SPINK5 and Netherton Syndrome: Novel Mutations, Demonstration of Missing LEKTI, and Differential Expression of Transglutaminases

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Netherton syndrome (NTS) is an autosomal recessive congenital ichthyosis featuring chronic inflammation of the skin, hair anomalies, epidermal hyperplasia with an impaired epidermal barrier function, failure to thrive and atopic manifestations. The disease is caused by mutations in the *SPINK5* gene encoding the serine proteinase inhibitor lympho-epithelial Kazal-type inhibitor (LEKTI). Sequence analyses of *SPINK5* in seven NTS patients from five different families allowed us to identify two known and three novel mutations all creating premature termination codons. We developed a monoclonal antibody giving a strong signal for LEKTI in the stratum granulosum of normal skin and demonstrated absence of the protein in NTS epidermis. Immunoblot analysis revealed presence of full length LEKTI and of LEKTI cleavage fragments in normal hair roots, whereas in NTS hair roots LEKTI and its cleavage products were completely missing. Transglutaminase1 activity was present throughout almost the entire suprabasal epidermis in NTS, whereas in normal skin it is restricted to the stratum granulosum. In contrast, immunostaining for transglutaminase3 was absent or faint. Moreover, comparable with the altered pattern in psoriatic skin the epidermis in NTS strongly expressed the serine proteinase inhibitor SKALP/elafin and the antimicrobial protein human β-defensin 2. These studies demonstrate LEKTI deficiency in the epidermis and in hair roots at the protein level and an aberrant expression of other proteins, especially transglutaminase1 and 3, which may account for the impaired epidermal barrier in NTS.

Key words: elafin/epidermal barrier/genetics/human β-defensin 2/mutation/Netherton syndrome/serine proteinases/skin/transglutaminase
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Netherton syndrome (NTS), a rare autosomal recessive genodermatosis, combines chronic and severe inflammation of the skin with hair shaft anomalies and atopic features. The degree of skin involvement varies, some patients show severe congenital ichthyosiform erythroderma (CIE), whereas most present with ichthyosis linearis circumflexa (ILC)—polycyclic migrating plaques with characteristic double-edged scales (Traupe, 1989). Often patients display at birth CIE, which in some of them improves and evolves into ILC. The finding of trichorrhexis invaginata ("Bamboo hairs") is diagnostic (Sybert, 1997). These and other hair anomalies (pili torti, trichorrhexis nodosa), however, are not always present and even repeated hair samples may be negative. Other conditions often associated with NTS are delayed growth, recurrent infections, urticaria, angioedema, failure to thrive, aminoaciduria, and in some instances slight mental retardation (Greene and Muller, 1985).

Abbreviations: AD, atopic dermatitis; hBD-2, human β-defensin 2; LEKTI, lympho-epithelial Kazal-type inhibitor; NTS, Netherton syndrome; PTC, premature termination codon

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Because of the variable expression of skin signs and delayed onset of characteristic hair abnormalities the diagnosis of NTS is often delayed. Frequently, the concomitant atopic diathesis with elevated IgE levels, asthma, and hay fever leads to misdiagnosis of severe atopic dermatitis (AD). Other cases are initially misclassified as Leiner disease—severe seborrhoic generalized dermatitis (Hausser and Anton-Lamprecht, 1996). Histopathology does not allow to distinguish NTS from disorders such as psoriasis, because both conditions display focal parakeratosis with psoriasiform epidermal hyperplasia, focal absence of the granular layer, intraepidermal neutrophil accumulation and at the functional level an impaired epidermal barrier (Hausser and Anton-Lamprecht, 1996). The list of differential diagnoses also includes erythrodermic psoriasis, erythrokeratoderma variabilis, and acrodermatitis enteropathica because of the perioral and perianal accentuation of erythema and scaling (Greene and Muller, 1985).

Recently, the diagnosis of NTS by molecular genetic analysis became possible due to the mapping of the respective gene locus on chromosome 5q32 (Chavanas *et al*, 2000a) and subsequent identification of mutations in the *SPINK5* gene (Chavanas *et al*, 2000a, b; Sprecher *et al*,

2001; Komatsu *et al*, 2002; Bitoun *et al*, 2002a, b). *SPINK5* encodes a serine protease inhibitor called LEKTI (lympho-epithelial Kazal-type inhibitor) (Mägert *et al*, 1999), a cysteine-rich protein consisting of 15 Kazal-type-like inhibitory domains (Mägert *et al*, 2002a). Recombinant human LEKTI inhibits *in vitro* a battery of serine proteases including plasmin, trypsin, subtilisin A, cathepsin G, and elastase (Mägert *et al*, 2002b; Mitsudo *et al*, 2003), but the exact assignment of specific inhibitory activity to individual domains remains to be determined. LEKTI mRNA is present in epidermis, pilosebaceous units (Komatsu *et al*, 2002) and other tissue, but its precise biological function *in vivo* remains unclear. We developed a novel monoclonal antibody to assess the effects of *SPINK5* mutations in NTS patients on the expression of epidermal LEKTI. In addition, we wondered how LEKTI mutations disturb keratinization and in view of the epidermal hyperplasia studied the behavior of transglutaminases which play a key role for cross-linking of the cornified envelope and thus for epidermal differentiation. In view of the susceptibility to severe infections we analyzed the expression of the antimicrobial peptides human β-defensin 2 (hBD-2) and SKALP/elafin. Here, we report two known and three novel *SPINK5* mutations, all of them resulting in epidermal LEKTI deficiency. In addition, we found a differential and remarkably discordant expression of transglutaminases1 and 3 in NTS, which may explain the disturbed epidermal barrier, and we observed the unexpectedly strong presence of hBD-2 and elafin in NTS.

Results

Mutation analyses Mutation analysis by direct sequencing revealed three novel and two published mutations (Table I). The novel mutations are an insertion of T in patient 1, a two-bp deletion in family H (patient 3.1 and 3.2; Fig 1) and a recurrent acceptor splice site mutation in patient 5, and siblings 2.1/2.2, who are homozygous for this putative mutation (Fig 2). Patient 1 is homozygous for 715insT, which leads to a frameshift resulting into a premature termination codon (PTC) after six codons. The two-bp deletion 398delTG in exon 5 immediately results into a PTC. This mutation in family H was transmitted to both patients 3.1 and 3.2 by their father. The novel mutation 1432–13 G>A affects intron 15 near the intron–exon boundary to exon 16. A detailed analysis of this mutation using the Splice Site Prediction Programm by Neural Network (http://www.fruitfly.org/seq_tools/splice.html and http://www.cbs.dtu.dk/services/NetGene2/) showed that the wild-type sequence cggtttcttaaagTCAA (splice site underlined) was changed to cagtttcttaaagTCAA. This predicts an additional very strong acceptor splice site 10 bp upstream of the wild-type aAG acceptor splice site used for exon 16 (Fig 2). This mutation was found in the heterozygous and homozygous state in two independent patients of different ethnic origins (Austria, Bosnia). In the family from Bosnia this mutation was transmitted by both (non-consanguineous) parents. It was not found in 80 normal chromosomes. All novel mutations predict a significant truncation of the LEKTI polypeptide.

Table I. Netherton syndrome, mutations in *SPINK5*

Patient	Mutations	Location	Nucleotide change	Predicted consequence	Mutation published
1[a]	715insT homozyg.	Exon 9 within domain 4	C(T)6G → C(T)7G	Frameshift (PTC + 6)	*Novel*
2.1	1432-13 G > A homozyg.	Exon 16 after domain 7	cggtttcttaaagTCAA → cagtttcttaaagTCAA	putative splice site upstream	*Novel*
2.2	1432-13 G > A homozyg.	Exon 16 after domain 7	cggtttcttaaagTCAA → cagtttcttaaagTCAA	putative splice site upstream	*Novel*
3.1	398delTG	Exon 5 within domain 2	TGTGCT → TGCTGA	Frameshift (PTC + 1)	*Novel*
	R790X	Exon 25 within domain 12	ACTCGA → ACTTGA 2368	PTC	Chavanas *et al* (2000b)
3.2	398delTG	Exon 5 within domain 2	TGTGCT → TGCTGA	(PTC + 1)	*Novel*
	R790X	Exon 25 with domain 12	ACTCGA → ACTTGA 2368	PTC	Chavanas *et al* (2000b)
4[a]	2468insA homozyg.	exon 26 end of domain 12	G(A)10G → G(A)11G	Frameshift (PTC + 4)	Chavanas *et al*, 2000b
5	1432-13 G>A	Exon 16 after domain 7	cggtttcttaaagTCAA → cagtttcttaaagTCAA	putative splice site upstream	*Novel*
	2468insA	Exon 26 end of domain 12	T(A)10G → T(A)11G	Frameshift (PTC + 4)	Chavanas *et al* (2000b)

[a]consanguineous parents: first cousins

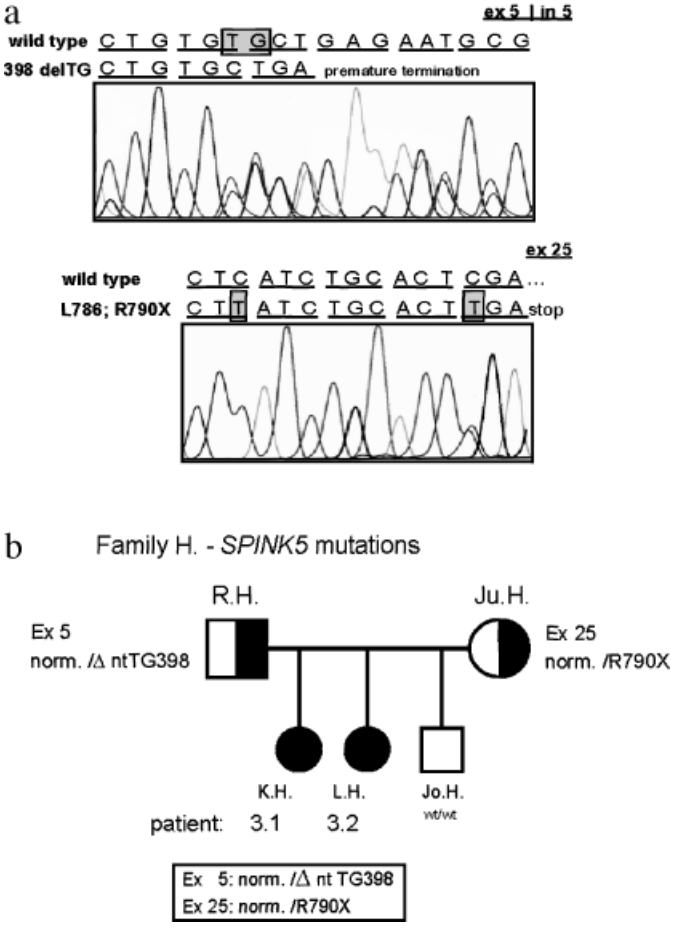

Figure 1
(*a*) Direct sequencing results of the *SPINK5* gene obtained in patient 2.1. The patient is heterozygous for a novel two bp deletion in exon 5 that bridges two codons. This results immediately in a premature termination codon (PTC). Exon 25 shows two changes: the heterozygous and conservative polymorphism L786, and a missense mutation leading to a PTC. This mutation has been reported earlier in other NTS patients (Chavanas *et al*, 2000b). (*b*) Representative pedigree of NTS-family H. The novel 398delTG was inherited from the father, R790X from the mother. The unaffected brother Jo.H. harbored neither mutation and was homozygous for the wild-type.

LEKTI antibody From one fusion, 24 positive hybridoma clones were selected and subcloned by three to four rounds of limiting dilution. Eight surviving subclones were isotyped and characterized by immunoblotting or immunocytochemistry. Anti-LEKTI (1D6G8, 4G12B1, 2A3A12, 1C11G6, 2A7C3, and 3B12F6) belonged to the IgG_1 subclass. The remaining MAbs 3E5H4 and 2A7C4 were of IgG_2 subclass. Because of its robust immunoreaction in immunoblotting 1C11G6 was subsequently used as the detecting antibody in immunohistochemistry. To identify the LEKTI domains recognized by 1C11G6, we performed a western blot analysis on purified LEKTI domains 6-9 or Sf9 cell lysates containing rLEKTI domains 1–6, 9–12, or 13–15 (Fig 3). 1C11G6 reacted strongly with precursor LEKTI, LEKTI domains 1–6, 6–9, 9–12, and weakly with 13–15. These data suggested recognition of a repetitive or recurring epitope that is present at least on domains 6–9.

LEKTI immunoblot analysis Detection of LEKTI in hair roots by immunoblot analysis showed a strong signal in healthy individuals ($n = 5$) visualizing the full length protein at 140 kDa. At least three further LEKTI fragments could be seen at approximately 110, 90, and 60 kDa, respectively (Fig 4; *lanes 1* and *2*). In NTS patients (patient 2.1; 2.2; 5 and 1) neither the full length form of LEKTI nor the cleaved products were present (Fig 4; *lanes 3–6*) demonstrating absence of full length protein and of cleaved LEKTI products in NTS hair roots.

Histochemical transglutaminase activity Skins of normal adults showed pericellular linear transglutaminase activity confined to the uppermost two or three nucleated cell layers (Fig 5*a*). In NTS, however, the activity was enhanced in terms of intensity of fluorescence after visualization of incorporated substrate and there was a very broad zone of enzymatic activity including most of the suprabasal layers (Fig 5*b–f*).

Immunohistochemistry Immunological detection of LEKTI in normal skin (Fig 6*a–f*) and in skin from psoriasis vulgaris patients (Fig 6*g*) was confined to the granular layer giving a predominantly cytoplasmic reaction occasionally mixed with a pericellular signal. Hair follicles stained positive (Fig 6*f*). Immunhistochemical double staining of involucrin and LEKTI in normal hair follicles revealed strong presence of LEKTI in the inner root sheath (Fig 7*a–d*). All NTS patients showed a striking absence of LEKTI immunoreactivity in the epidermis (Fig 6*h–l*).

Transglutaminase1 protein (as detected by immunohistochemistry) showed a pericellular distribution of the uppermost two to three nucleated epidermal cell layers and no immunoreactivity in the cornified layer in normal skin. In contrast NTS skin consistently showed a grossly enlarged distribution zone including most of the suprabasal cell layers. The histochemical localization of transglutaminase1 activity at pH 7.4 in normal and NTS skin was highly comparable with the respective distribution of the protein (not shown).

Immunoreactive transglutaminase3 in normal skin appeared as a blurred band along the border of granular and cornified layer in normal skin (Fig 8*a*). Only the uppermost nucleated layer was involved showing a mixed pericellular and cytoplasmic staining. In contrast to transglutaminase1, transglutaminase3 was also present in the cornified layer occupying the lower third of the stratum corneum. NTS skin, however, showed very faint or absent transglutaminase3 immunoreactivity (Fig 8*c–h*). As an exception, patient 3.1 (Fig 8*h*), the clinically less affected sister of patient 3.2, showed a transglutaminase3 expression comparable with that of normal skin. Elafin was absent in normal epidermis (Fig 9*a*). In contrast, psoriatic and NTS skin (Fig 9*a–f*) showed a clear pericellular linear expression of the uppermost three to five nucleated keratinocyte layers below the cornified layer. Again, patient 3.1 (Fig 9*f*) showed elafin absence comparable with normal skin. With hBD-2, no immunostaining was achieved in normal skins (Fig 10*a*). In contrast, psoriatic skins showed multilamellar expression strictly confined to the cornified layer (Fig 10*b*). NTS skins also expressed hBD-2 in the cornified layer, albeit less intense than seen with psoriatic skins (Fig 10*c–e*). Again, as

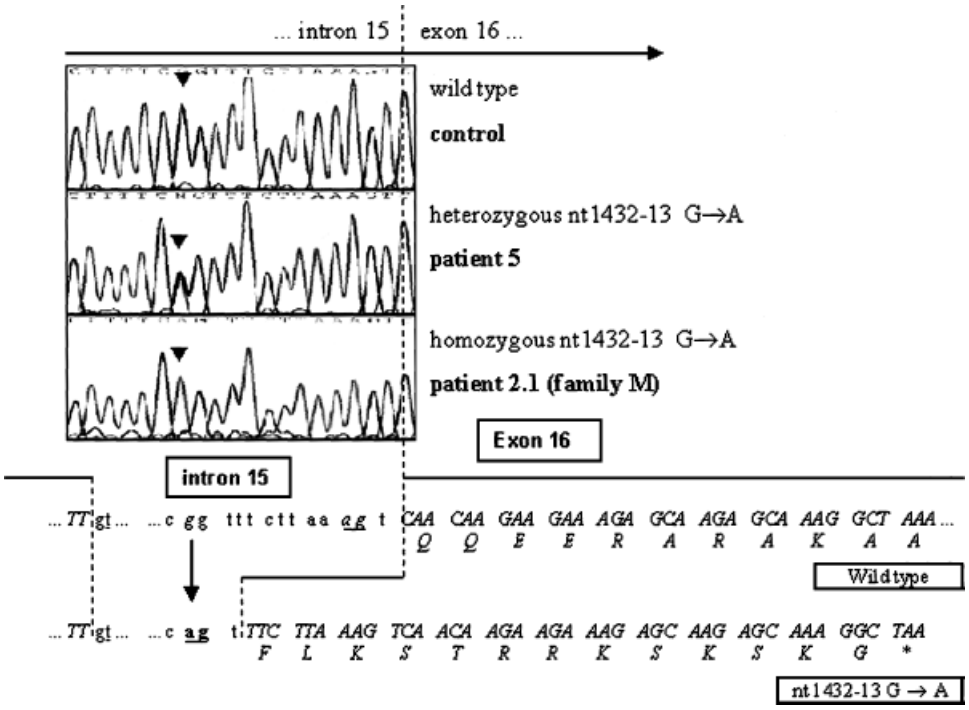

Figure 2
Direct sequencing results of the *SPINK5* gene obtained in patient 5, 2.1 and 2.2. The novel mutation 1432–13 G>A in intron 15 is heterozygous in patient 5 and homozygous in patient 2.1 and his sister 2.2. Schematic representation of the proposed effect of the novel putative mutation (below). The base exchange creates a strong additional splice site upstream of the proper/obligatory correct splice site. Alternative utilization of this novel splice site would lead to a 3'extension of exon 16 and a frameshift resulting in an premature termination within exon 16.

an exception patient 3.1 (Fig 10*f*), showed only very faint expression of hBD-2.

Other differentiation markers Involucrin in normal skin was restricted to the granular layer with a granular pericellular staining and interspersed perinuclear patches (not shown). Psoriatic skins expressed involucrin in up to six cell layers below the cornified layer in a granular pericellular fashion. NTS skins showed a mixture of both patterns (not shown). Annexins I and II showed a patchy pericellular immunostaining of basal and some suprabasal keratinocytes (not shown). In contrast, psoriatic skin showed a pericellular expression of almost all suprabasal cell layers. NTS patients showed a mixture between normal and psoriatic skin (not shown). No significant difference was noted between the siblings 3.1 and 3.2 with any of the above markers (not shown).

Discussion

We have identified two published (Chavanas *et al*, 2000b) and three novel *SPINK5* mutations in NTS patients. Two novel mutations 398delTG (exon 5) and 715insT (exon 9) are frameshift mutations, which immediately or after six codons, respectively, cause a PTC. The PTC in exon 5 predictably truncates the LEKTI polypeptide in the second of 15 inhibitor domains and represents an equivalent to a functional null allele. 715insT in exon 9 predictably truncates the peptide within the inhibitory LEKTI domain 4. The third

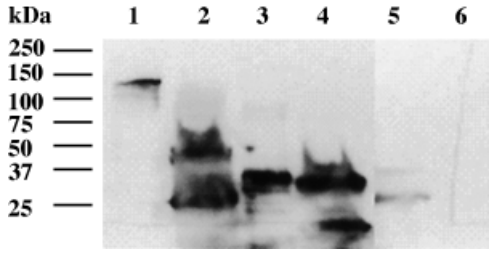

Figure 3
Specificity of LEKTI monoclonal antibody in immunoblot analysis. Full length recombinant LEKTI or lysates from Sf9 cells expressing different LEKTI fragments were subjected to SDS-PAGE and electroblotting onto nitrocellulose and detected with LEKTI mAb 1C11G6. (*Lane 1*) precursor rLEKTI (125 kDa) (1.0 μg); (*lane 2*) cell lysate containing LEKTI domains 1–6 (43 kDa); (*lane 3*) purified rLEKTI domains 6–9 (35.5 kDa) (1.0 μg); (*lane 4*) cell lysate containing rLEKTI domains 9–12 (35.2 kDa); (*lane 5*) cell lysate containing (50 μg) rLEKTI domains 13–15 (30.6 kDa); (*lane 6*) cell lysate from uninfected cells (50 μg). The additional lower Mw bands in *lanes 2* and *4* may reflect proteolytic breakdown products of rLEKTI fragments.

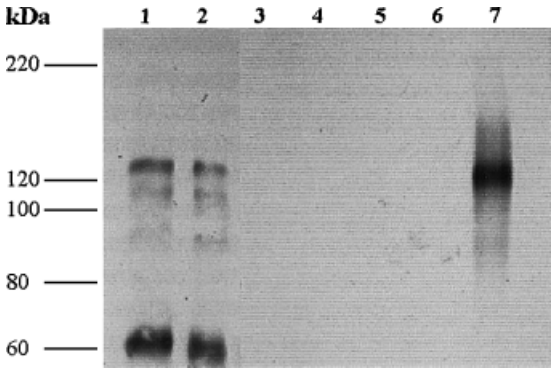

Figure 4
Immunoblot results of LEKTI in human hair roots. The figure shows the immunoblot analysis in hair roots of two normal individuals (*lanes 1* and *2*) and of four NTS patients (*lanes 3–6*: patient 5; 2.1; 2.2; 1). *Lane 7* shows rLEKTI (130 ng). In hair roots from normal individuals full length protein is visualized at 140 kDa and at least three further cleaved products can be seen. In contrast in the four NTS patients neither the full length LEKTI nor the cleaved products are present.

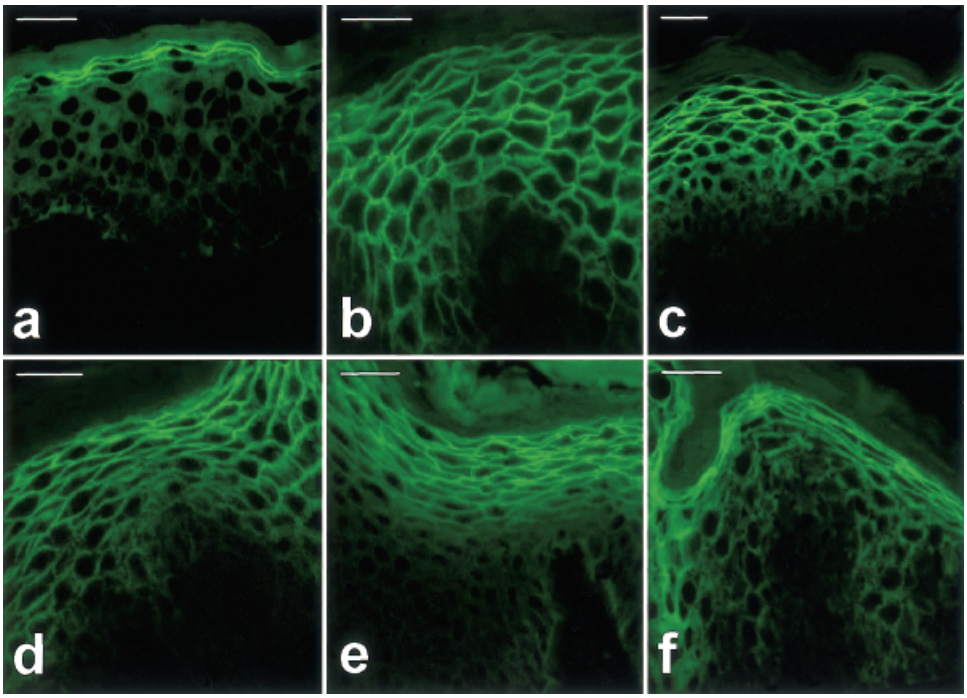

Figure 5
Visualization of transglutaminase1 activity in skin sections of Netherton syndrome patients. (*a*) Strong pericellular epidermal transglutaminase1 at pH 7.4 in normal skins spanning 2–3 layers of the str. granulosum. In contrast skin from NTS patients (*b–f*: patient 4; 1; 3.2; 3.1; 2.1) shows a grossly enlarged activity zone. The pericellular linear distribution pattern, however, is well preserved, the activity appears enhanced. (Magnification bar corresponds to 30 μm.)

novel mutation in intron 15—1432-13 G>A—creates a putative acceptor splice site only 10 bp upstream of the orthodox splice site. Usage of the alternative splice site will result in a frame shift and a PTC in exon 16 predictably leading to a truncation after Kazal-type-like domain 7. Since we had no cell cultures of NTS patients available we could not evaluate the effect of the splice site mutation at mRNA level, but immunoblot analysis of NTS hair roots demonstrated absence of SPINK5 gene products in all four NTS patients analyzed. In contrast, immunoblot analysis of healthy hair roots gave a strong signal of full length LEKTI and of at least three smaller LEKTI fragments. These data provide evidence for a proteolytic processing of LEKTI occurring *in vivo* and are in agreement with *in vitro* results obtained in keratinocyte cultures likewise showing cleavage of LEKTI (Bitoun *et al*, 2003). Furthermore, all mutations were associated with a dramatic lack of immunhistochemical LEKTI-staining in the epidermis of the respective NTS patients. The newly developed antibody against recombinant LEKTI presumably recognizes already epitopes in LEKTI domain 1–6, but—except for patient 1—all NTS patients show PTC mutations after domain 6. The homozygous mutation 2468insA in exon 26 in patient 4 even predicts a polypeptide of 11 complete domains and a nearly complete domain 12. The striking lack of epidermal LEKTI staining in all NTS patients and our immunoblot results therefore suggest nonsense-mediated decay, a rapid and complete proteolytic breakdown of truncated LEKTI protein, or possibly, both. Moreover, it emphasizes the potential usefulness of the antibody for future diagnosis of NTS in clinical practice utilizing either immunhistochemistry of skin biopsies or immunoblot analysis of plucked hair roots.

Surprisingly, we observed a differential expression of transglutaminases in the epidermis of NTS patients. Although transglutaminase1 activity was found in a much enhanced zone affecting almost the entire suprabasal epidermis, a dramatic decrease was found for transglutaminase3. In view of the eminent role of transglutaminases for the assembly of the cornified envelope and for epidermal differentiation, the behavior of transglutaminase1 was expected and a similar pattern was observed by us in psoriatic skin likewise featuring epidermal hyperplasia. Rather unexpected was the dramatic lack of transglutaminase3, which may account for the markedly impaired epidermal barrier in NTS. We performed a preliminary immunfluorescence study of transglutaminase3 expression in a small set of five patients with AD (data not shown). There was a strong signal of transglutaminase3 in the stratum corneum of all patients which may implicate a different pathophysiology of the epidermal barrier defect in AD and NTS. A recent study comparing transglutaminase5 expression with proliferating (keratin 14) and differentiating (transglutaminase3) markers in different diseases such as psoriasis, ichthyosis vulgaris, and lamellar ichthyosis, found a completely misregulated expression of transglutaminases3 and 5 in Darier's disease (Candi *et al*, 2002). These authors simultaneously observed total absence or overexpression of transglutaminase3 in different areas of the same lesion. Obviously, NTS is the first skin disorder reported showing consistent and complete lack of transglutaminase3 expression. Initially, we attributed the lack of transglutaminase3 to a disturbance of epidermal differentiation; however, other differentiation markers and cornified envelope proteins such as involucrin showed only subtle or no expression differences.

Interestingly, we found proteins expressed in NTS epidermis that are normally absent in normal skin, namely, elafin and hBD-2. Elafin/SKALP, a small serine proteinase inhibitor directed against polymorphonuclear leukocyte (PMN)-derived enzymes such as elastase and proteinase

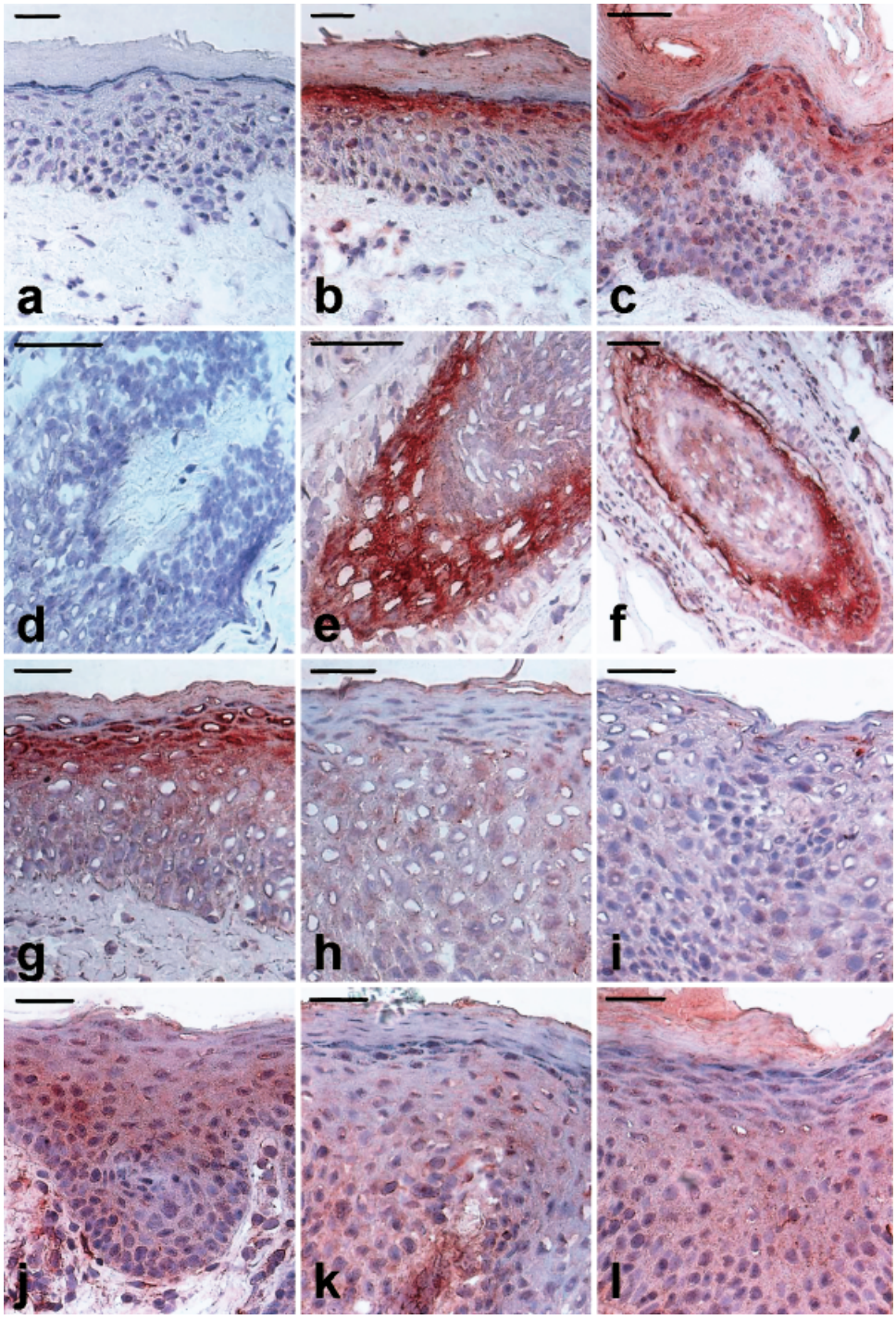

Figure 6
Visualization of LEKTI distribution in normal and NTS skin. Normal skin (*a–f*) shows a predominantly cytoplasmic, partially pericellular presence along the stratum granulosum (*b* and *c*). Hair follicles show a mixed pericellular/cytoplasmic immunostaining in the inner root sheath (*e* and *f*). Application of preimmune serum gave negative results (*a* and *d*). Psoriatic skin shows pronounced cytoplasmic staining in a broadened subcorneal zone (*g*). In contrast, NTS-patients (*h–l*: patient 4; 2.1; 1; 3.1; 3.2) showed absent or only faint patchy cytoplasmic staining indicating a dramatic reduction of epidermal LEKTI. (Magnification bar corresponds to 30 μm.)

3, contains transglutaminase substrate domains for cross-linking to extracellular and cell envelope proteins (Schalkwijk *et al*, 1999). Elafin/SKALP is constitutively expressed in several epithelia that are continuously subjected to inflammatory stimuli, such as the oral cavity and the vagina where it colocalizes with transglutaminase1. Pseudostratified epithelia, simple/glandular epithelia and normal epidermis, however, are negative (Pfundt *et al*, 1996). Certain skin conditions, however, show epidermal elafin expression with psoriasis being the most prominent (Schalkwijk *et al*, 1993; Nonomura *et al*, 1994; Streit *et al*, 1995). Remarkably, the NTS patient 3.1 showed only very faint immunostaining for elafin. Since she appeared to be clinically much less affected than her elafin-expressing sister this difference might reflect her current lower disease activity. In fact, decreasing elafin/SKALP serum levels have been reported in psoriasis in correlation with the improvement of the PASI score during cyclosporin A treatment (Alkemade *et al*, 1995). It is currently believed that inflammatory mediators such as IL-1β or TNF-α secreted by dermal neutrophils may be involved in overexpression of elafin in keratinocytes; this could protect the epidermis from degradation by dermal neutrophil infiltration. Recent findings suggest that elafin cannot only be induced by bacterial lipopolysaccharides

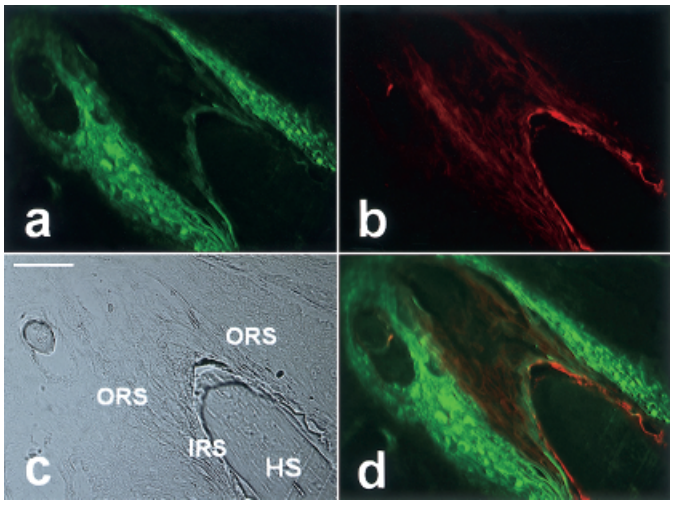

Figure 7
Immunohistochemical visualization of involucrin and LEKTI in hair follicles of normal individuals. (*a*) FITC anti-rabbit: Immunofluorescence staining with polyclonal rabbit anti-involucrin antibody shows both staining of the outer root sheath (ORS) and of the inner root sheath (IRS) of the hair follicle. (*b*) TexasRed-anti-mouse: A strong signal for LEKTI is seen in the IRS. (*c*) Translucent microscope of the hair follicle (HS $\sim$ hair shaft). (*d*) Overlay image FITC + TexasRed: Double staining of involucrin and LEKTI demonstrates that LEKTI is predominantly present in the IRS. (Magnification bar corresponds to 30 μm.)

but it also exerts microbicidal action, comparable with anti-microbial defensin-like peptides (reviewed in Sallenave, 2002). The obvious upregulation of elafin in NTS skin might be regarded as a compensatory mechanism to provide both anti-microbial activity and compensation for lost proteinase inhibitor activity due to LEKTI deficiency.

Similarly, the expression of the professional anti-microbial peptide hBD-2 is intriguing. It plays an important role in the skin innate immune system and is markedly upregulated in psoriatic skin (Harder *et al*, 1997; Huh *et al*, 2002). In contrast, skin of patients with AD shows no or faint expression of hBD-2 (Ong *et al*, 2002). Since psoriasis patients have a strikingly reduced rate of viral and bacterial skin infections in comparison with patients with AD (7% *vs* 30%) (Christophers and Henseler, 1987) it is currently assumed that the overexpression of hBD-2 and other anti-microbial peptides protect psoriatic skin from infection despite chronic inflammation (Ong *et al*, 2002). NTS is currently believed to be related to AD due to its chronic dermatitis and atopic features. A number of reports concerning large series of patients with NTS (Traupe, 1989; Bitoun *et al*, 2002a and b) mention that like in AD NTS patients suffer frequently from skin infections, often caused by *Staphylococcus aureus*. A recent report suggests that this property may be related to selective antibody deficiency to bacterial polysaccharide antigens (Stryk *et al*, 1999). This was not investigated in the present study, but it is obvious that the high epidermal expression of elafin and hBD-2 in NTS does not seem to prevent skin infections effectively although its expression pattern resembles that of psoriasis.

The marked lack of LEKTI staining in all five NTS patients and missing of LEKTI in hair roots suggests that LEKTI deficiency is a hallmark of NTS. These findings are consistent with a recent study (Bitoun *et al*, 2003). The consequences of the LEKTI deficiency, however, remain an enigma. Komatsu *et al* (2002) demonstrated elevated hydrolytic activity in the stratum corneum in NTS patients and suggested a major role for LEKTI in normal desquamation by controlled breakdown of desmosomal proteins. Accordingly, LEKTI deficiency would cause enhanced proteolytic activity in the epidermis, subsequent enhanced breakdown of desmosomal proteins and hyperdesquamation (Komatsu *et al*, 2002). Although it is true that inflamed areas in NTS skin show coarse flaking we would like to point out that the fine desquamation found in most patients even in ILC does not appear to fit the above pathomechanism. LEKTI inhibits *in vitro* a battery of proteases (Mitsudo *et al* 2003) some of which have the potential to process and activate proinflammatory cytokines. Given the prominent inflammatory features of NTS it appears equally possible that LEKTI deficiency primarily causes proteinase-mediated inflammation with epidermal hyperplasia and impaired

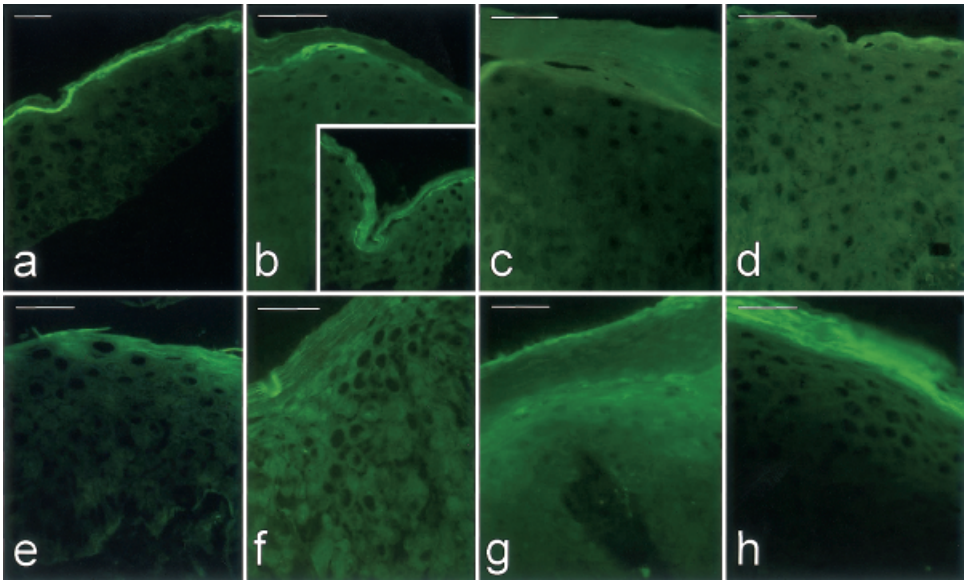

Figure 8
Immunohistochemical detection of transglutaminase3 protein in normal skin and NTS. (*a*) Normal skin shows a narrow band between granular and cornified layer. (*b*) Psoriatic skin shows a similar pattern with interruptions. In contrast, NTS skins show a clear lack of transglutaminase3 expression (*c*,*d*,*f*: patient 5; 4; 2.1) or very faint and scant intracellular immunostaining (e.g., patient 1; 3.2). Remarkably, patient 3.2 (*g*), the more severly affected sister of patient 3.1 (*h*), showed intracellular scanty staining in 3–5 cell layers below the cornified layer, whereas in patient 3.1 (*h*) intense immunostaining is seen at the boarder between granular and cornified layer and within the cornified layer. (Magnification bar corresponds to 30 μm.)

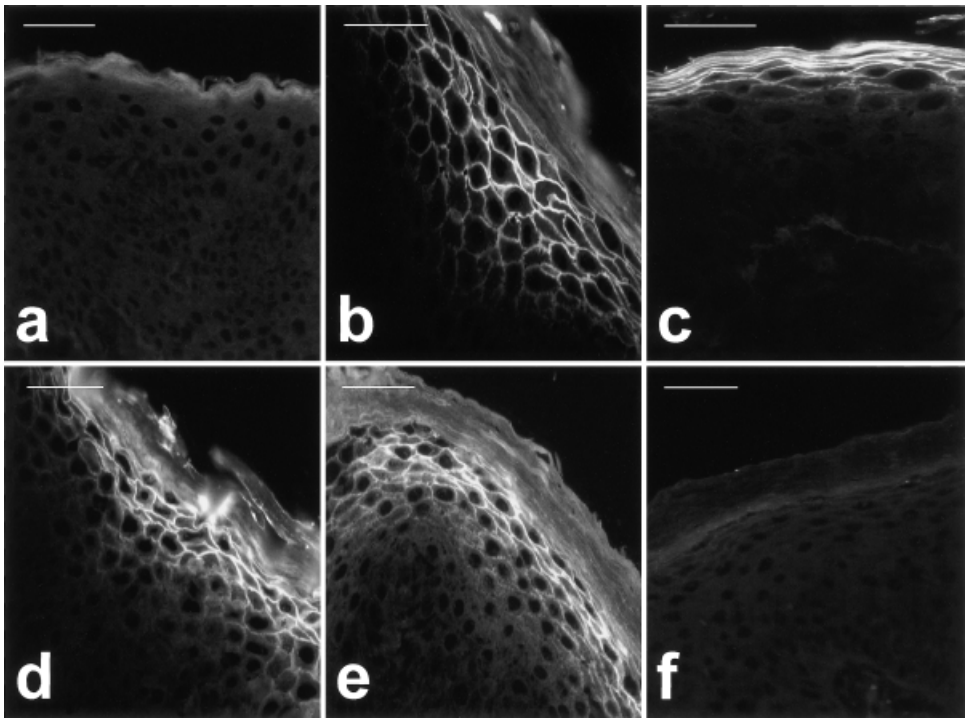

Figure 9
Immunohistochemical detection of elafin in normal skin, psoriatic skin and NTS. (*a*) Normal skin: no significant immunostaining. (*b*) Psoriatic skin shows pericellular linear and continuous localization in broad subcorneal zone layer (three to five keratinocyte layers). (*c–e*: patient 1; 4; 3.2) NTS skin shows moderate to strong pericellular elafin expression in a subcorneal zone comprising maximally three cell layers and slight staining in the cornified layer. (*f*) Patient 3.1 showed no significant elafin expression in contrast to her clinically more severely affected sister 3.2 (compare with (*e*)). (Magnification bar corresponds to 30 μm.)

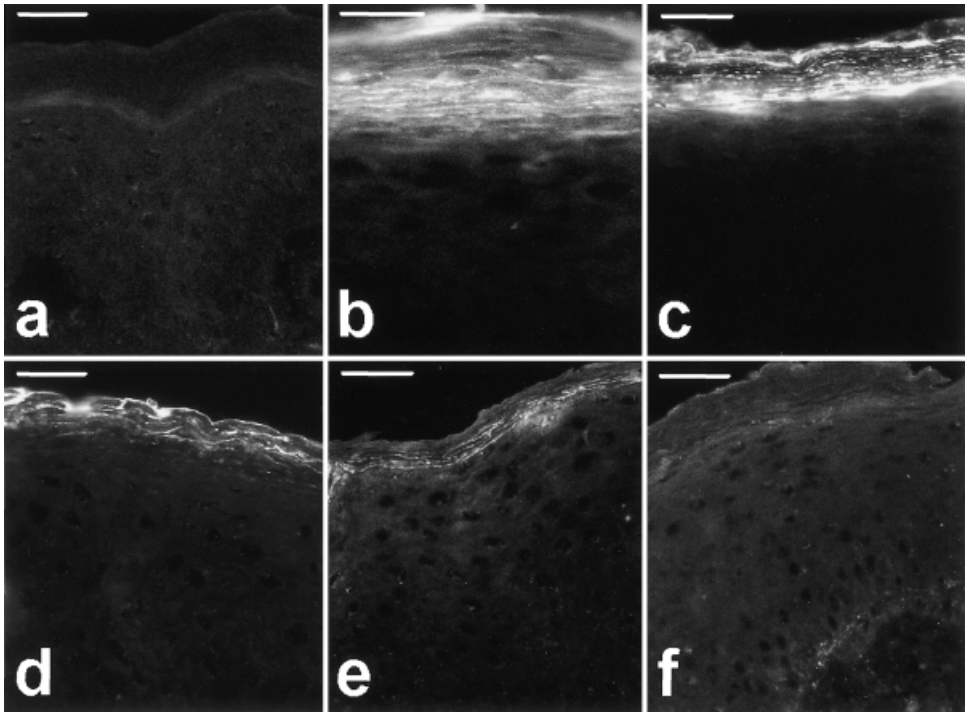

Figure 10
Immunohistochemical detection of human β-defensin 2 in normal skin, psoriatic skin and NTS. (*a*) Normal skin: no significant immunostaining. (*b*) Psoriatic skin shows strong expression of hBD2 in a multilamellar sharply defined fashion restricted to the cornified layer. (*c–e*: patient 1; 4; 3.2) NTS skin shows moderate hBD2 expression. (*f*) Patient 3.1 showed no significant hBD2 expression in contrast to her clinically more severely affected sister L.H. (compare with (*e*)). (Magnification bar corresponds to 30 μm.)

epidermal barrier as secondary features. This speculation however, warrants further investigation.

In conclusion, we have shown that LEKTI can be detected by immunoblot analysis of normal human hair roots but it was missing in four NTS patients and that *SPINK5* mutations in NTS are associated with epidermal LEKTI deficiency at protein level. The surprising differential expression of transglutaminases discloses a profound alteration of epidermal differentiation and may account for the impaired epidermal barrier.

Materials and Methods

Patients We ascertained five different NTS families including seven affected individuals. Local ethical committee approval and informed consent was obtained for the study. The diagnosis of NTS was based on the presence of CIE-like ichthyosis or ILC, hair shaft abnormalities including trichorrhexis invaginata or nodosa, or pili torti, elevated IgE levels, allergies, history of recurrent bacterial infections, malnutrition, failure to thrive, and neonatal dehydration (online Table S1). All patients exhibited typical skin involvement and trichorrhexis invaginata. Four mm punch biopsies were taken and

results obtained compared with skin biopsies from 30 normal adults, three normal newborns, and five patients with classical psoriasis vulgaris.

***SPINK5* mutation analysis** Genomic DNA was prepared from peripheral blood leukocytes by standard procedures (Sambrook *et al*, 1989). All 33 exons of the *SPINK5* gene were amplified using most of the published primer pairs (Chavanas *et al*, 2000b; Sprecher *et al*, 2001). New primers were designed from the *Homo sapiens* chromosome 5 partial sequences (GenBank accession no. AC116334, AC008722) and from the partial sequences of *SPINK5* (GenBank accesion no. Aj276578, Aj391235–Aj391241, AF295783, AF295784) for exons 7–16 (online Table S2). Individual PCR reactions were performed using the above specified primers with *Taq* DNA polymerase recombinant (Invitrogen Life Technologies, Karlsruhe, Germany), 5% dimethyl sulfoxide (Sigma-Aldrich Chemie GmbH, Steinheim, Germany), 0.5 M Betaine (dto). Genomic DNA (100 ng per μL) of affected individuals was amplified using the Biometra Thermocycler TGradient. For all exons PCR conditions were as follows: 94°C 4.30 min followed by 35 cycles at 56°C 1 min, 72°C 1 min, 94°C 45 s; and a final extension step at 72°C for 10 min. Mutation analysis was performed by direct sequencing of amplified exons using Big Dye Terminator Protocol and Reaction Clean-up for 96-well Microtiter Plates with Sephadex G-50. Sequences were directly read out in an ABI 3700 (Applied Biosystems, Foster City, California). The following electronic databanks were used: http://www.ncbi.nlm.nih.gov/PubMed/; http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi; http://genius.embnet.dkfz-heidelberg.de/menu/; http://www.cbs.dtu.dk/services/NetGene2/; http://www.fruitfly.org/seq_tools/splice.html. Data analysis was performed using Chromas Version 1.43 (Conor Mc Carthy, Griffith University, Brisbane, Queensland, Australia).

Histochemical transglutaminase assay This screening assay allows to differentiate between full, residual and lost epidermal transglutaminase1 activity (Raghunath *et al*, 1998). Briefly, cryosections were incubated with the transglutaminase substrate biotinylated cadaverine (Molecular Probes, Leiden, The Netherlands) in Tris buffer pH 7.4 in the presence of calcium ions. Incorporated substrate was visualized using streptavidin DTAF. Slides were viewed with an Axioscope 2 and digital images taken using an Axiocam HR video camera and Axiovision 3.0 software (all Carl Zeiss, Jena, Germany).

Immunohistochemical localization of transglutaminase1, transglutaminase3 and other proteins Acetone fixed cryosections (−20°C for 10 min) were incubated with mouse MAb BT-621 against human keratinocyte transglutaminase1 (Biomedical Technologies, Stoughton, Massachusetts) 1:40 for 16 h at 4°C or rabbit anti-transglutaminase3 diluted 1:100 (kindly provided by Dr Soo-Youl Kim, NIH). Transglutaminase1 antibody was detected by a two-step procedure using biotinylated goat anti-mouse 1:400 for 90 min (Jackson ImmunoResearch Laboratories, Inc., West Grove, Pennsylvania) and streptavidin DTAF (Jackson ImmunoResearch Laboratories, Inc., West Grove, Pennsylvania) 1:100 for 30 min. The transglutaminase3 antibody was visualized using swine anti-rabbit FITC 1:20 (Jackson ImmunoResearch Laboratories, Inc., West Grove, Pennsylvania). For the detection of other proteins (except elafin) cryosections were fixed in methanol. Sections were either incubated with rabbit-anti-involucrin (CellSystems Biotechnologie Vertrieb GmbH, St. Katharinen, Germany) diluted 1:200, rabbit antibodies against elafin 1:2000 (Peptide Institute, Inc., Minoh-shi Osaka, Japan), rabbit-anti-annexin I 1:200, and mouse-anti-annexin II 1:400 (both: Zymed, San Francisco) or rabbit-anti-hBD-2 1:2000 (Peptide Institute, Inc., Minoh-shi Osaka, Japan), respectively, for 16 h at room temperature. Rabbit-antibodies were detected with FITC-goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories); mouse-antibodies with FITC-goat anti-mouse IgG (Jackson ImmunoResearch Laboratories) both diluted 1:100.

Production and characterization of monoclonal LEKTI antibody Eight-wk-old BALB/c mice (Harlan Technology, Chicago) were primed i.p. with 50 μg of recombinant LEKTI (Mitsudo *et al*, 2003) in complete Freund Adjuvant and boosted at 3-wk intervals with 25 μg of antigen in complete Freund Adjuvant. Spleen cells harvested 3 d after the fourth immunization were fused with myeloma cells (P3 × 63-Ag8.653) and antibody-secreting hybrids were identified 1 wk later by a capture ELISA using purified rLEKTI as the antigen. Clones secreting desirable antibody were subjected to limiting-dilution cloning to isolate monoclones and isotyped (Zymed Labs, Inc., S. San Francisco); ascites was produced in BALB/c mice. To determine the LEKTI regions recognized by different Mabs, immunoblot analysis was done using purified rLEKTI domains 6–9 or Sf9 cell lysates containing rLEKTI domains 1–6, 9–12, or 13–15. Briefly, proteins were mixed with 2 × gel loading buffer (4% SDS, 20% glycerol, 120 mM Tris–HCl, pH 6.8%, 0.01% bromophenol blue, 2% β-mercaptoethanol), heated to 95°C for 5 min, and separated by SDS-PAGE (6%) (Mitsudo *et al*, 2003). Proteins were transferred from polyacrylamide gel onto nitrocellulose membranes (Schleicher & Schull BioScience, Inc., Keene, New Hampshire) using a mini-transblot electrophoretic cell (BioRad, Hercules, California) at 25 V for 16 h at 4°C. After a blocking step (16 h with 3% BSA in Tris buffered saline) membranes were subsequently incubated with mouse ascites 1:1000 for 2 h at room temperature, horseradish peroxidase-conjugated goat-anti-mouse IgG (H + L) 1:2500 (Jackson ImmunoResearch Laboratories, Inc., West Grove, Pennsylvania). Finally, bound LEKTI antibody was visualized by exposure to Kodak X-AR5 films (Eastman Kodak Company Scientific Imaging Systems, Rochester, New York) using chemiluminescence (ECL system, Amersham Bioscience Corporation, Piscataway, New Jersey).

Immunoblot analysis of human hair samples Twenty scalp hairs were each plucked from five healthy voluntary individuals and from four NTS patients (patient 1; 2.1; 2.2 and 5) and hair roots were transferred into 40 μL lysis buffer containing 1% Triton X-100 in PBS, 1 μM Pepstatin, 1 μM E64, 2 mM Pefabloc (all proteinase inhibitors from Sigma, Deisenhofen, Germany). Subsequently, these samples were kept at 4°C for 20 min, heated at 99°C for 10 min and then stored at −20°C before western blot analysis.

For immunoblot analysis samples were subjected to electrophoresis using 25 μL of sample-mixture from each normal control individual and 40 μL of samples from each NTS patient. For electrophoresis 7.5% acrylamide gels under reducing conditions were used and afterwards proteins were transferred onto a PVDF-membrane (Immobilon P, Millipore, Eschborn, Germany) by western blotting. For detection with LEKTI-specific antibodies the membrane was saturated with 3% BSA in TBS for 1 h, incubated with the primary antibody (mAb 1C11G6 1:1.000) for 2 h and afterwards with peroxidase-conjugated secondary anti-mouse antibody (Sigma, Deisenhofen, Germany) for 1 h at room temperature. Detection was performed using chemiluminescence detection reagent Rotilumin (Roth, Karlsruhe, Germany) following manufacturer's instructions.

Immunhistochemical localization of LEKTI Acetone-fixed cryosections were airdried and blocked with normal goat serum 10%, or with BSA 2% in PBS for blocking of endogenous peroxidase, respectively. Sections were then incubated with mAb 1C11G6 1:200 for 16 h at RT. LEKTI antibody was either detected with FITC-goat anti-mouse antibody 1:100 (Jackson ImmunoResearch Laboratories, Inc., West Grove, Pennsylvania) or a peroxidase procedure using DAKO EnVision System (DAKO Corp., Carpinteria, California) and counterstaining with Mayer's hemalum. For involucrin/LEKTI double staining sections were incubated with mAb 1C11G6 1:100 and polyclonal rabbit anti-involucrin 1:1000 (BAbCO, Richmond, California) and antibodies were detected with both TexasRed-goat anti-mouse antibody 1:100 and FITC-goat anti-rabbit (both: Jackson ImmunoResearch Laboratories, Inc., West Grove, Pennsylvania).

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Supplementary Material

The following material is available from http://www.blackwellpublishing.com/products/journals/suppmat/JID/JID23220/JID23220sm.htm

Table S1. Netherton Syndrome patients: synopsis of clinical findings

Table S2. Designed new primers: Exon 7 to 16

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