Ishida-Yamamoto *et al*., 2004, 2005). KLKs are capable of cleaving corneodesmosomes, and their enzymatic activities are suppressed by partial recombinant LEKTI domains (Simon *et al*., 2001; Caubet *et al*., 2004; Egelrud *et al*., 2005; Schechter *et al*., 2005; Borgono *et al*., 2007). Overall trypsin-like and/or chymotrypsin-like activities are considerably elevated in the skin of *SPINK5*-deficient mice (Descargues *et al*., 2005) and in NS patients (Komatsu *et al*., 2002; Hachem *et al*., 2006). The elevated activities cause increased degradation of corneodesmosomal cadherins in NS patients (Descargues *et al*., 2006). Altogether, these accumulating data strongly suggest that, in skin, (1) KLKs are desquamation-related serine proteases; (2) LEKTI may be a negative regulator of KLKs/other proteases and overall protease activities; and (3) the balance between serine proteases and inhibitors, for example, KLKs and LEKTI, may be essential, not only for steady desquamation but also for normal skin barrier function.

A unique characteristic of NS patients is that premature stop codon mutations in *SPINK5* are sufficient to cause this disease. NS patients are thought to synthesize truncated LEKTI with fewer inhibitory domains (Sprecher *et al*., 2001; Bitoun *et al*., 2002; Komatsu *et al*., 2002), and they always lack some domains encoded downstream from the premature stop codons. Since different mutations are predicted to generate different truncated LEKTI variants, each NS patient possesses a different truncated LEKTI. It is thus conceivable that there might be a correlation between genotype and phenotype in NS, depending on the location of the mutations. For example, an NS patient with a shorter LEKTI (fewer inhibitory domains) might present with a severe phenotype, while a patient with further downstream mutations (and longer LEKTI) could present with a milder phenotype. In fact, our previous study hinted to a correlation between genotype and severity of skin lesions and SC protease activity in Japanese NS patients (Komatsu *et al*., 2002).

Two new studies have demonstrated a correlation between cutaneous phenotype (the severity of skin lesion) and serine protease activities in skin, residual LEKTI expression and degradation of desmoglein1/desmocollin1 in the skin of NS (Descargues *et al*., 2006; Hachem *et al*., 2006). However, a correlation between genotype and phenotype was not established. Therefore, this study aimed to (1) elucidate a possible genotype and phenotype correlation in NS; (2) reveal which clinical features are linked to the genotype; (3) verify the correspondence between the inhibitory profiles of LEKTI domains and the SC protease activities in NS patients; (4) clarify a potential correlation between genotype and KLK levels; and (5) propose individualized therapy according to patient's genotype.

RESULTS

Correlations between *SPINK5* gene mutations and clinical manifestations in NS patients

Five patients diagnosed as NS due to gene mutations in *SPINK5*, from five unrelated Japanese families, were studied (Patients A–E) (Table 1). All *SPINK5* mutations led to a premature stop codon, and predicted a production of truncated LEKTI. Since we were unable to demonstrate truncated proteins in tissue from patients with NS, we used recombinant protein to test the hypothesis that the length of LEKTI correlated with protease inhibitory activity. Patients A–D or Patient E displayed compound heterozygous or homozygous mutations, respectively. Each patient was predicted to possess a different number of LEKTI domains (Table 1). To date, 10 different and non-related families have been diagnosed/reported as NS according to *SPINK5* gene mutations in Japan (data not shown). Eight out of 10 families displayed heterozygote Y126X mutation in exon 5 (including our Patients A–D). Six families (including Patient D) and one other family (Patient E) had heterozygote or homozygote R790X mutation in exon 25, respectively. The most frequent pattern of compound heterozygote mutations was Y126X/R790X, which were found in four families (including Patient D). All other mutations have been detected only once (data not shown).

Various clinical symptoms can accompany NS, for example, growth retardation, bamboo hair, or atopic features (Griffiths *et al*., 1998). We examined whether these clinical features are correlated with the genotype, that is, the number of remaining LEKTI domains. We found the following: (1) clinical features that could be correlated with the genotype were cutaneous severity, including the palms/soles region (Figure 1), pain/irritation in denuded skin, growth retardation, frequency of skin infections, and general condition after birth; (2) clinical features that could not be correlated with the genotype were occurrence or severity of allergic diseases (including the levels of eosinophils and IgE in blood), bamboo hair, pruritus, sweat secretion, and temperature instability (Table 1).

In these patients, cutaneous bleeding and blistering was not experienced; oral mucosa, nails, and teeth were intact and no apparent physical, neurological, or mental dysfunction was reported. Sexual dysfunction was not determined (Table 1). All patients were aware of low sweat secretion and temperature instability, especially during the summer.

Inhibitory profile of partial recombinant LEKTI domains toward various SC enzymatic activities

Several clinical manifestations were found to correlate with the genotype of NS patients, indicating that some of the missing inhibitory functions of downstream LEKTI domains could not be compensated by the remaining (upstream) domains. To further examine this issue, we prepared four different partial recombinant LEKTI domains as follows: LEKTI domains 1–6, domains 6–9′ (full domains 6–8 plus partial domain 9), domains 9–12, and domains 12–15. Five different SC activities were selected for evaluation, for the following reasons: the overall SC trypsin-like (Phe-Ser-Arg-; FSR-) and chymotrypsin-like (Arg-Pro-Tyr-) activities are required for desquamation (Suzuki *et al*., 1996). LEKTI domain 15 displays anti-plasmin activity (Egelrud *et al*., 2005), suggesting the presence of SC plasmin-like (Val-Leu-Lys-) activity. Several KLKs exhibit differences in their kinetic preferences between trypsin-like FSR- and trypsin-like

Table 1. Clinical features of NS patients from five unrelated Japanese families

Netherton syndrome (age at time studied, gender)	Patient A (10 years, female)	Patient B (1 year, female)	Patient C (21 years, female)	Patient D (7 years, male)	Patient E (7 years, male)
Stop codon mutations in *SPINK5*	375delAT/715insT	375delAT/966insC	375delAT/1621G to T	375delAT/2368C to T	2368C to T
	Exon 5/exon 9	Exon 5/exon 11	Exon 5/exon 18	Exon 5/exon 25	Exon 25/exon 25
Stop codons in LEKTI	Y126X/C-D239-244L-X	Y126X/G-E323-342R-X	Y126X/E541X	Y126X/R790X	R790X/R790X
	Domain 2/domain 4	Domain 2/domain 5	Domain 2/domain 8	Domain 2/domain 12′	Domain 12′/domain 12′
LEKTI domains retained	Domains 1–4	Domains 1–5	Domains 1–8	Domains 1–12′	Domains 1–12′
Cutaneous features					
Non-palm/sole region	Consistent erythroderma	Consistent erythroderma	Consistent erythroderma	Focal (occasionally erythroderma)	Focal (occasionally erythroderma)
Palms/soles	Severe peeling	Mild peeling	No lesion	No lesion	No lesion
Lichenification	No	Yes (joints)	Yes (joints)	Yes (joints)	Yes (joints)
Pain/irritation in denuded Skin	Yes	Yes	Yes	Slight	Slight
Percutaneous absorption	Over-absorption	Unknown	Unknown	Unknown	Unknown
Frequent skin infection	Yes	Yes	Yes	Occasionally	No
General condition	Good or lethal	Good	Good	Good	Good
Growth retardation	<-3 SD	~-3 SD	<-3 SD	~-2 SD	~-2 SD
Height (cm) at 7.5 years	95	—	98	112	104
Weight (kg) at 7.5 years	16	—	17	21	17
Current height and weight					
Age at time measured (years)	10.2	0.6	18	7.5	7.5
Current height (cm)	105	58	131	112	104
Current weight (kg)	20	5.5	38	21	17
Allergic diseases	Asthma	Urticaria	Not apparent	Food allergy	Not apparent
Laboratory findings					
Eosinophil (mm^3)	91	1,206	400	1,089	3,280
IgE (IU per ml)	32,488	17	8,497	5,590	3,411
Clinical course	No improvement	Improved with age	Improved with age	Improved with age	Improved with age
Reference	Hanakawa *et al.*, 2005	Mizuno *et al.*, 2006	Komatsu *et al.*, 2002	Tsukamoto *et al.*, 2003	Komatsu *et al.*, 2002

LEKTI, lymphoepithelial Kazal-type-related inhibitor; NS, Netherton syndrome; SD, standard deviation; *SPINK5*, serine protease inhibitor Kazal-type 5. Patient A experienced life-threatening systemic infections around her neonatal to early infantile period; however, her general condition is recently good. Patient A only has experienced the death of her sibling around neonatal phase by NS with the complication of systemic infections (Hanakawa *et al.*, 2005).

PFR- (Pro-Phe-Arg-) activities (Oka *et al.*, 2002; Magklara *et al.*, 2003; Michael *et al.*, 2005; Luo *et al.*, 2006). Furin-like (Arg-X-Lys-Arg-) activity may contribute to the processing of LEKTI pro-protein to individual LEKTI domains (Seidah and Chretien, 1999; Komatsu *et al.*, 2002; Mitsudo *et al.*, 2003). Thus, the inhibitory properties of each LEKTI fragment toward the overall SC protease activities were investigated using normal SC samples (Table 2), and the aforementioned substrates.

When 125–150 ng per mg of SC dry weight of the specified LEKTI fragment was co-incubated with SC samples, FSR activity declined to $\sim$50%, compared to control activity (Table 2). A higher amount of LEKTI domains 6–9′ and 9–12 (250 ng mg^{-1}) reduced the activity to $\sim$30%. PFR activity was significantly attenuated by domains 12–15 in a dose-dependent manner, whereas other domains suppressed the activity only slightly. Plasmin-like activity was inhibited significantly by domains 12–15 only (residual activity $\sim$60%), but without a dose–response effect. Furin-like activity was not significantly suppressed by any of the domains. Chymotrypsin-like activity was suppressed significantly by all domains, in a dose-dependent manner and with a similar magnitude of inhibition (residual activity $\sim$70–40%) (Table 2).

We then examined if the observed inhibition by LEKTI fragments was due to unprocessed LEKTI fragments (comprising

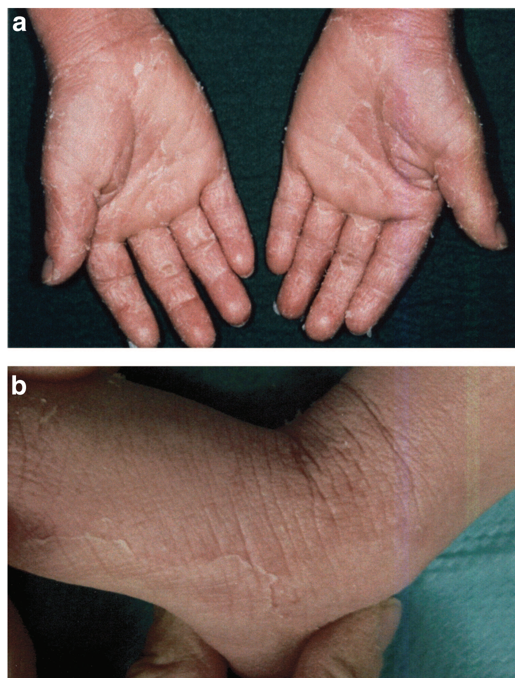

Figure 1. Palm and plantar lesions in NS patients. (**a**) Patient A (7 years). Patients A and B have consistently displayed scaling and peeling in their palms and soles. The lesion in Patient B was much milder that that in Patient A. In contrast, Patients C–E had no palm/plantar lesions (Table 1). (**b**) Patient E (2 years). Generally, the lesions in palms and soles were absent or much milder than those in non-palm and plantar lesions. Thus, NS patients are highly likely to display a ''linear scaling'' at the border between the palms/soles (the SC appears to be almost intact) and non-palms/soles (the SC is over-desquamated). We confirmed the ''linear scaling'' for all patients (Patients A–E); therefore, the ''linear scaling'' could be a potential specific finding in NS.

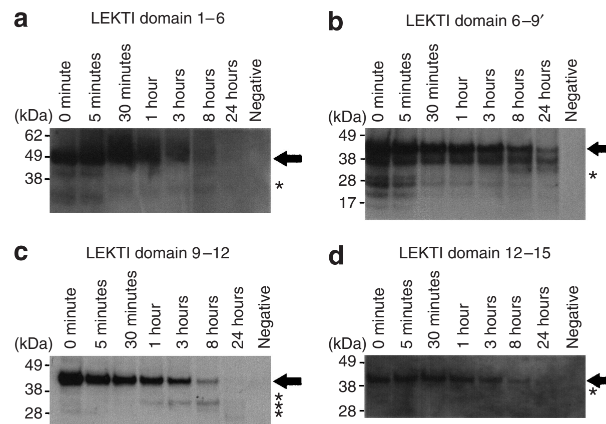

Figure 2. Proteolytic degradation of LEKTI domains by SC proteases. The specified LEKTI domain, domains 1–6 (**a**), domains 6–9′ (**b**), domains 9–12 (**c**), and domains 12–15 (**d**), was incubated with normal SC. The amount of LEKTI added was 500 ng per mg dry weight for LEKTI domains 6–9′, 9–12, and 12–15 and 600 ng mg^{-1} for domains 1–6. Endogenous LEKTI (no recombinant protein was added) was undetectable (negative). When each LEKTI domain was incubated without SC sample, degradation was negligible at any time point (Borgono *et al.*, 2007). Arrows indicate the full length of each domain and asterisks show the fragments which did not exist at 0 minute. The detection Abs were monoclonals 1C11G6 (for domains 1–6, 6–9′, and 9–12) and 1D6G8 (for domains 12–15) (Raghunath *et al.*, 2004). The smaller fragments appeared after incubation, suggesting presence of specific LEKTI cleavage sites by SC proteases.

several domains) or by individual domains, after LEKTI proteolysis (Figure 2). LEKTI domains were not significantly processed up to 3 hours of incubation, an interval that is as long as the duration of the enzymatic assay. Thus, the inhibitory activities shown in Table 2 are likely mediated by unprocessed LEKTI. Proteolytic processing of LEKTI to individual domains may not be necessary for its inhibitory function. LEKTI fragments were significantly degraded after 24 hours, yielding smaller fragments (Figure 2).

Correlation between genotypes and overall SC protease enzymatic activities in NS patients

The diversity of LEKTI inhibitory functions demonstrated in Table 2 suggests that NS patients could display variable overall SC protease activity, corresponding to their genotype (due to lack of downstream LEKTI inhibitory domains). To test this possibility, we measured the overall SC protease activities in skin extracts from NS patients (Table 3).

Trypsin-like (FSR- and PFR-) activities were high in patients with upstream *SPINK5* mutations (Patients A–C) (Table 3). The chymotrypsin-like activity was not elevated in any patients, except for the aggravated skin lesion in Patient B. Plasmin- and furin-like activities were significantly elevated in all patients, except Patient A (on two occasions, these activities were not determined due to sample depletion). The aggravated lesion in Patient B seems to exhibit higher enzymatic activities than the stable lesion of the same patient or lesions of other patients (Table 3).

Correlation between genotypes and KLK levels in the SC or serum of NS patients

The balance of serine proteases and their inhibitors, including KLKs and LEKTI, is important for regulation of skin desquamation (Ekholm *et al.*, 2000; Descargues *et al.*, 2005; Komatsu *et al.*, 2005b; Hachem *et al.*, 2006). Since NS patients are deficient in some inhibitory LEKTI domains, they may present with alterations in KLK concentrations or activities in the SC or serum. Thus, KLK levels were measured in the SC and serum of these five patients by ELISAs (Tables 4 and 5).

In the SC (Table 4a), chymotrypsin-like KLK7 levels ranged widely among the five patients. In Patients A and B, the elevation of KLKs 5, 8, and 11 was quite significant. For Patients C–E, the most pronounced increases were for KLKs 6, 8, 10, and 13 (Table 4a). In the aggravated skin lesion (Table 4b), KLK levels were, in general, higher than those in the stable lesion. The differences were particularly notable for KLKs 6, 10, 13, and 14 (Table 4b).

In serum (Table 5), where both KLKs and LEKTI are present (Magert *et al.*, 1999; Yousef and Diamandis, 2001), KLKs in NS patients tended to be higher than those in the normal subjects. The elevation of total trypsin-like KLKs was more pronounced than that of chymotrypsin-like KLKs. KLK11 levels did not differ significantly among all the patients

Table 2. Inhibition of the overall SC enzymatic activities by LEKTI domains

Substrate	Amount of LEKTI domain (ng per mg dry weight)	Residual overall stratum corneum enzymatic activity (mean ± SEM (%)) LEKTI domain: Domains 1–6	Domains 6–9′	Domains 9–12	Domains 12–15
Trypsin-like activity	125–150	58.1 ± 3.5[#]	46.3 ± 4.0[#]	53.9 ± 3.3[#]	59.5 ± 2.5[#]
Phe-Ser-Arg-AMC	250–300	46.3 ± 4.1[#]	28.4 ± 2.5[*1,*2,#]	29.8 ± 2.5[*1,*2,#]	52.6 ± 4.0[#]
	500–600	NC	NC	NC	NC
Trypsin-like activity	125–150	NC	NC	NC	75.3 ± 5.5[#]
Pro-Phe-Arg-AMC	250–300	83.9 ± 1.9[#]	84.3 ± 4.0[#]	88.6 ± 5.8[#]	64.5 ± 3.1[*3,#]
	500–600	NC	NC	NC	38.7 ± 4.4[*4,#]
Chymotrypsin-like activity	125–150	67.5 ± 4.4[#]	59.3 ± 2.1[#]	69.8 ± 2.2[#]	56.9 ± 4.0[#]
Arg-Pro-Tyr-pNA	250–300	37.0 ± 7.4[#]	36.6 ± 3.6[#]	39.1 ± 3.9[#]	39.8 ± 6.6[#]
	500–600	NC	NC	NC	NC
Plasmin-like activity	125–150	NC	NC	NC	59.7 ± 7.2[#]
Val-Leu-Lys-AMC	250–300	90.9 ± 3.3	96.5 ± 5.6	88.0 ± 2.5[#]	66.4 ± 5.9[*5,#]
	500–600	NC	NC	NC	60.1 ± 6.3[#]
Furin-like activity	125–150	NC	NC	NC	NC
Pyr-Arg-Thr-Lys-Arg-AMC	250–300	92.6 ± 1.8	91.3 ± 2.1	96.1 ± 1.6	92.4 ± 2.6
	500–600	NC	NC	NC	NC

Residual overall SC enzymatic activities (released AMC or pNA with LEKTI domain divided by released AMC or pNA without LEKTI domain (control)) are indicated as (mean ± SEM (%)). The measurement was performed at 3 or 4 hours time point, for AMC or pNA, respectively. The amount of LEKTI domain to be added in the assay was determined according to the molecular weight (Figure 2); 250 or 300 ng per mg dry weight for LEKTI domains 6–9′, 9–12, and 12–15, or domains 1–6, respectively. When SC sample or LEKTI domain was boiled for 5 minutes before the assay started, the protease activity or inhibitory function was completely lost, respectively (data not shown).
[#]Significant differences by *t*-test between the activity with the specified LEKTI domain and the activity without the domain ($P<0.01$). Significant differences (*t*-test or one-way analysis of variance with Bonferroni's multiple comparison test as a *post hoc* test, $P<0.05$) were detected as follows: (*1), the residual FSR activities using the specified LEKTI domain (250 ng mg^{-1} vs 125 ng mg^{-1}); (*2), the residual FSR activities using the specified domain (250 ng mg^{-1}) vs domains 1–6 (300 ng mg^{-1}) or 12–15 (250 ng mg^{-1}); (*3), the residual PFR activity using domains 12–15 (250 ng mg^{-1}) vs the other domains (250–300 ng mg^{-1}); (*4), the residual PFR activity using domains 12–15 (>500 ng mg^{-1} vs 125 or 250 ng mg^{-1}); (*5), the residual plasmin-like activity using domains 12–15 (250 ng mg^{-1}) vs the other domains (250–300 ng mg^{-1}). NC, no change in comparison to 250–300 ng mg^{-1} LEKTI.
AMC, 7-amino-4-methyl-coumarin; LEKTI, lymphoepithelial Kazal-type-related inhibitor; PNA, para-nitroanilide; SC, stratum corneum.

compared to the normal subjects. Also, the elevation of serum KLKs in Patient E was milder than that of the other patients (Table 5).

DISCUSSION

In Japanese NS patients, cutaneous severity seems to correlate with the genotype (Table 1). The generalized erythroderma of variable intensity in NS (Griffiths *et al.*, 1998) may be explained by the diversity of *SPINK5* mutations in NS. A sister of Patient A with NS (with stop codon mutations in exon 2/exon 4) (Hanakawa *et al.*, 2005) and members of Turkish families with a homozygote mutation in exon 3 (Bitoun *et al.*, 2002) died from the disease. In contrast, Patients B–E did not have any life-threatening complication so far. Despite the potential lethality of *SPINK5* mutations in Patient A, the SC protease activities or KLK levels were not necessarily much different in comparison to the other patients (Tables 3–5). Presumably, Patient A might have developed an alternative regulatory pathway of protease activities during her life.

Allergic reactions, and/or eosinophil counts and IgE levels in blood do not seem to be linked to the genotype of NS patients. Patient C and her brother (who also suffered from NS) never experienced any allergic reactions, and a significant elevation of eosinophil/IgE did not appear until adolescence (Komatsu *et al.*, 2002). Therefore, the emergence of allergic features could be due to secondary factors.

The pathogenesis of growth retardation in NS is not known. A common finding is absence of a significant abnormality in the serum level of human growth hormone (hGH). It is known that hGH is proteolytically processed by serine proteases (such as plasmin and thrombin) in both the pituitary and the periphery (Baumann, 1991; Garcia-Barros *et al.*, 2000). *KLKs* (*KLKs* 5–8 and 10–14) and *SPINK5* are

Table 3. SC enzymatic activities in NS patients

Substrate (released AMC or pNA; nmol per mg dry weight)	Normal	Netherton syndrome Patient A	Patient B	Patient C	Patient D	Patient E	Patient B Aggravated
Trypsin-like activity							
Phe-Ser-Arg-AMC	15.5±1.5	41.5[1]	52.0[1]	54.5[1]	12.5	25.0[1]	ND
Pro-Phe-Arg-AMC	5.7±3.1	13.0[1]	8.4	13.7[1]	4.2	8.3	17.9[1]
Chymotrypsin-like activity							
Arg-Pro-Tyr-pNA	13.9±5.5	9.2	ND	5.9	13.8	11.7	75.3[1]
Plasmin-like activity							
Val-Leu-Lys-AMC	1.7±1.0	2.1	5.5[1]	ND	3.7[1]	4.9[1]	10.6[1]
Furin-like activity							
Pyr-Arg-Thr-Lys-Arg-AMC	3.0±1.3	4.4	ND	21.7[1]	27.4[1]	23.4[1]	43.6[1]

AMC, 7-amino-4-methyl-coumarin; pNA, para-nitroanilide; SC, stratum corneum; SD, standard deviation; NS, Netherton syndrome; ND, not determined due to the sample depletion.
[1]Significant differences ($P<0.05$) between the mean of normal samples and each patient at the specified time (Smirnov test for extreme values). The data for normal controls were referred from a previous study (Komatsu *et al*., 2005b, 2006b). For Patient B, two different samples were used for the assay, obtained at different times: (1) at a stable phase and (2) an aggravated phase. The SC samples in the aggravated lesion were not available for the other four patients.

expressed in the pituitary and appear to be localized to hGH-producing cells. KLKs 4–6, 8, 13, and 14 were able to cleave hGH, yielding various isoforms, *in vitro*. Inhibitor LEKTI fragments were able to suppress the activity of KLKs 4, 5, and 14 *in vitro* (Komatsu *et al*., 2007a). Based on these data, we recently proposed that LEKTI and KLKs might be involved in the regulation of growth hormone processing. In NS, hGH may be proteolytically over-processed by proteases, due to LEKTI inhibitory dysfunction (deficiency) in the pituitary. This could lead to over-fragmentation of the hormone and loss of its bioactivity (Komatsu *et al*., 2007a). According to this suggestion, patients with upstream SPINK5 mutations should present with more severe growth retardation. In support of this, Patient A displayed the most severe growth retardation among the five patients (Table 1). Thus, the involvement of LEKTI in the regulation of hGH proteolysis is likely.

In the enzymatic assays of overall SC protease activities (Table 2), LEKTI domains, particularly domains 6–9′ and 9–12, displayed strong inhibitory properties against trypsin-like FSR-activity. Patients D and E (who possess domains 6–12′) displayed only a slight elevation of FSR-activity (Table 3). We could thus conclude that LEKTI domains 6–12 are essential for the inhibition of FSR-activity in the SC. Hence, a therapeutic intervention aiming to suppress FSR-activity in the SC may be effective in patients who possess <12 LEKTI domains, that is, Patients A–C.

Both FSR- and PFR-substrates represent trypsin-like activity. However, the inhibition by LEKTI fragments was different (Table 2), suggesting that the proteases representing each activity may be different.

Based on their kinetic properties, KLKs 5, 6, 8, 11, and 13 may affect both FSR- and PFR-activities (Oka *et al*., 2002; Magklara *et al*., 2003; Kapadia *et al*., 2004; Michael *et al*., 2005; Luo *et al*., 2006). The inhibitory profile of LEKTI domains toward KLKs 5, 6, and 13 (Schechter *et al*., 2005; Borgono *et al*., 2007) was comparable to the profile toward FSR-activity, but dissimilar to the activity toward PFR-activity (Figure 3). Therefore, in the SC, the enzymatic activities of these KLKs may be contributing mainly to FSR-activity. In addition, KLK enzymatic activities seem to be regulated by upstream LEKTI domains (domains 1–12) with the exception of KLK5 (Figure 3).

The responsible enzymes for PFR-activity are not known, but all five patients still lacked domains 12–15, which predominantly inhibit PFR-activity (Table 2). Hence, therapeutically, it may be justified to control the PFR-activity in the SC of these patients.

LEKTI domains 12–15 exhibit significant inhibition toward PFR- and plasmin-like activities, unlike the other domains (Table 2). We thus hypothesize that domains 12–15 may possess a specific inhibitory role, uncompensated by other LEKTI domains.

LEKTI domain 15, but not domain 6, is able to suppress plasmin (Egelrud *et al*., 2005); most patients displayed a significant elevation of plasmin-like activity, probably due to absence of domain 15 (Table 3). Suppression of plasmin-like activity in the SC may be a fruitful therapeutic strategy for all NS patients.

None of LEKTI domains significantly suppressed the SC furin-like activity (Table 2), which may be responsible for the

Table 4. KLK levels in the SC of NS patients by ELISA

(a)

KLK (ng per mg dry weight)	Normal Mean±SD	Patient A	Patient B	Patient C	Patient D	Patient E
Chymotrypsin-like KLK						
KLK7	10.9±6.0	57.8[1]	11.0	34.7[1]	57.5[1]	13.1
Trypsin-like KLKs						
KLK11	8.7±4.1	20.2[1]	20.7[1]	16.0	8.1	15.3
KLK8	5.8±1.8	21.8[1]	21.8[1]	16.8[1]	8.7	11.5[1]
KLK5	3.1±1.4	17.8[1]	11.8[1]	4.2	7.4[1]	3.6
KLK10	0.67±0.41	0.20	1.2	4.2[1]	5.8[1]	1.4
KLK14	0.34±0.13	0.24	0.38	0.28	0.10	0.41
KLK6	0.28±0.12	0.11	0.16	49.4[1]	25.4[1]	0.84[1]
KLK13	0.17±0.14	0.83[1]	0.04	20.1[1]	7.2[1]	1.1[1]
Total of Trypsin-like KLKs	19.1±5.4	61.2[1]	56.1[1]	111.0[1]	62.7[1]	34.2[1]

(b)

KLK (ng per mg dry weight)	Normal	Patient B		Patient E	
	Mean±SD		Aggravated		Aggravated
Chymotrypsin-like KLK					
KLK7	10.9±6.0	11.0	32.9[1]	13.1	14.1
Trypsin-like KLKs					
KLK11	8.7±4.1	20.7[1]	22.1[1]	15.3	12.4
KLK8	5.8±1.8	21.8[1]	20.6[1]	11.5[1]	36.8[1]
KLK5	3.1±1.4	11.8[1]	9.3[1]	3.6	0.64
KLK10	0.67±0.41	1.2	28.7[1]	1.4	2.1[1]
KLK14	0.34±0.13	0.38	4.5[1]	0.41	4.8[1]
KLK6	0.28±0.12	0.16	74.6[1]	0.84[1]	10.2[1]
KLK13	0.17±0.14	0.04	41.3[1]	1.1[1]	16.4[1]
Total of Trypsin-like KLKs	19.1±5.4	56.1[1]	201.0[1]	34.2[1]	83.3[1]

KLK, kallikrein; NS, Netherton syndrome; SD, standard deviation; SC, stratum corneum.
Data for normal subjects were referred from a previous study (Komatsu *et al.*, 2005b). KLKs were subdivided into chymotrypsin-like KLK (KLK7) and trypsin-like KLKs (the rest of KLKs) (Yousef and Diamandis, 2001).
[1]Smirnov test detected significant differences between the normal SC subjects and individual patients ($P<0.05$). For Patients B and E, data from two different samples are shown, that is, (1) stable and (2) aggravated lesions (Table 4b), which correspond to data described in Table 3. SC samples in aggravated phase were not available for the other three patients.

proteolytic processing of pro-LEKTI into 15 individual inhibitory domains (Seidah and Chretien, 1999; Komatsu *et al.*, 2002; Mitsudo *et al.*, 2003). Hence, the processing of pro-LEKTI may be independent from LEKTI inhibition of protease activity. The data of Figure 2 suggest that proteolysis of pro-LEKTI to individual domains is not essential for the domains' inhibitory functions. The increased furin-like activity in NS patients (Table 3) may help in the release of more LEKTI domains, which might provide a more efficient inhibitory activity compared to unprocessed LEKTI.

The SC chymotrypsin-like activity was not significantly elevated in NS patients (Table 3), whereas *SPINK5*-deficient mice displayed significant elevation of chymotrypsin-like activity in their skin (Descargues *et al.*, 2005). KLKs 7 and 14 may be the major players of chymotrypsin-like proteases/activity in the SC (Felber *et al.*, 2005; Komatsu *et al.*, 2005b, 2007a). Altogether, it is possible that (1) the same chymotrypsin-like enzymes (e.g., KLKs 7 and 14) might be targeted by all LEKTI domains tested. Thus, upstream LEKTI domains may be sufficient in regulating their activity; and/or (2) another inhibitor (e.g., α2-macroglobulin family) (Galliano *et al.*, 2006) may be compensating for the missing LEKTI functions in these patients. These interpretations might also explain the normal chymotrypsin-like activity (Table 3), despite increased KLK7 levels (Table 4a) in the SC of these patients. It seems that in NS patients, the SC chymotrypsin-like activity is not as important as other activities, such as FSR-, PFR-, or plasmin-like activities.

In the SC (Table 4a), the levels of trypsin-like KLKs 5, 8, and 11 were particularly high in Patients A and B. Also, trypsin-like

Table 5. KLK levels in the serum of NS patients by ELISA

KLK (ng ml^{-1})	Normal Mean±SD	Patient A	Patient B	Patient C	Patient D	Patient E
Chymotrypsin-like KLK						
KLK7	5.1±2.1	16.4[1]	32.0[1]	14.4[1]	26.0[1]	11.6[1]
Trypsin-like KLKs						
KLK6	4.4±1.5	15.9[1]	10.6[1]	15.3[1]	9.6[1]	5.0
KLK8	1.9±0.77	15.8[1]	27.7[1]	15.8[1]	17.6[1]	9.4[1]
KLK10	1.2±0.56	6.0[1]	11.8[1]	8.2[1]	10.8[1]	2.0
KLK5	0.68±0.15	2.9[1]	3.6[1]	2.9[1]	3.4[1]	1.4[1]
KLK11	0.54±0.16	0.64	0.56	0.34	0.36	0.76
KLK14	0.22±0.091	1.2[1]	1.2[1]	0.40[1]	0.50[1]	0.74[1]
KLK13	<0.01	0.75[1]	0.57[1]	1.6[1]	0.89[1]	0.40[1]
Total of Trypsin-like KLKs	9.0±1.6	43.3[1]	56.0[1]	44.6[1]	43.1[1]	19.8[1]

KLK, kallikrein; NS, Netherton syndrome; SD, standard deviation.
[1]Smirnov test showed significant differences between the normal subjects and individual patients ($P<0.05$). The amount KLK13 in normal samples is very low or undetectable; KLK13 in normal serum was reported as <0.01 ng ml^{-1} without SD. Total of trypsin-like KLKs indicates the sum of trypsin-like KLKs.

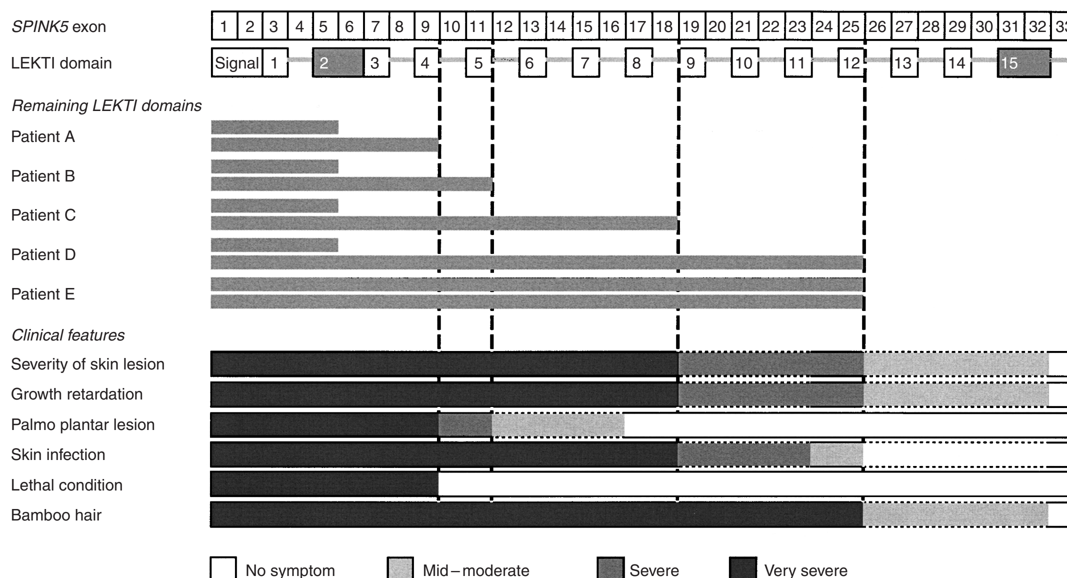

Figure 3. Genotype and phenotype correlations in NS patients. The *SPINK5* gene consists of 33 exons, encoding 15 LEKTI inhibitory domains (Magert *et al.*, 1999; Chavanas *et al.*, 2000). LEKTI domains 2 and 15 (highlighted) are characterized as Kazal-type domains; the rest are non-Kazal-type domains (Magert *et al.*, 1999). In general, exons with odd numbers contain 128 nt (GenBank accession no. NP_006837) and encode the conserved inhibitory domain; the even number exons encode connecting amino-acid chains with the exceptions of Kazal-type domains 2 and 15. The remaining LEKTI domains for individual patients are indicated by the gray bars. The severity of clinical features was evaluated based on the information described in Table 1. Dotted lines are a predicted correlation.

KLKs 6, 10, and 13 tended to be low in Patients A–C. There might be some correlation between the SC trypsin-like KLK levels and disease genotype, although multiple LEKTI domains were able to suppress multiple KLKs (Figures 4 and 5).

KLKs 6, 13, and 14 were notably elevated in the aggravated skin lesion (Table 4b). Since these KLK elevations were also observed in psoriatic skin lesions (Komatsu *et al.*, 2007b), the elevations could represent a secondary phenomenon. In general, the aggravated skin tended to display higher SC protease activities and KLK levels, compared to the stable lesion (Tables 3 and 4b).

Most KLKs were lower in Patient E than Patient D (Tables 4 and 5), although both patients possess LEKTI domains 1–12′ (Table 1). Thus, compound heterozygote vs homozygote mutations in *SPINK5* could affect KLK expression.

In conclusion, we provide early evidence for genotype and phenotype correlations in Japanese NS patients. The correlations are between cutaneous severity, growth retardation,

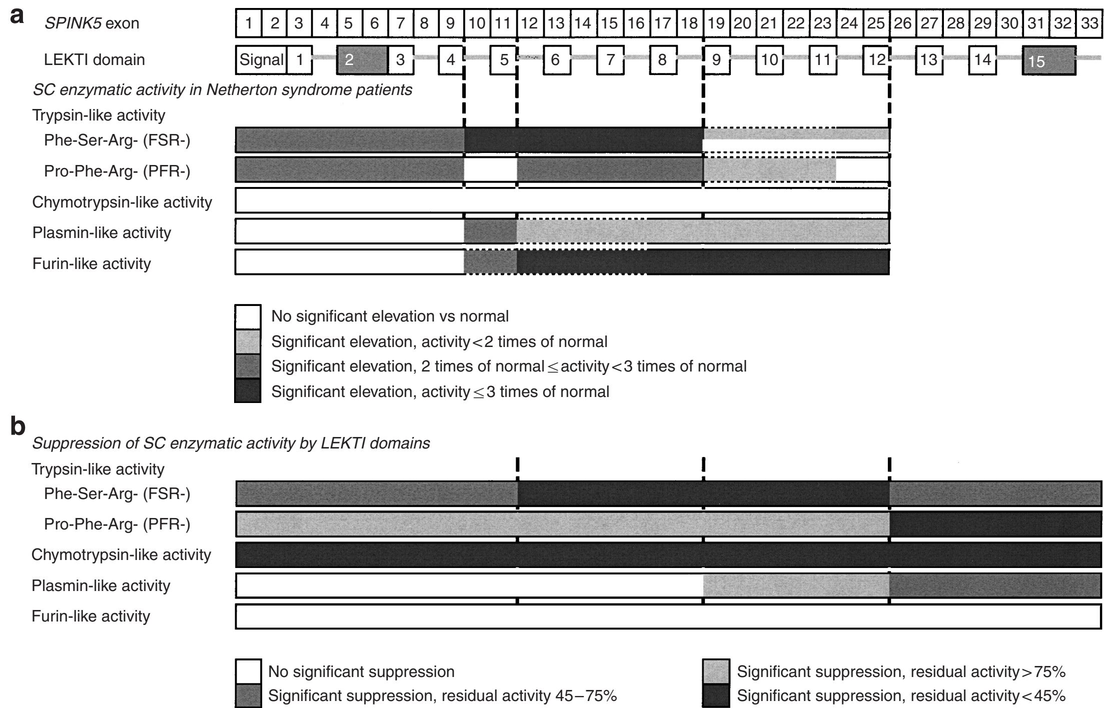

Figure 4. Correlation between LEKTI domain function and SC enzymatic activities in NS patients (see also Figure 3 for additional explanations). (**a**) Summary of SC enzymatic activities in NS patients, (**b**) and residual SC activities after suppression by LEKTI domains using normal SC samples. For the suppression of SC enzymatic activity, we present the results with the lowest residual activity, regardless of the amount of LEKTI domains added (Table 2). The distinct inhibitory function of partial LEKTI domains seems to be related to the SC protease activities in NS patients. For example, in the SC, some LEKTI domains, particularly domains 6–9′ and 9–12, displayed strong inhibitory properties against trypsin-like FSR-activity (**b**), suggesting that LEKTI domains 6–12 are essential for the inhibition of FSR-activity in the SC. The strong inhibition by LEKTI domains 6–12 against FSR-activity may explain why Patients D and E (who possess domains 6–12′) displayed only a slight elevation of FSR-activity (**a**). All five patients lacked domains 12–15, which predominantly inhibit trypsin-like PFR-activity (**b**). This may explain why PFR-activity is likely to show a significant elevation in NS patients (**a**).

frequency of skin infection, the overall SC protease activities, and KLK levels in the SC. The altered KLK expression in the SC of NS patients suggests presence of a regulatory system that controls gene expression of proteases and their inhibitors (*SPINK5* and *KLKs*) in skin tissue.

MATERIALS AND METHODS

Patients and *SPINK5* gene mutation analysis

Informed consent was obtained from all patients, their parents, and normal volunteers. Our studies were performed according to the Declaration of Helsinki Principal. The Medical Ethics Committee of the Graduate School of Medical Science, School of Medicine, Kanazawa University approved all described studies.

Five patients from five unrelated Japanese families were studied (Patients A–E) (Table 1) (Komatsu *et al*., 2002; Tsukamoto *et al*., 2003; Hanakawa *et al*., 2005; Mizuno *et al*., 2006). *SPINK5* gene mutation analysis was performed using standard methods for all the patients. Details of these gene mutations are described elsewhere (Komatsu *et al*., 2002).

Sample preparation of the SC and serum

The SC samples were obtained from the forearm of the patients and 25 normal volunteers (20–35 years of age; a mix of males and females), by stripping using Nichiban™ tape (Nichiban, Tokyo, Japan). The SC samples were washed and purified using toluene. After toluene treatment, the purified samples were air-dried and weighed. The detailed procedure for purification is described elsewhere (Komatsu *et al*., 2005b). The data for normal subjects (for both the SC and serum) shown in Tables 3–5 were referred from our previous studies (Komatsu *et al*., 2005b, 2006b).

Preparation of recombinant LEKTI domains and anti-LEKTI antibodies

Recombinant LEKTI domains containing intact LEKTI domains 1–6, domains 6–9′ (containing domains 6–8 and partial domain 9), domains 9–12, and domains 12–15 were produced in a baculovirus/insect cell line system as reported previously (Jayakumar *et al*., 2004; Schechter *et al*., 2005; Borgono *et al*., 2007). Anti-LEKTI mAbs 1C11G6 and 1D6G8 were produced as described previously (Raghunath *et al*., 2004).

Immunofluorometric ELISA for human tissue KKLs

With the exception of Fuso-FB6MA53 anti-KLK11 antibody (Ab), which was purchased (Fuso, Osaka, Japan), all other monoclonal and polyclonal anti-kallikrein Abs were developed in our laboratory (Komatsu *et al*., 2005a). Each of the Abs displayed negligible cross-reactivity with other KLKs (data not shown). The detailed procedures and conditions for sample preparation and each KLK ELISA assay are described elsewhere (Komatsu *et al*., 2005b).

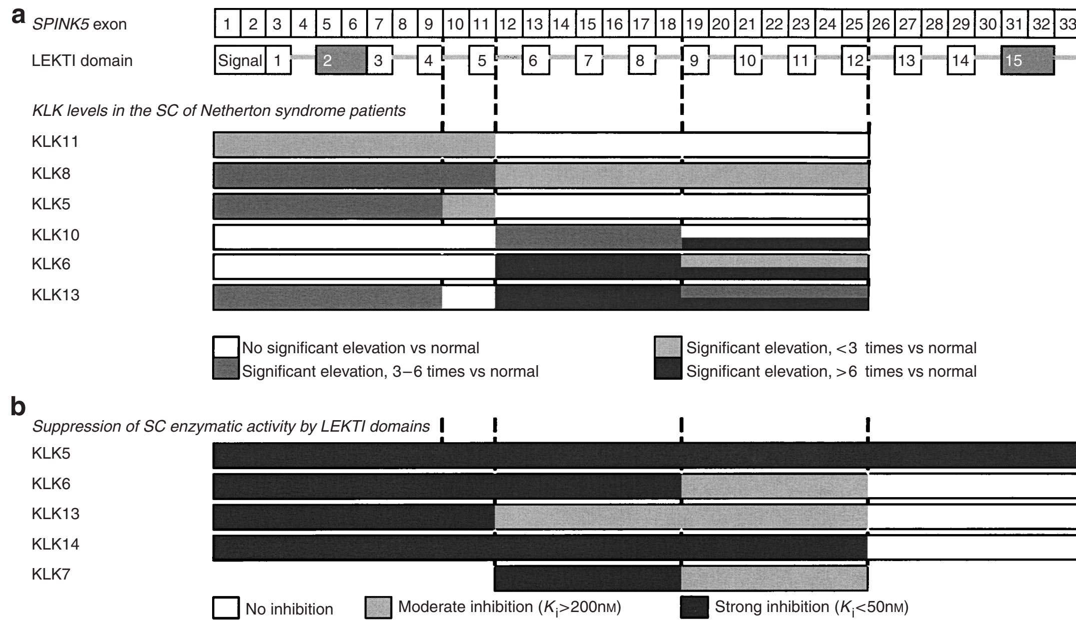

Figure 5. Suppression of KLK activity by LEKTI domains and KLK expression in NS patients (see also Figure 3 for additional explanations). (**a**) Summary of KLK levels in the SC of NS patients, (**b**) and suppression of KLK enzymatic activity by LEKTI domains. Data for suppression of KLK enzymatic activity were derived from previous studies (Schechter *et al.*, 2005 for KLK7 and Borgono *et al.*, 2007 for the rest of KLKs). Upstream LEKTI domains tend to show strong inhibition toward individual KLKs (**b**). However, no evident correlation was observed between inhibition by LEKTI domains toward KLKs and KLK expressions in NS patients (Table 5).

Assay of SC protease enzymatic activities

The synthetic peptide substrates Boc-Phe-Ser-Arg-AMC (AMC, 7-amino-4-methyl-coumarin), Boc-Pro-Phe-Arg-AMC, Boc-Pyr-Arg-Thr-Lys-Arg-AMC, and Boc-Val-Leu-Lys-AMC (BACHEM, Torrance, CA) were used at 0.1 mM final concentration. MeO-Suc-Arg-Pro-Tyr-pNA-HCl (pNA, para-nitroanilide) (Chromogenix, Milano, Italy) was used at 0.4 mM final concentration.

The SC samples (0.5 mg dry weight) were mixed with 10 μl of *N*,*N*-dimethylformamide, 240 μl of 0.1% Triton X-100, 175 μl of 0.2 M Tris-HCl buffer (pH 8.0), and 50 μl of either 1 mM AMC substrates or 4 mM of pNA substrate (Komatsu *et al.*, 2005b) at 4°C. Then, the mixture was incubated at 37°C with shaking for 1–4 hours (Table 3). Released AMC was measured using a fluorescence spectrophotometer (Wallac Victor^2 1420 Multilabel Counter, Perkin Elmer, Boston, MA). Released pNA was measured spectrophotometrically at 405 nm (Wallac Victor^2 1420 Multilabel Counter). Due to the limited amount of samples, each assay was performed only once per subject, but all measurements were repeated three times.

Inhibition assay of SC protease enzymatic activities

The mixture comprising 0.5 mg dry weight of SC sample, 10 μl of *N*,*N*-dimethylformamide, 240 μl of 0.1% Triton X-100, and 175 μl of 0.2 M Tris-HCl buffer (pH 8.0) was incubated at 4°C overnight with shaking. After incubation, the mixture was centrifuged, and the supernatant/extract was collected at 4°C.

Eighty-five microliters of the SC extract/supernatant (corresponding to the extraction of approximately 0.1 mg of the SC), the specified AMC substrate (0.1 mM final concentration), and the specified amount of LEKTI domain were all mixed at 4°C. Then, the volume was adjusted to 100 μl with 0.01% of phosphate-buffered saline at 4°C. The mixture was incubated at 37°C with shaking for 1–3 hours and the released AMC was measured. For the pNA substrate, a similar procedure as the measurement of SC protease activity was applied. That is, the specified LEKTI domain was added into the same mixture as the SC protease activity assay at 4°C. Then, the reaction mixture with LEKTI domain was incubated at 37°C with shaking for 2–4 hours, and the released pNA was measured. Each inhibition assay per each substrate, with the specified amount of LEKTI domain, was repeated at least four times. However, due to limited sample availability, the individuals tested with each substrate were not always the same.

The residual enzymatic activity (expressed as ratio of released AMC or pNA with LEKTI domain/released AMC or pNA without LEKTI domain) was calculated for each individual. We evaluated the residual activity at 1, 2, and 3 hours time points for AMC. The data at 2 and 3 hours were highly comparable, with a small standard error among samples, while the 1 hour data varied widely (data not shown). Hence, the 3 hours data were adopted for further evaluation. For pNA substrate, the 4 hours data were used, for the same reason.

Detection of LEKTI domains by immunoassay

The SC sample (0.5 mg dry weight) was mixed with 10 μl of *N*,*N*-dimethylformamide, 240 μl of 0.1% Triton X-100, and 175 μl of 0.2 M Tris-HCl buffer (pH 8.0) at 4°C. An amount of LEKTI domain (500 or 600 ng per mg dry weight for LEKTI domains 6–9′, 9–12 and 12–15, or domains 1–6, respectively) was added into the mixture, and the volume was adjusted to 500 μl with 0.01% phosphate-buffered saline at 4°C. The mixture with LEKTI domains was incubated for different time points ranging from 0 minute to 24 hours at 37°C with shaking. At each time point, the mixture was centrifuged, then 25 μl

(corresponding to ∼25 or 30 mg of undigested LEKTI domain) of the supernatant was collected. As primary Abs, 1C11G6 or 1D6G8 mAbs (Raghunath *et al*., 2004) were used for the detection of LEKTI domains 1–6, 6–9′, and 9–12, or 12–15, respectively. Further detailed procedures for western blotting are described elsewhere (Komatsu *et al*., 2005a). For each LEKTI domain, the assay was performed three times using three different individual samples. However, due to a limited amount of samples, the individuals tested with different domains were not always the same.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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