Clinical Expression and New *SPINK5* Splicing Defects in Netherton Syndrome: Unmasking a Frequent Founder Synonymous Mutation and Unconventional Intronic Mutations

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Netherton syndrome (NS) is a severe skin disease caused by loss-of-function mutations in *SPINK5* (serine protease inhibitor Kazal-type 5) encoding the serine protease inhibitor LEKTI (lympho-epithelial Kazal type-related inhibitor). Here, we disclose new *SPINK5* defects in 12 patients, who presented a clinical triad suggestive of NS with variations in inter- and intra-familial disease expression. We identified a new and frequent synonymous mutation c.891C>T (p.Cys297Cys) in exon 11 of the 12 NS patients. This mutation disrupts an exonic splicing enhancer sequence and causes out-of-frame skipping of exon 11. Haplotype analysis indicates that this mutation is a founder mutation in Greece. Two other new deep intronic mutations, c.283-12T>A in intron 4 and c.1820+53G>A in intron 19, induced partial intronic sequence retention. A new nonsense c.2557C>T (p.Arg853X) mutation was also identified. All mutations led to a premature termination codon resulting in no detectable LEKTI on skin sections. Two patients with deep intronic mutations showed residual LEKTI fragments in cultured keratinocytes. These fragments retained some functional activity, and could therefore, together with other determinants, contribute to modulate the disease phenotype. This new founder mutation, the most frequent mutation described in European populations so far, and these unusual intronic mutations, widen the clinical and molecular spectrum of NS and offer new diagnostic perspectives for NS patients.

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INTRODUCTION

Netherton syndrome (NS, OMIM 256500) is one of the most severe inherited skin diseases of children and young adults (Comel, 1949; Netherton, 1958). It is a rare (1 in 200,000 newborns) autosomal-recessive genodermatosis, which classically associates congenital scaly erythroderma, a specific hair shaft defect (*trichorrhexis invaginata*, TI), and constant atopic manifestations (Traupe, 1989; Sybert, 1997; Ong and Harper, 2006). NS newborns often present with extensive exfoliative erythroderma of variable intensity. Erythroderma can persist throughout life in most severe cases or more often evolves into the typical pattern of *ichthyosis linearis circumflexa* (ILC; Comel, 1949; Hausser and Anton-Lamprecht, 1996). Dehydration, infections, and failure to thrive are frequent complications during the neonatal period. NS patients suffer from a broad range of allergic manifestations including atopic dermatitis-like lesions with elevated serum IgE levels, urticaria, asthma, angioedema, and food allergy (Judge *et al.*, 1994; Van Gysel *et al.*, 2001). Pruritus and lichenification are common. The disease can show a fluctuating course throughout childhood and adulthood, contributing to misdiagnosis.

We previously identified *SPINK5* (serine protease inhibitor Kazal-type 5) as the defective gene in NS, encoding the serine protease inhibitor LEKTI (lympho-epithelial

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Abbreviations: ESE, exonic splicing enhancer; LEKTI, lympho-epithelial Kazal type–related inhibitor; NS, Netherton syndrome; SPINK5, serine protease inhibitor Kazal-type 5

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Kazal-type-related inhibitor), which is a secreted multi-domain inhibitor, expressed in the granular layer of the epidermis and in the inner root sheets of hair follicles (Chavanas *et al.*, 2000; Bitoun *et al.*, 2003). Proteolytic fragments of LEKTI efficiently and specifically inhibit epidermal proteases such as kallikrein (KLK)5, KLK7, and KLK14 (Bitoun *et al.*, 2003; Egelrud *et al.*, 2005; Borgono *et al.*, 2007; Deraison *et al.*, 2007; Fortugno *et al.*, 2011). In NS, loss-of-function mutations in *SPINK5* result in unopposed protease activity in the epidermis, leading to premature (corneo)desmosome cleavage, abnormal filaggrin maturation, and proteinase-activated receptor 2 activation (Descargues *et al.*, 2005; Hachem *et al.*, 2006; Briot *et al.*, 2009; Bonnart *et al.*, 2010). This induces severe skin barrier impairment, and production of proallergic and proinflammatory molecules, which have a key role in the development of allergic manifestations in NS (Descargues *et al.*, 2005; Briot *et al.*, 2009; Renner *et al.*, 2009; Bonnart *et al.*, 2010). Loss of LEKTI expression is a diagnostic feature of NS, allowing for rapid and early diagnosis of the disease (Bitoun *et al.*, 2003; Ong *et al.*, 2004).

More than 60 distinct *SPINK5* loss-of-function mutations have been reported (Sprecher *et al.*, 2001; Bitoun *et al.*, 2002a, b; Raghunath *et al.*, 2004; Mizuno *et al.*, 2005; Shimomura *et al.*, 2005; Descargues *et al.*, 2006; Lin *et al.*, 2007; Zhao *et al.*, 2007). Here, we report significant inter- and intra-familial variations in disease expression in NS patients with no detectable LEKTI in the skin. We unmask new and non-classical mutations affecting *SPINK5* pre-mRNA splicing, which were initially not recognized as causative mutations because of their nature and/or position.

RESULTS

Clinical features of NS and *SPINK5* mutations

We report 12 individuals with NS (in the age group of 9 months to 38 years), from 10 non-consanguineous families (Table 1). The classical triad of congenital scaly erythroderma, TI, and eczematous-like rashes was seen in 41% of the patients only (5/12). Specifically, scaly erythroderma was absent at birth in 59% of patients (7/12), TI was observed in 83.3% of patients (10/12), and all patients developed red eczematous-like plaques (Figure 1). Hair abnormalities were clinically noted in 66% (8/12) of the patients. Four patients (1, 5, 6, and 7), who displayed TI on microscopic examination, showed dense, thin but otherwise apparently normal hair with no alopecia at the time of examination (Figure 1). All patients except two (4.1 and 8) developed ILC. Asthma was found in 50% (6/12) of the patients and angioedema in 25% (3/12) of the patients. Food allergy was the most frequent non-cutaneous allergic manifestation, present in 58.3% (7/12) of the patients. High IgE levels were detected in 66.6% (8/12) of the patients (Table 1). Additional findings included dehydration, recurrent infections, moderate-to-severe pruritus, and growth retardation (Supplementary Table S1 online).

The clinical presentation showed differences between patients and varied over time. Both multiplex families (NS3 and NS4) showed intra-familial variability: NS3.1 developed extensive ILC on the trunk, whereas his brother NS3.2 had limited skin lesions and partial alopecia (Figure 1); patient NS4.1 displayed minor eczematous-like lesions with no alopecia, whereas his younger brother NS4.2 had ILC and alopecia sparing the vertex; none of them had detectable TI (Table 1).

SPINK5 direct sequencing identified only one of the two mutations in 10 of these NS patients (3 to 12), and no mutation in patients 1 and 2 (Table 1). A new nonsense c.2557C>T (p.Arg853X) mutation was identified in exon 27 in NS patient 9, and four previously described mutations in patients 3 to 8, and 10 (Table 1). To identify the second or both *SPINK5* mutations (NS1 and NS2), we carefully analyzed genetic variations throughout *SPINK5.* Patients 1 and 2 both displayed a c.891C>T (C297(TGC>TGT)) variation in exon 11, in addition to a c.1820+53G>A (c.IVS19+53G>A) change in intron 19, and a c.283-12T>A (c.IVS4-12T>A) change in intron 4, respectively. The other NS patients of this study were subsequently tested and all displayed the c.891C>T nucleotide change (Table 1).

The recurrent and founder c.891C>T mutation triggers skipping of exon 11 through disruption of a SF2/ASF-binding site in an ESE sequence

We investigated the consequences of the c.891C>T change by reverse transcription (RT)-PCR analysis of mRNA from NS1 keratinocytes (Supplementary Figure S1A online), using primers flanking exon 11, and showed two bands of reduced but similar intensity, one of normal size and a second of smaller size (Figure 2a). Sequencing of the smaller amplimer revealed skipping of the entire exon 11 (Figure 2b), changing the phase and leading to a premature termination codon (TGA) in exon 12 of the mutant *SPINK5* transcript (Supplementary Figure S1B online). We next tested the possibility that this mutation occurred in an exonic splicing motif and scanned the wild-type (c.891C) and mutant (c.891T) sequence using different web-based programs. Exonic splicing enhancer (ESE) finder (Cartegni *et al.*, 2003), automated splice site analyses (Nalla and Rogan, 2005), and human splicing finder (Desmet *et al.*, 2009) software indicated that the c.891T nucleotide indeed completely abolished a SF2/ASF-binding motif, but also predicted the creation of several new potential exonic splicing silencers (Figure 2c), as well as an additional hnRNPA1-binding site at the site or surrounding the mutation (data not shown). These findings strongly suggest that the c.891C>T mutation affects *SPINK5* mRNA splicing through disruption of the consensus ESE sequence and the creation of a new exonic splicing silencer.

Among the 10 families carrying the c.891C>T mutation, 5 were of Greek origin and the others, except for NS1 (The Netherlands), were also from Mediterranean background. Haplotype analysis using seven *SPINK5* microsatellite markers revealed that mutation c.891C>T segregated with the same combination of alleles in all Greek families, and also in the families originating from France (NS3), Kosovo (NS7 and NS8), and Southern Italy (NS9), suggesting a founder effect in the Greek families and raising the possibility of distant Mediterranean ancestors in these families (Figure 2d).

Table 1. Clinical features and *SPINK5* mutations identified in the 12 NS patients in this report

Family	Age (years)[1]	Sex	SE	Diagnostic parameters			LEKTI detection	*SPINK5* mutations[2]					
				ILC	TI	Atopic manifestations		Geographic origin	Mutation	Nucleotide change	Consequence	Location	Reference
1	38	F	–	+	+	E, AO, H-IgE, U, A	Negative	The Netherlands	c.1820+53G>A*	catcgg>catcag	Intronic sequence retention	Intron 19	Present study
									p.Cys297Cys*	**c.891C>T**	**Exon skipping**	**Exon 11**	Present study
2	16 mo	M	+	+	+	E, H-IgE, He	Negative	Greece	c.283-12T>A*	atcatg>atcaag	Intronic sequence retention	Intron 4	Present study
									p.Cys297Cys*	**c.891C>T**	**Exon skipping**	**Exon 11**	Present study
3.1	10	M	–	+	+	E, FA, AO, H-IgE, A	Negative	France	p.Arg371X	c.1111C>T	Nonsense (PTC)	Exon 13	Bitoun *et al.* (2002a, b)
									p.Cys297Cys*	**c.891C>T**	**Exon skipping**	**Exon 11**	Present study
3.2	2	M	–	+	+	E, FA, A	Negative	France	p.Arg371X	c.1111C>T	Nonsense (PTC)	Exon 13	Bitoun *et al.* (2002a, b)
									p.Cys297Cys*	**c.891C>T**	**Exon skipping**	**Exon 11**	Present study
4.1	5	M	–	–	–	E, FA, H-IgE	Negative	Greece	p.Ala80GlyfsX18	c.238dup1	Frameshift (PTC+18 aa)	Exon 4	Chavanas *et al.* (2000)
									p.Cys297Cys*	**c.891C>T**	**Exon skipping**	**Exon 11**	Present study
4.2	9 mo	M	–	+	tn	E, FA, H-IgE	Negative	Greece	p.Ala80GlyfsX18	c.238dup1	Frameshift (PTC+18 aa)	Exon 4	Chavanas *et al.* (2000)
									p.Cys297Cys*	**c.891C>T**	**Exon skipping**	**Exon 11**	Present study
5	2	F	–	+	+	E, FA	Negative	Greece	c.410+1G>A	TGCgt>TGCat	Altered splicing	Intron 5	Renner *et al.* (2009)
									p.Cys297Cys*	**c.891C>T**	**Exon skipping**	**Exon 11**	Present study
6	25	M	+	+	+	E, H-IgE, A	Negative	Greece	ND				Present study
									p.Cys297Cys*	**c.891C>T**	**Exon skipping**	**Exon 11**	Present study
7	26	M	–	+	+	E	Negative	Kosovo	p.Phe51PhefsX6	c.153del1	Frameshift (PTC+6 aa)	Exon 3	Chavanas *et al.* (2000)
									p.Cys297Cys*	**c.891C>T**	**Exon skipping**	**Exon 11**	Present study
8	10 mo	M	+	–	+	E, FA, H-IgE, He	Negative	Kosovo	p.Phe51PhefsX6	c.153del1	Frameshift (PTC+6 aa)	Exon 3	Chavanas *et al.* (2000)
									p.Cys297Cys*	**c.891C>T**	**Exon skipping**	**Exon 11**	Present study
9	13	M	+	+	+	E, H-IgE, He, A	ND	Italy	p.Arg853X*	c.2557C>T	Nonsense (PTC)	Exon 27	Present study
									p.Cys297Cys*	**c.891C>T**	**Exon skipping**	**Exon 11**	Present study
10	12	M	+	+	+	E, FA, AO, H-IgE, U, A	ND	Greece	p.Ala80GlyfsX18	c.238dup1	Frameshift (PTC+18 aa)	Exon 4	Chavanas *et al.* (2000)
									p.Cys297Cys*	**c.891C>T**	**Exon skipping**	**Exon 11**	Present study

Abbrevations: A, asthma; AO, angioedema; E, eczematous-like rashes; cDNA, complementary DNA; F, female; FA, food allergy; H-IgE, hyper IgE; He, hypereosinophilia; ILC, *ichthyosis linearis circumflexa*; M, male; ND, not determined; NS, Netherton syndrome; PTC, premature termination codon; SE, scaly erythroderma at birth; TI, trichorrhexis invaginata; tn, trichorrhexis nodosa; U, urticaria.
[1]The age, expressed in years or in months (mo), corresponds to the patient's age when referred to our center.
[2]Amino acid and nucleotide numbering refers to the cDNA sequence, with nucleotide position 1 assigned to the first nucleotide of the ATG initiation codon in exon 1. Bases in exons are noted by upper-case letters and bases in introns by lower-case letters. The Cys297Cys mutation shared by all patients in this study is indicated in bold. (PTC + *n* aa) indicates that a PTC is created *n* amino acids downstream of the mutation. Asterisks (*) refer to new *SPINK5* mutations.

c.283-12T>A and c.1820+53G>A mutations cause partial intronic retention in *SPINK5* transcripts

We first studied NS2 who is a compound heterozygote for mutations c.283-12T>A in intron 4 and c.891C>T in exon 11 (Supplementary Figure S1C online). RT-PCR analysis, using FAM-labeled primers surrounding intron 4, showed a 400-bp amplimer corresponding to the normal *SPINK5* band in NHK, whereas a larger additional species was present in lower amounts in NSK2 (Figure 2e). Sequence analysis of this larger amplimer identified transcripts carrying the inclusion of the last 10 nucleotides of intron 4 (Figure 2f), showing that c.283-12T>A impairs *SPINK5* pre-mRNA splicing and leads to the retention of an intronic sequence (Supplementary Figure S1D online). In a similar manner, NS1, who is a compound heterozygote for c.1820+53G>A mutation in intron 19 and c.891C>T in exon 11, showed on RT-PCR analysis a wild-type band and a larger *SPINK5* transcript of reduced intensity (Figure 2g). Sequencing of the larger band showed retention of the first 54 nucleotides of intron 19 (Figure 2h), confirming that c.1820+53G>A mutation alters

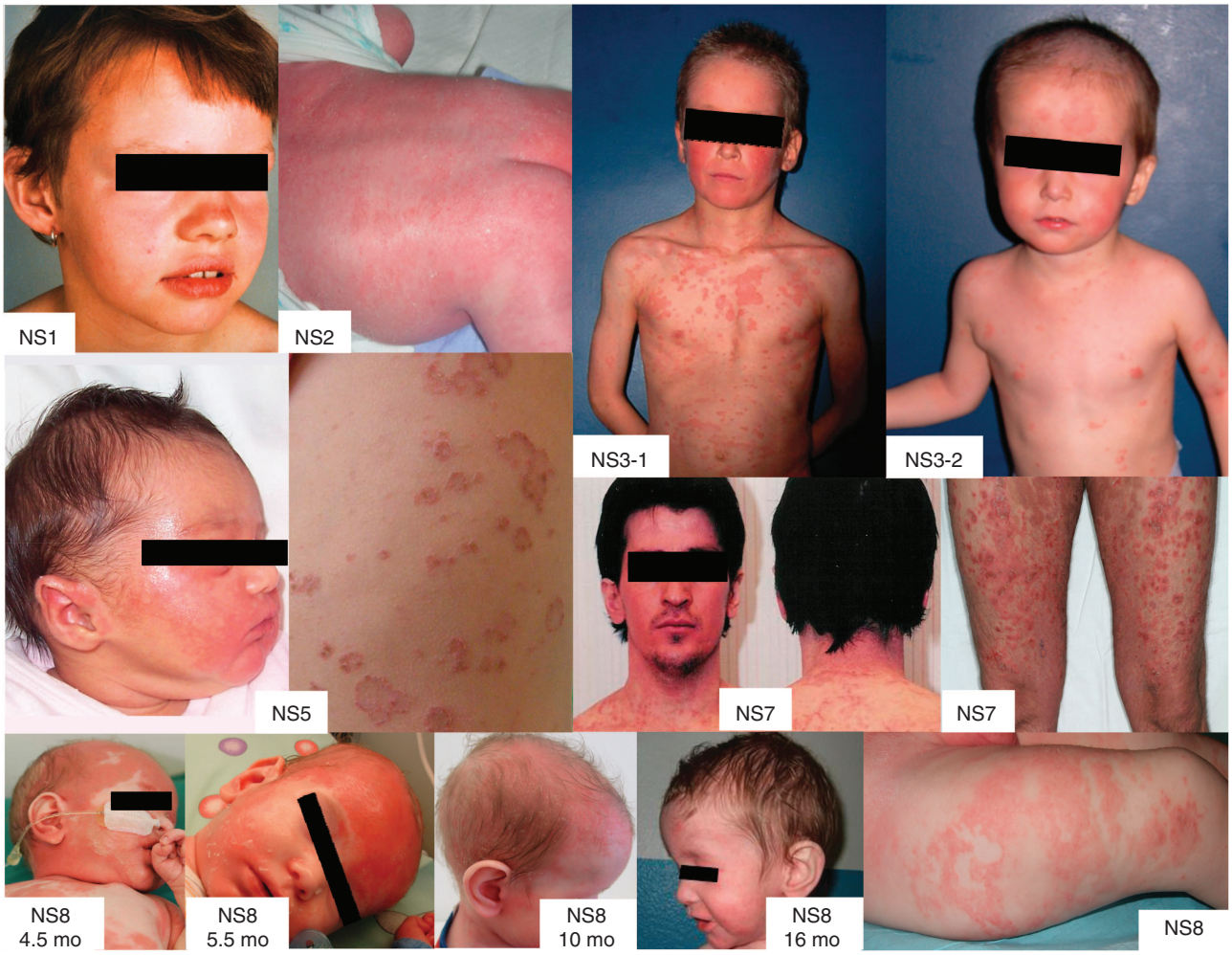

Figure 1. Clinical variability in patients with Netherton syndrome. NS1 presented with thin and dense hair despite the presence of TI. NS2 had marked scaly erythroderma at birth, which quickly evolved into a moderate diffuse eczematous-like eruption. NS3.1 had spiky hair, red cheeks, and extensive *ichthyosis linearis circumflexa* (ILC) on his chest. NS3.2, his younger affected brother, had partial alopecia and only a few eczematous-like plaques. NS5 had long, thin, slow-growing and brittle hair with TI, eczematous-like lesions of her cheeks, and developed ILC on her trunk and on her cheeks. NS7 had dense, normal-looking hair with TI. He developed extensive and very inflammatory lesions of ILC, made of confluent plaques prominent on the trunk and the thighs, with scaly, double-edged and crusty lesions. NS8 presented with neonatal exfoliative erythroderma, which evolved into sharply delineated and extensive red plaques on the entire body at 4.5 and 5.5 months (mo). Seborrheic dermatitis-like lesions of the scalp were noted at 10 months and resolved at 16 months. His hair was thin, sparse, and long, with TI present on dermoscopy. He developed inflammatory, urticaria-like annular plaques of the thighs with no definite ILC.

correct *SPINK5* pre-mRNA splicing (Supplementary Figure S1F online).

NS patients 1 and 2 exhibit residual LEKTI expression in cultured keratinocytes

As normally spliced *SPINK5* transcripts were also detected in NS1 and NS2 cultured keratinocytes, we investigated the consequences of these new splicing events on LEKTI production. We first confirmed that LEKTI immunostaining was negative on skin cross-sections in NS1 and NS2 compared with healthy control skin (Figure 3a). We next performed western blotting on the supernatant of keratinocytes using LEKTI antibodies raised against N terminus (D1–D6) or C terminus domains (D13–D15) of the protein. An NS patient homozygous for the c.2471_2475delAAGAGinsT *SPINK5* mutation, leading to a premature termination codon without impairing splicing (Goujon *et al.*, 2010), showed complete absence of LEKTI (Figure 3b). In contrast, NSK1 and NSK2 displayed weak expression of LEKTI fragments compared with NHK (Figure 3b). *In situ* zymography using casein conjugated to FITC as a substrate showed that NS1 and NS2 had an increased proteolytic activity compared with normal epidermis, but to a lesser extent than in the skin of a LEKTI-null NS patient (Figure 3c). Analysis by quantitative RT-PCR of the expression levels of downstream targets of the KLK5–proteinase-activated receptor 2 pathway revealed that *TSLP*, *TNF-α*, and *IL-8* induction in NSK1 and NSK2 was significantly reduced as compared with NSK (Figure 3d). Taken together, these results provide evidence that NS1 and NS2 patients produce residual levels of LEKTI domains that retain functional activities, although no LEKTI is detectable on skin sections.

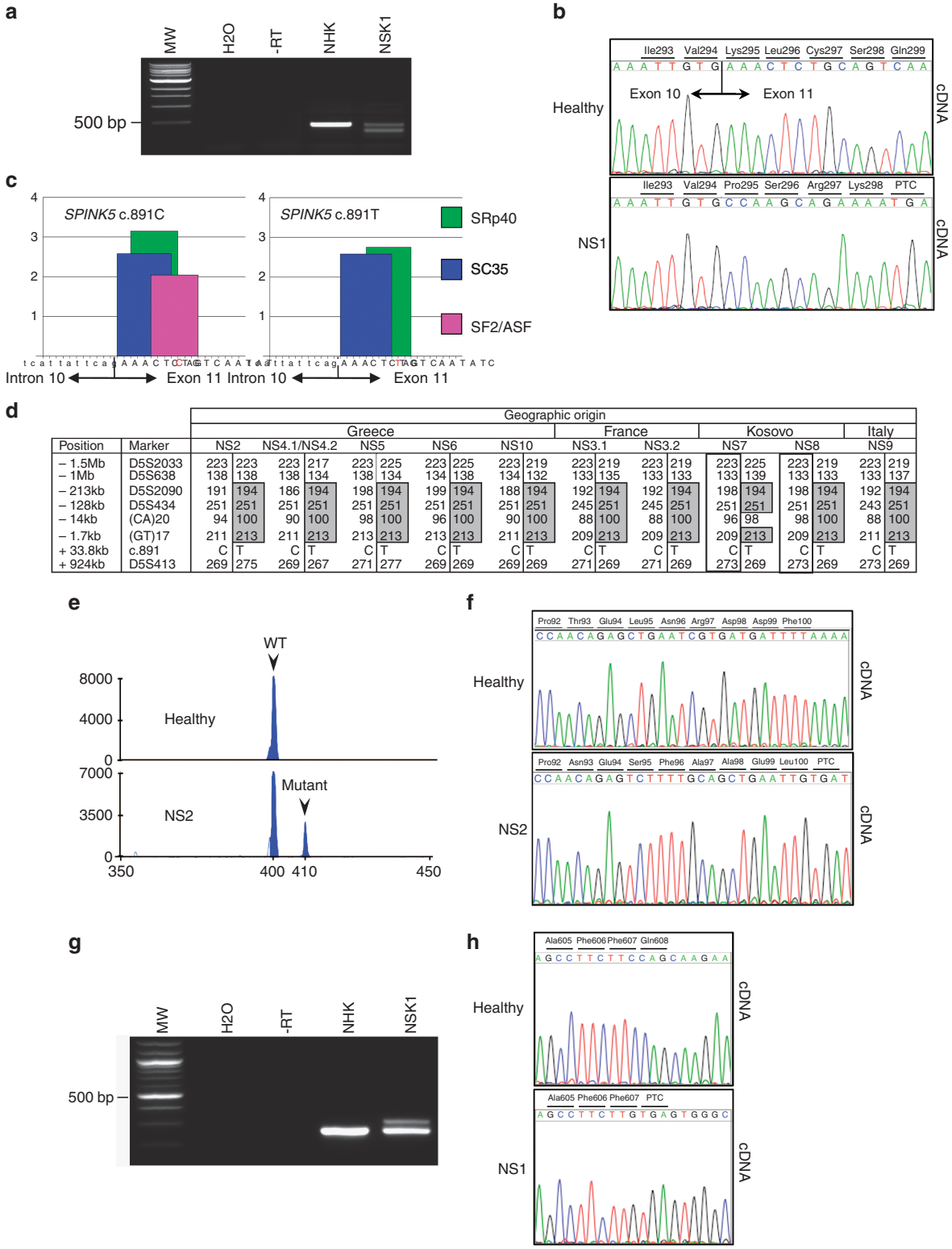

Figure 2. Consequences of c.891C>T, c.283-12T>A, and c.1820+53G>A mutations on *SPINK5* pre-mRNA splicing. (**a**) Reverse transcription (RT)-PCR analysis of mRNA from control and NS1 keratinocytes (NHK and NSK1, respectively). (**b**) Sequence analysis of RT-PCR amplimers from NSK1. (**c**) Analysis of wild-type (WT; c.891C) and mutant (c.891T) *SPINK5* intron 10-exon 11 junction with exonic splicing enhancer finder website (Cartegni *et al.*, 2003). Note that T is shown instead of U for consistency with the designation of the mutation. (**d**) Haplotype analysis of families NS2 to NS10. The *SPINK5* haplotype carrying the c.891C>T mutation is shaded in gray. (**e** and **g**) RT-PCR analysis performed on total RNA from NS2 (NSK2) and NS1 (NSK1) keratinocytes, separated by capillary electrophoresis for c.283-12T>A, and by agarose gel for c.1820+53G>A mutation. (**f** and **h**) Sequence analysis of *SPINK5* mutant transcripts in NS2 and NS1. Premature termination codon (TGA) codons are underlined. CDNA, complementary DNA; MW, molecular weight; NS, Netherton syndrome.

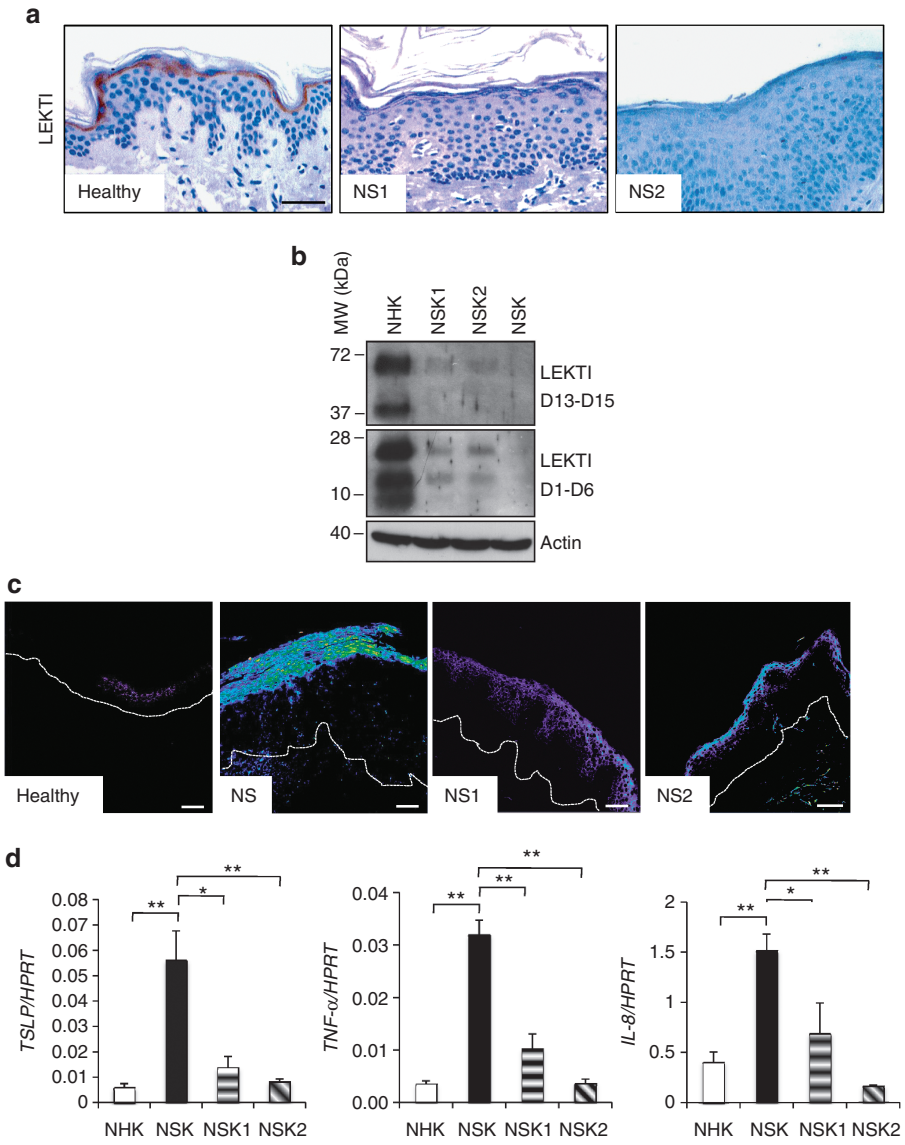

Figure 3. LEKTI expression in skin and cultured keratinocytes from NS patients 1 and 2. (**a**) Immunohistochemistry experiment performed on NS1, NS2, and healthy control skin sections. Bar = 50 µm. (**b**) Western blotting of keratinocyte supernatant from NS1, NS2 (NSK1 and NSK2), healthy control (NHK), and a Netherton syndrome (NS) patient with a homozygous *SPINK5*-null mutation (NSK), using LEKTI antibodies raised against D1–D6 and D13–D15 domains. (**c**) *In situ* zymography analysis. Protease activity is represented by a pseudo-color gradient, which represents the intensity values of fluorescence. The values of signal range from 0 (dark) to 255 (white). The dotted line represents the basal membrane of the epidermis. Bar = 50 µm. (**d**) Quantitative RT-PCR analysis on cultured primary keratinocytes from NS patients 1 (NSK1) and 2 (NSK2), three healthy individuals (NHK), and two NS patients with LEKTI-null expression (NSK). *, $P<0.05$; **, $P<0,01$. The results are the mean of two independent experiments. HPRT, hypoxanthine-guanine phosphoribosyl transferase; MW, molecular weight; TNF, tumor necrosis factor; TSLP, thymic stromal lymphopoietin.

DISCUSSION

Netherton syndrome is caused by *SPINK5* mutations, for which, to date, at least 60 different loss-of-function mutations have been reported (http://www.hgmd.cf.ac.uk). Among these, almost 29% alter *SPINK5* transcript splicing by disrupting consensus acceptor or donor splice sites. Here, we describe new unusual *SPINK5* causative mutations, which could have been misdiagnosed as silent polymorphisms because of their nature and/or position. We also show evidence for inter- and intra-familial variability in disease expression despite negative LEKTI in the skin.

Clinical presentation differed from the classical congenital scaly erythroderma in seven patients who showed no exfoliative erythroderma at birth, but showed extensive redness of the skin or eczematous-like plaques instead. Eczematous-like plaques varied in size, aspect, and location, but developed in all patients studied. Hair abnormalities also showed a wide range of variations between patients and during the course of the disease, with several patients having a history of transitory hair loss in the neonatal period or during infancy followed by slow hair re-growth. Two patients (4.1 and 4.2) showed no detectable TI at the age of 5 years

and 9 months, respectively, which delayed the diagnosis of NS in the absence of typical skin manifestations. ILC, a highly specific feature of NS, was seen in every patient except two (4.1 and 8), but its clinical aspect was highly variable between patients, ranging from minor and localized scaly lesions to extensive, inflammatory, and ichthyotic plaques (Figure 1). Pruritus was a constant, often disabling and aggravating feature. The disease showed a fluctuating course, adding considerable heterogeneity in patient clinical presentation.

We and others previously reported a correlation between epidermal protease activity, LEKTI detection, and disease severity in rare NS patients with residual LEKTI expression in the skin (Descargues *et al.*, 2006; Hachem *et al.*, 2006). In these unusual patients, reduced expression of LEKTI fragments, retaining some functional activity, is thought to attenuate disease severity. In this study, LEKTI immunostaining was negative in all patients, although two NS patients (NS1 and NS2) displayed residual LEKTI expression in cultured keratinocytes. These patients carried the deep intronic c.1820+53G>A and c.283-12T>A mutations in introns 19 and 4, which induced partial intron retention due to the activation of cryptic splice sites. Transcript analysis revealed that abnormally spliced forms containing intron 19 or 4 sequences were less abundant than normally spliced *SPINK5* transcripts, suggesting that mutations c.1820+53G>A and c.283-12T>A did not completely abolish normal splicing and/or may lead to nonsense-mediated mRNA decay. In fact, we showed that residual LEKTI fragments secreted by NS1 and NS2 keratinocytes retained some functional activity as shown by *in situ* zymography and cytokine expression profiles. This is consistent with these mutations being ''leaky'' mutations, allowing for the production of some level of normal protein, which is not sufficient to prevent the development of the disease. It is possible that residual LEKTI may attenuate the phenotype in patients NS1 and NS2, who did not show a severe form of NS. However, additional genetic and/or environmental factors are likely to affect disease expression, as shown by inter- and intra-familial variations seen in the other families. Finally, the clinical spectrum of NS appears to be much wider than classically described, and atypical forms are likely to be further diagnosed by LEKTI and *SPINK5* testing.

All NS patients included in our study displayed the c.891C>T sequence variation in exon 11 on one *SPINK5* allele. This nucleotide change was translationally silent but changed the balance between ESE and exonic splicing silencer sequences, inducing out-of-frame skipping of exon 11 and a premature termination codon in exon 12. This altered splicing mechanism is the first described splicing mechanism in NS involving an ESE sequence. The mutant transcripts lacking exon 11 were expressed at a similar level than the transcripts containing exon 11, suggesting that mutation c.891C>T was fully penetrant. In fact, c.891C>T is the most common *SPINK5* mutation described so far in the European population (10 out of 105 families (i.e., 9.5%) from our international NS cohort). Although additional cases need to be studied, our findings suggest that the high frequency of this mutation may result from a founder effect in families originating from Mediterranean countries.

Mutations c.1820+53G>A and c.283-12T>A, together with the previously described c.1431-12G>A mutation (Raghunath *et al.*, 2004), are the first *SPINK5* mutations to our knowledge to be located so distant from a consensus splicing site. This may have delayed the recognition of this sequence variation as being a disease-causing mutation before.

In conclusion, our results widen the clinical and molecular spectrum of NS, revealing considerable clinical variations in the disease phenotype, and open new diagnostic perspectives to identify causative mutations in NS patients with uncharacterized *SPINK5* allele.

MATERIALS AND METHODS

Patients and clinical material

Our study included 12 patients with a non-ambiguous diagnosis of NS. The diagnosis was based on clinical criteria and was confirmed by negative LEKTI immunostaining (Table 1). Written informed consent was obtained from all NS patients (or parents) from whom blood was collected. This work was approved by the Local Ethical Committee (DEC 04005) and conducted according to the Declaration of Helsinki Principles.

Primary keratinocyte culture and western blotting

Healthy human and NS primary keratinocytes were isolated and cultured as previously described (Bitoun *et al.*, 2003). Secreted proteins of cultured keratinocytes were analyzed with LEKTI antibodies raised against D1–D6 or D13–D15 domains (Deraison *et al.*, 2007).

Reverse transcription-PCR analysis and complementary DNA sequencing

RNA was extracted from keratinocytes, and first-strand complementary DNA synthesis was carried out using superscript III reverse transcriptase (Invitrogen, Carlsbad, CA; Briot *et al.*, 2009). Primers used are described in Supplementary Table S2 online. For c.283-12T>A, PCR products were analyzed by capillary electrophoresis using FAM-labeled primer. The complementary DNA amplimers were cloned into PGEM-T vector and sequenced using the T7 and SP6 primers.

Genotype analysis

Polymorphic microsatellite markers D5S2033, D5S638, D5S2090, D5S434, $(CA)_{20}$ (AJ304417) and $(GT)_{17}$ (AJ304416), and D5S413, surrounding *SPINK5* ATG, were used (Supplementary Table S2 online).

Prediction of pre-mRNA splicing sites and exonic splicing sequences

Genomic environment of the mutation and exonic splicing sequences were analyzed with Automated Splice Site Analysis (https://splice.cmh.edu/; Nalla and Rogan, 2005), Human Splicing Factor (http://www.umd.be/HSF/; Desmet *et al.*, 2009), and ESE Finder (http://rulai.cshl.edu/tools/ESE/; Cartegni *et al.*, 2003).

Immunostaining and *in situ* zymography

Skin cross-sections were stained with LEKTI mAb, which recognizes fragment D1–D6, as previously described (Bitoun *et al.*, 2003). *In situ* zymography was performed as reported earlier (Bonnart *et al.*, 2010).

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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