

The Spectrum of Pathogenic Mutations in SPINK5 in 19 Families with Netherton Syndrome: Implications for Mutation Detection and First Case of Prenatal Diagnosis

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The Comèl-Netherton syndrome is an autosomal recessive multisystemic disorder characterized by localized or generalized congenital ichthyosis, hair shaft abnormalities, immune deficiency, and markedly elevated IgE levels. Life-threatening complications during infancy include temperature and electrolyte imbalance, recurrent infections, and failure to thrive. To study the clinical presentations of the Comèl-Netherton syndrome and its molecular cause, we ascertained 19 unrelated families of various ethnic backgrounds. Results of initial linkage studies mapped the Comèl-Netherton syndrome in 12 multiplex families to a 12 cM interval on 5q32, thus confirming genetic homogeneity of Comèl-Netherton syndrome across families of different origins. The Comèl-Netherton syndrome region harbors the SPINK5 gene, which encodes a multidomain serine protease inhibitor (LEKTI) predominantly expressed in epithelial and lymphoid tissues. Recently, recessive mutations in SPINK5 were identified in several Comèl-Netherton syndrome patients from consanguineous families. We used heteroduplex analysis

followed by direct DNA sequencing to screen all 33 exons and flanking intronic sequences of SPINK5 in the affected individuals of our cohort. Mutation analysis revealed 17 distinct mutations, 15 of which were novel, segregating in 14 Comèl-Netherton syndrome families. The nucleotide changes included four non-sense mutations, eight small deletions or insertions leading to frameshift, and five splice site defects, all of which are expected to result in premature terminated or altered translation of SPINK5. Almost half of the mutations clustered between exons 2 and 8, including two recurrent mutations. Genotype-phenotype correlations suggested that homozygous nucleotide changes resulting in early truncation of LEKTI are associated with a severe phenotype. For the first time, we used molecular data to perform prenatal testing, thus demonstrating the feasibility of molecular diagnosis in the Comèl-Netherton syndrome. **Key words:** atopic dermatitis/congenital recessive ichthyosis/hair abnormalities/linkage analysis/serine protease inhibitors. *J Invest Dermatol* 117:179–187, 2001

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Abbreviations: NTS, Comèl-Netherton syndrome; LEKTI, serine protease inhibitor Kazal type 5; CIE, nonbullous congenital ichthyosiform erythroderma; ILC, ichthyosis linearis circumflexa; CSGE, conformation-sensitive gel electrophoresis; gDNA, genomic DNA.

The Comèl-Netherton syndrome (NTS; MIM 256500) is a rare autosomal recessive disorder of the skin, hair and immune system first reported by Comèl in 1949 and Netherton in 1958 (Comèl, 1949; Netherton, 1958). The syndrome presents at or soon after birth with generalized erythroderma, scaling, and/or continuous peeling of the skin resembling nonbullous congenital ichthyosiform erythroderma (CIE; OMIM 242100) or peeling skin syndrome (OMIM 270300). In about 20% of patients the neonatal period is complicated by hypernatremic dehydration, electrolyte imbalances, perturbed thermoregulation, failure to thrive and recurrent

infections (Jones *et al*, 1986; Hausser *et al*, 1989; Traupe, 1989), which may result in neonatal demise (de Wolf *et al*, 1995; Judge *et al*, 1994; Smith *et al*, 1995; Judge and Harper, 1996). These complications might, at least in part, be attributed to the collapse of the epidermal barrier function (Fartasch *et al*, 1999). Whereas in individuals with severe disease the generalized CIE-like features persist throughout life, the ichthyosis in most patients gradually evolves into migratory, serpiginous, scaling, and erythematous plaques, which are bordered by a peculiar double-edged scale descriptively named ichthyosis linearis circumflexa (ILC) (Greene and Muller, 1985; Traupe, 1989). The skin lesions are often pruritic, resemble atopic eczema, and show an unstable, undulating course. They are usually accompanied by hair shaft abnormalities that develop during early childhood and may result in diffuse alopecia. The hallmark of NTS is trichorrhexis invaginata (bamboo hair), but other abnormalities, including pili torti (twisted hair) and trichorrhexis nodosa (hair of varying diameter), have been observed. Markedly elevated IgE levels, allergic reactions to food and common antigens, malnutrition, and increased susceptibility to skin, respiratory tract or systemic infections are also characteristic (Sybert, 1997).

Although there are no more than 150 cases of NTS reported in the literature, its true incidence might be as high as 1/50,000 (Traupe, 1989) due to challenging diagnostic problems during infancy and early childhood and overlapping features with atopic dermatitis (ILC type) and other recessive ichthyoses (CIE type). A recent report indicating that up to 18% of congenital erythrodermas are attributable to NTS lends support to this assumption (Pruszkowski *et al*, 2000). Most patients with NTS are sporadic cases; however, there are reports of affected sibships (Dupre *et al*, 1978; Caputo *et al*, 1984; Kassis *et al*, 1986) and of consanguinity in about 10% of the families with NTS, which is consistent with autosomal recessive inheritance (Greene and Muller, 1985).

The pathogenesis of NTS has engendered considerable interest because of the peculiar association of skin, hair, and immunologic abnormalities in the context of a monogenic disorder. A mouse mutant with lance head-shaped hair, scaly skin, and elevated IgE levels termed "lanceolate hair" (*lah*), which maps in the vicinity of a cluster of desmosomal genes on mouse chromosome 18q, was thought to be an animal model for NTS (Montagutelli *et al*, 1996; Sundberg *et al*, 2000); however, linkage analysis in five NTS families excluded the homologous region on human chromosome 18q12 as well as other candidate gene regions (Sprecher *et al*, 2000). NTS was finally mapped by genome-wide screening to a 4 cM interval on chromosome 5q32 (Chavanas *et al*, 2000a), and several patients, most resulting from consanguineous matings, were found to carry mutations in SPINK5 (Chavanas *et al*, 2000b). This gene, 61 kb in size, consists of 33 exons and encodes a recently discovered Kazal-type serine protease inhibitor (designated LEKTI) (Chavanas *et al*, 2000b; Mägert *et al*, 1999). We studied an international cohort of 19 NTS families and report here novel and recurrent mutations with significant implications for the development of successful mutation detection strategies as well as the first prenatal diagnosis of NTS.

MATERIALS AND METHODS

Patients and clinical material We ascertained 19 NTS families, including 21 affected individuals, with their informed consent. The diagnosis of NTS was based on the presence of the following features: (i) CIE-like ichthyosis or ILC (**Fig 1A, B**); (ii) hair shaft abnormalities, including trichorrhexis invaginata (**Fig 1C**), trichorrhexis nodosa, or pili torti; and (iii) elevated IgE levels, and/or history of recurrent bacterial infections, malnutrition, neonatal dehydration, and allergies. To compare the severity of the disease between individuals, we developed a severity score (see **Table I**) based on the following criteria: skin involvement (ILC or eczema of <30% of body surface = 1, ILC or eczema of >30% of body surface = 2, generalized scaling erythema or eczema = 3); hair shaft abnormalities (none = 1, mild = 1, severe = 2); IgE levels

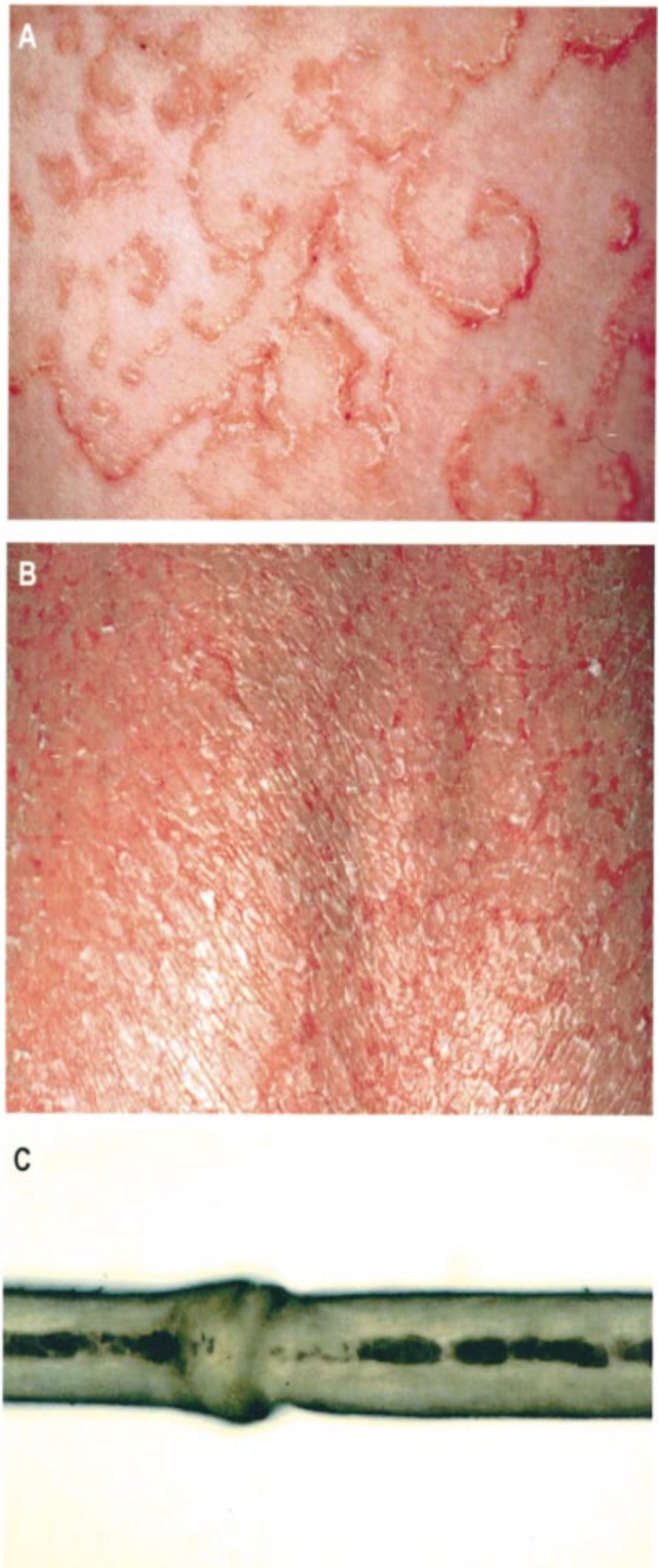


Figure 1. Clinical features of NTS. (A) Ichthyosis linearis circumflexa. Note the circinate hyperkeratotic plaques bordered by a characteristic double-edged scale. (B) Generalized CIE-like ichthyosis in NTS. (C) Trichorrhexis invaginata. Note the typical ball-and-socket appearance of the hair shaft.

Table I. Clinical features and severity of NTS

Family	Ethnic origin	Age	Skin involvement			Hair shaft abnormalities (TI, PT, TN)		Serum IgE levels (IU/ml)			Associated Features					Severity score
			1	2	3	1	2	1	2	3	Postnatal Hypernatr. Dehydration	Postnatal Failure to Thrive	Growth Retardation/ Enteropathy	Severe Recurr./ Systematic Infections	Allergic reactions (food/ pollen)	
			ILC/ Eczma (<30%)	ILC/ Eczma (>30%)	Gener. CIE-like ichthyosis	Mild	Severe	100– 1,000	>1,000– 10,000	>10,000						
1	Italian	5		2		1			2		0	0	0	1	1	7
2	German/ Irish	16	1			1			2		1	0	0	0	1	6
3	English/ Italian	7		2			2		2		0	0	0	1	1	8
4	Northern European	5		2		1			2		0	1	0	1	1	8
5	Irish/ Scottish	12			3		2			3	1	1	1	1	1	13
6	Dominican Republic	18			3		2			3	0	1	1	1	1	13
7c	Puerto-Rican	8			3		2	N/A			0	1	1	1	0	[8]
8c	Iranian	3			3	1		N/A	2		0	1	0	0	1	[8]
9	African-American	17			3		2			3	0	1	0	1	1	11
		2		2			2		2		0	1	0	1	0	8
10	Northern European	13			3	1			2		1	1	1	1	1	11
11	Italian/Finnish	33		2			2		2		0	0	0	1	1	8
12	Northern European	4	1				2	1			0	1	0	1	1	7
		6	1				2	1			0	0	0	1	1	6
13	Argentinian	12	1			1			2		1	1	0	1	1	8
14	German	55		2			2		2		0	0	0	0	1	7
15	German/ Northern European	13		2			2		2		1	1	0		1	9
16	Polish/ Romanian	6	1			1			2		0	1	0	1	0	6
17	German/Northern European	5			3		2			3	0	1	0	1	1	11
18	Northern European	3			3	1		1			1	0	0	1	1	8
19	Northern European	2			3	1		1			1	1	0	1	1	9

ILC, ichthyosis linearis circumflexa; CIE, congenital ichthyosiform erythroderma; TI, trichorrhexis invaginata; TN, trichorrhexis nodosa; PT, pili torti; c, consanguineous family; N/A, not analyzed.

Table II. Genomic primer sequences for amplification of SPINK5 (exons 1-33)^a

Exon	Upstream primer	Downstream primer	Amplicon size (bp)	Annealing temperature	Taq PM system ^b
1	CAATGCATGGAGTGGACCTGT	AGACATTTTCAGGATTATACATGC	229	60	Q
2	GTGCCCTTCTTTTATTTGCCATG	AAAGCTATTAGTACCTACCAG	248	60	Q
3	TATCTACTATGTATCAGGCATTG	ATTTACCAGTTCAGAGACTAGC	371	60	Q
4	ATCTGGGGTTCTGTGTCCAC	CAGGTATGACCTAGTAATTAAG	327	57	Q
5	TATTAGCTCAATGTAGCCTTC	TGTAGGGAAAATTGTGTGTCATG	349	60	Q
6	TAAAGGAGAATGACAATGCAATG	TACTATTTGAAGTTAGCCTCCC	215	60	Q
7	AGTACTTACTGAGCTACATCTAC	TTATCTCTCTGCTGAGTGATTC	358	60	Q
8	CGTAGCAGAGGATATGAAAGTG	GAGAAACACAGGTGGAAACGTCAC	393	60	Q
9	GGAAGGATCTCTGAGCCTAG	GCATTATGATTGTGGAGGGC	424	60	Q
10	CTAAAACCTCAGGACAACCTTAG	CAATAGCTACCCACACATCGAAGAC	521	57	Q
11	GAAAACAGAATATATCTCAACT	CTGTCTTGAGCCACAGTCTC	272	57	AG
12	GAAGAAATCATAGCACCATAC	GCAAAATCTCACCCTTTAGGC	349	60	Q
13	GAGATGTAACATTAGTTTCTG	ATGTCTCCAATCAGACAGTTTCTC	370	60	Q
14	TGCAATTGTGAGGATTTCCAG	CCTGAACATGATCTGTGGATC	304	60	Q
15	AATCCATGCCTTCAAAGTTAATC	CCAAGACTGAATGCTACACTG	392	60	Q
16	TGAGGCAGGAAGAATTGCTTG	GTGAAATAAAATTGGTACTCTGGACAC	244	57	AG
17	CTGATTGATGACGGAACCTTTG	CTCACGGTCTATACTCTAC	389	60	Q
18	CAAAAGCACCTCTCAGACTAG	AGTACTCTTGATATATGGGGATC	380	60	Q
19	TTCCAAATGTGTGACCTAGTTC	CAAACCTCAGTGCAAAGACAGC	432	60	Q
20	GTTTACCTTTCCACTCCAAAGC	TGAACCCCTAGTTCCCTCAC	319	60	Q
21	GAATACAGCCACCTTCTTAAG	AATACATATTATTTGCCTGGCTC	392	60	Q
22	AGAGAAGGCCTGCAGCATAG	AGCACTGCCTTTACCATAGAC	344	60	Q
23	CCACCGTACCCGGCAATG	TGCATCCAAGGAGCCATA	378	60	Q
24	GTGCCAGAATCCTAGGAAGAC	CTAGCTTGATCATGTTGACACC	332	60	Q
25	ATTATTTTGCTATCACAGCAAG	TCATTAATTACCAGATCTGCTTC	372	60	Q
26	TGACTGTGAGTCTTAAAGTAC	GGGACAGAGTCAGCATTTTAC	252	60	Q
27	TCTGTTTTTTTCTGTGTTATGAGT	GTGTGATGCCAAGTATCTTAGG	335	60	Q
28	CCAACAATCAGAAGTATTAGC	CAAAGTGACAAGACAAGAAAAATC	389	60	Q
29	GGCCTCTGTTGCCAGGATG	TATACAACCTTATTCAAGCAGCAG	347	60	Q
30	GCCTGGGCCTTCCAAAAT	ACTTCAGGCTGCACTGAATCA	379	60	Q
31	GTGCAAAATCAATCTTTGAGTTTG	CTAAACTGGAAGATATGATGGG	324	60	Q
32	GACACCTGGATGATACCTAC	CCAGATAAATGTCCATTACTCAG	365	60	Q
33	CTAGCATCTAACCTACCCATC	GCAATAAGACAAGCTCCGAAG	148	60	Q

^aThe primers were placed on flanking intronic sequences at least 25bp from the exon-intron borders.

^bQ = Qiagen Taq; AG = Perkin Elmer Ampli Taq Gold.

(< 100 IU per ml = 0, 100–1000 IU per ml = 1, > 1000–10,000 IU per ml = 2, > 10,000 IU per ml = 3), associated features (absent = 0, present = 1). Our cohort comprised 12 multiplex families and seven families with a single affected child, including two consanguineous families. The families were of northern European, African-American, Mediterranean, Iranian, South American, and Puerto-Rican descent. Peripheral blood samples and/or buccal swabs of all participants were collected and prepared for DNA analysis following standard procedures.

Microsatellite analysis Twelve multiplex NTS families (57 individuals, 18 affected) were initially included in the linkage studies. We selected four polymorphic microsatellite markers spanning 12 cM on 5q derived from the GDB database, including: cen-D5S436–3 cM–D5S2090–2 cM–D5S434–7 cM–D5S410–tel. Genotypes of all family members were determined by polymerase chain reaction (PCR) amplification of genomic DNA (gDNA) using fluorescently labeled (6-FAM, HEX, NED phosphoramidite), tailed primers and Ampli Taq Gold (PE Applied Biosystems, Foster City, CA), according to the manufacturer's instructions. The amplicons were separated by polyacrylamide gel electrophoresis on an ABI Prism 377 sequencer, and genotypes were established using the GeneScan 3.1 and GenoTyper 2.0 software (PE Biosystems). Genotypes were used to construct the most parsimonious haplotype for each family member. Multipoint LOD scores were computed with GENEHUNTER (Kruglyak *et al*, 1996). Allele frequencies for markers were obtained from the GDB database or our data. NTS was modeled as an autosomal recessive disorder with complete penetrance and disease allele frequency of 0.00001.

Mutation analysis Mutation analysis of SPINK5 was performed using primer pairs as previously described (Chavanas *et al*, 2000b) and/or newly designed (exons 1, 9, 11, 16, 31, and 33) from the partial sequences of SPINK5 (GenBank accession no. AJ270994, AJ391230–AJ391254, AJ276577–AJ276580). To design primers for exons 8 and 10,

we established the intronic boundaries of those exons (sequences are deposited in GenBank under accession nos AF295783 and AF295784) by amplifying gDNA with the Expand Long Template PCR system (Boehringer Mannheim, Indianapolis, IN) and intraexonic primers of exon 10 (forward 5'-GTA ACA TGA AGA TCG GAA GCG TCT C-3'), exon 8 (forward: 5'-GAA AAT GCC AAG CGA GAG GGT GAA AC-3'; reverse: 5'-CTT TTC AGC ATT TCG TCG AAT TCT AG-3') as well as primers located in introns 6 (forward), 8 (reverse) and 10 (reverse) (Table II). The gDNA of affected individuals was PCR amplified using the above specified primers with Taq polymerase and Q solution (Qiagen, Valencia, CA) or AmpliTaq Gold, GeneAmp PCR Buffer II and 2% dimethyl sulfoxide (Table II). Cycling conditions were 95°C 5 min followed by 35 cycles at 95°C 30 s, 57–60°C 45 s, 72°C 90 s; and a final extension step at 72°C for 7 min.

These PCR products were mixed 1 : 1 with amplicons obtained from healthy controls before screening for sequence alterations by heteroduplex analysis using conformation-sensitive gel electrophoresis (CSGE, 6% polyacrylamide gels) (Körkkö *et al*, 1998). Amplicons with divergent migration patterns on CSGE were gel-purified (QIAquick gel extraction kit, Qiagen) and subjected to bi-directional DNA sequencing using the BigDye terminator system on an ABI Prism 377 sequencer (PE Applied Biosystems). If no evidence for mutations was obtained by heteroduplex analysis, we subsequently analyzed all fragments by direct DNA sequencing. Each sequence variant was confirmed by restriction fragment analysis, CSGE or sequencing, and excluded from 50 to 120 alleles of unrelated control individuals.

Electronic database information GDB database, <http://gdbhttp://www.gdb.org>; GenBank, <http://www.ncbi.nlm.nih.gov> (accession numbers for SPINK5: AJ270994, AJ391230–AJ391254, AJ276577–AJ276580, AF295783, and AF295784); Online Mendelian Inheritance in Man (OMIM), <http://www.ncbi.nlm.nih.gov/Omim/>.

RESULTS

Clinical features of NTS We examined a total of 21 affected individuals aged 2–55 y with NTS from 19 families of diverse ethnic backgrounds (**Table I**). In all but one patient NTS manifested at birth with scaling erythroderma. In one family, a sibling diagnosed with “ichthyosis” and severe failure to thrive died at 2 y of age of unknown cause (family 8, **Table I**). The dermatologic features at the time of examination covered a wide spectrum ranging from a few localized, erythematous plaques with scaling borders to generalized erythroderma with fine, white scales reminiscent of CIE. All affected individuals had also hair shaft abnormalities, including trichorrhexis invaginata (**Fig 1C**), trichorrhexis nodosa, and pili torti. Patients from nine families had severe manifestations, including CIE-like, generalized skin involvement, short, sparse hair, a history of failure to thrive and/or growth retardation, postnatal dehydration, enteropathy, and systemic infections (**Table I**). Their cutaneous features included generalized erythema, fine white scaling (**Fig 1B**) often associated with sheet-like peeling of the stratum corneum resembling peeling skin syndrome. A distinct group of 12 patients had only localized skin lesions, ILC (**Fig 1A**) and/or eczematous plaques and flexural lichenification reminiscent of atopic dermatitis, less often associated with other organ manifestations. The type of disease was consistent for affected siblings in our cohort despite some phenotypic variability in severity. There was considerable overlap with symptoms of the atopic spectrum. All patients reported persistent pruritus, 18 had a history of allergic reactions to food and/or pollens, and five had asthma. Whereas most patients had elevated IgE levels above 1000 IU per ml, only patients with CIE-like ichthyosis exhibited extreme elevations (> 10,000 IU per ml).

Mutation analysis of SPINK5: Altered protein translation due to splice site, nonsense or frameshift mutations On account of the observed phenotypic heterogeneity of NTS, we decided to test our cohort for linkage to the NTS locus in 12 multiplex families amenable to this approach. Using multipoint linkage analysis, we confirmed mapping of NTS to 5q32 (maximal LOD score of 4.4 at locus D5S434), providing convincing evidence for locus homogeneity across families of diverse genetic backgrounds (data not shown).

Using heteroduplex analysis and direct DNA sequencing, we analyzed the gDNA of 19 unrelated NTS patients for sequence variations in SPINK5 as exemplified in **Fig 2**. We identified 17 distinct mutations, 15 of which had not been reported previously (**Table III**, **Fig 3**). The mutations were distributed across the entire gene, although eight of 17 (47%) clustered between exons 2 and 8, which encode the signal peptide and the first three putative inhibitory domains of LEKTI. The majority were private mutations, although we observed three recurrent nucleotide alterations: 2264insA occurred in two families of African-American and northern European origin, and 81+2T→A segregated in two unrelated families of northern European descent (**Fig 3**); patients of two consanguineous families from Iran and Puerto-Rico were homozygous for 238insG, whereas another affected individual of Italian background was heterozygous for this mutation.

The mutational spectrum included small deletions and insertions (n = 8), splice site (n = 5) and nonsense mutations (n = 4) (**Table III**). All these mutations can be predicted to disrupt significantly the amino acid sequence of the translated LEKTI protein either by introducing a premature termination codon as result of a frameshift or nonsense mutations, or by altering conserved consensus RNA splicing signals. Similar to previously reported data (Chavanas *et al.*, 2000b), we obtained evidence for nonsense mediated mRNA decay using semiquantitative reverse transcription-PCR from RNA of cultured keratinocytes of a patient in family 6 (**Table III**) (data not shown). Although RNA samples of patients with splice site defects were not available for analysis, all mutations eliminated invariant splice donor or acceptor sites and are, therefore, expected to produce abnormal splice products.

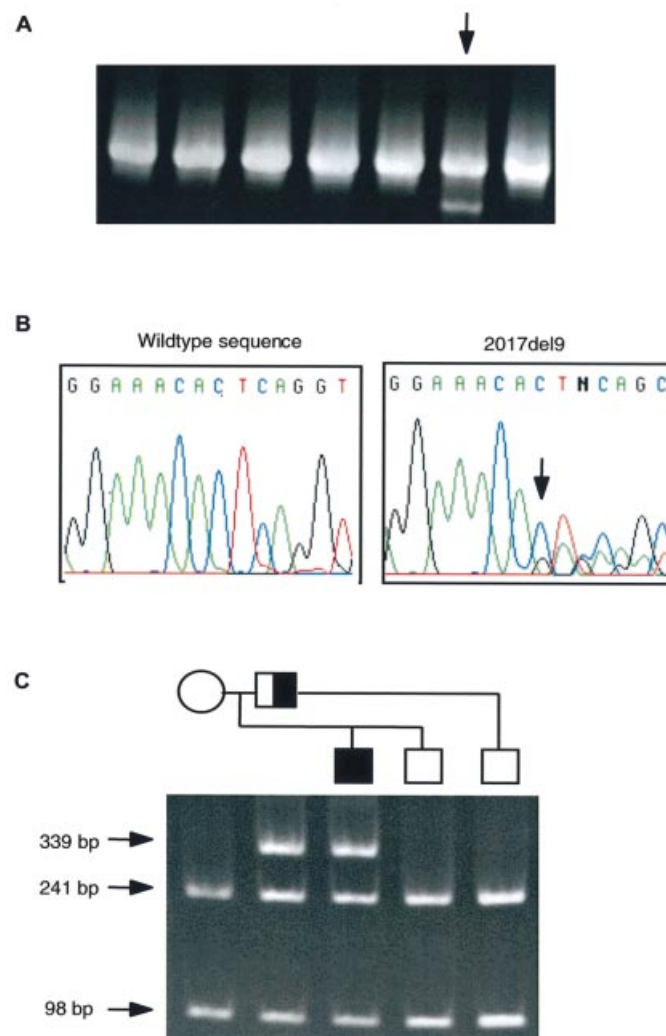


Figure 2. Representative example of mutation screening of SPINK5. (A) DNA obtained from affected individuals of different families was amplified with primers specific for exon 22 of SPINK5. PCR products were analyzed using CSGE as described in *Materials and Methods*. Results show abnormal migration of PCR product obtained with DNA from an affected individual of family 2 (arrow). (B) Direct sequencing of this PCR product revealed a heterozygous 9-bp deletion across the 3' intronic boundary of exon 22 (arrow) predicted to eliminate the splice donor site and leading to partial translation of intron 22 (premature termination codon 20 residues downstream of the mutation). (C) Segregation of the mutation throughout the family was verified using digestion with *MspI* DNA endonuclease. As the mutation abolishes a restriction site for this enzyme, the patient (filled symbol) as well as his father demonstrate both an uncut fragment (339 bp) and two cut fragments (241 bp and 98 bp).

Only 12 of 25 mutant alleles were detected by CSGE screening (**Fig 2**), reflecting a sensitivity of about 50% for this screening method. Subsequent direct DNA sequencing of the entire coding region and flanking splice sites of SPINK5 in the remaining disease alleles revealed 13 additional mutations and a large number of nonconsequential polymorphisms mainly in the noncoding regions of SPINK5 (data not shown). Nevertheless, we did not identify any pathogenic sequence variation in 13 disease alleles of patients from eight unrelated NTS families. It is possible that these patients carry mutations in the 5' regulatory region of the gene, within introns, or in the carboxy-terminal untranslated region, which may alter expression or function of the protein.

We compared our molecular data with the clinical presentation of NTS in our cohort (**Tables I and III**). Our analysis revealed that

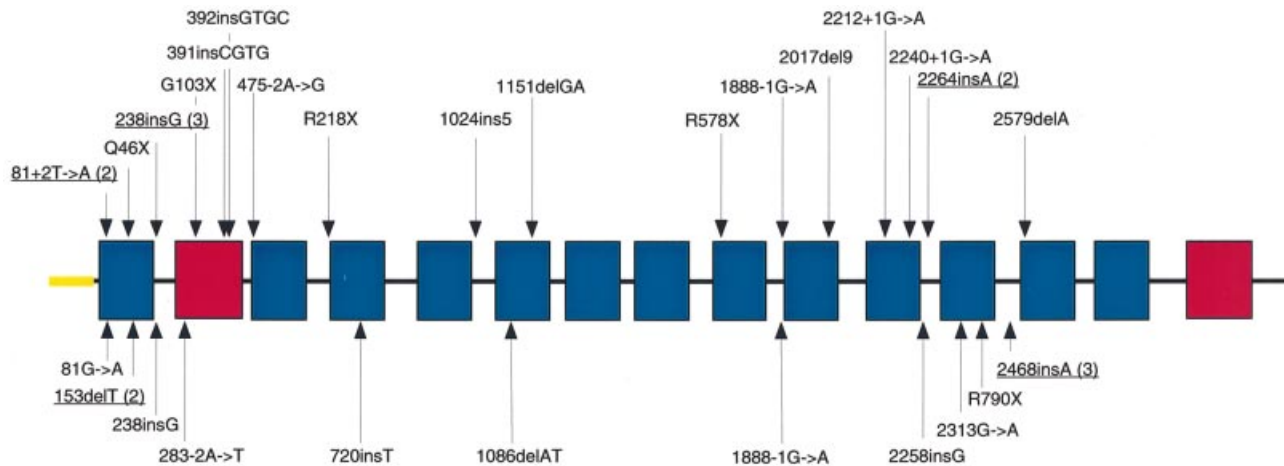


Figure 3. Spectrum of mutations in SPINK5. Mutations are indicated along a schematic representation of the LEKTI polypeptide. Typical Kazal-type domains are represented in red, other Kazal-like domains are depicted in blue. The N-terminal secretory signal peptide is colored in yellow. Mutations reported in the present study appear above the diagram, and previously reported mutations (Chavanas *et al*, 2000b) appear below the diagram. Mutations that were observed more than once are underlined, and the number of unrelated families carrying this mutation is shown in parenthesis.

the presence of homozygous frameshift and splice site mutations toward the 5' end of SPINK5 (81 + 2T→A, 238insG, 391insCGTG) resulted in a severe phenotype with generalized, CIE-like skin manifestation (families 5, 6, 7, and 8). These mutations are predicted to interrupt LEKTI and to eliminate almost all KAZAL-like domains rendering the protein nonfunctional (null alleles); however, the phenotype of NTS patients who were compound heterozygotes for a premature termination codon or splicing mutation was variable, and did not allow drawing any definitive genotype–phenotype correlations.

Prenatal diagnosis of NTS A 32 y old healthy mother of two children, one of which has NTS (family 4 in **Table I**), was referred to our study in the seventh gestational week of her third pregnancy. Mutation analysis of SPINK5 demonstrated that the affected child was compound heterozygous for two SPINK5 mutations, each inherited from one parent (**Table III**). The maternal mutation 475-2 A→G alters a consensus splice acceptor site and is predicted to result in skipping of exon 7. The paternal mutation, 2264insA results in a frameshift introducing a premature termination codon 1 amino acid downstream of the mutation site (**Fig 4A**). This mutation could lead to the synthesis of a truncated translation product or to the absence of protein (null allele) as a consequence of non-sense mediated mRNA decay. In addition to these mutations, each parent carried a heterozygous nonconsequential sequence polymorphism, the father a T→C substitution in intron 23 at position 2241–26, and the mother a silent polymorphism R177R (531G→A transition) in exon 7. In contrast to the affected child, who inherited both mutations but neither polymorphisms, the unaffected sibling carried only the polymorphisms. Therefore, we were able to establish the phase and determine that the nondisease associated polymorphisms were located on the parental wild-type allele (**Fig 4B**). Prenatal diagnosis was performed on gDNA extracted from fetal chorionic villi obtained by CVS at the 12th week of pregnancy. Direct DNA sequencing of SPINK5 amplicons harboring the known sequence variants revealed the absence of both pathogenic mutations but presence of both sequence polymorphisms in the fetus (**Fig 4A**). These results ruled out a possible contamination of the tested sample with maternal cells, and conclusively determined that the fetus was unaffected (**Fig 4B**). As predicted, the mother gave birth to a healthy child. Our data demonstrate the feasibility of prenatal diagnosis for NTS and also underscore that intragenic polymorphic sites can be valuable assets assisting genetic testing.

DISCUSSION

Protease inhibitors play a central part in the maintenance of many physiologic functions such as food digestion, blood coagulation, and extracellular matrix remodeling by limiting unwanted proteolytic activities and thereby regulating cell migration, proliferation, and differentiation. Serine protease inhibitors comprise four families, serpins, leuco-proteases, Kunitz type and Kazal type serine protease inhibitors (Bode and Huber, 1992; Roberts *et al*, 1995). The latter family is characterized by three conserved disulfide bonds and a reactive site loop of each inhibitory domain, for which pancreatic secretory trypsin inhibitor is a prototype (Roberts *et al*, 1995). Pancreatic secretory trypsin inhibitor has only a single active domain that targets trypsin, whereas other avian Kazal-type serine protease inhibitors have multiple inhibitory domains (up to seven) with different specificities for trypsin, chymotrypsin, elastase, and subtilisin, and might have the potential to inhibit these simultaneously (Saxena and Tayyab, 1997). LEKTI contains an unprecedented number of 15 inhibitory domains, two of which represent classical Kazal sites. The remaining 13 Kazal-like domains, one of which was shown to inhibit trypsin *in vitro*, contain only two intramolecular disulfide bonds (Mägert *et al*, 1999). Nevertheless, the actual physiologic substrates of LEKTI are currently unknown.

The results of this study (**Table III**) confirm the recent report by Chavanas *et al* (2000b) and unequivocally establish that mutations in SPINK5 and subsequent elimination/inactivation of the serine protease inhibitor LEKTI are the molecular cause of NTS. The pathomechanisms leading to this complex disease, however, remain to be elucidated. As SPINK5 is highly expressed in the thymus (Mägert *et al*, 1999), defective LEKTI expression might have a deleterious effect on T-cell differentiation, thus explaining the unbalanced Th2 immune response with markedly elevated IgE levels and the increased susceptibility to infections characteristic for NTS. It is also possible that like other serine protease inhibitors LEKTI is expressed as part of a cytokine-activated acute-phase response to trauma and against bacterial and chemical assaults (Yasuda *et al*, 1993; Roberts *et al*, 1995).

A wide variety of protease inhibitors, including LEKTI are expressed in the skin (Abts *et al*, 1999; Alkemade *et al*, 1994; Scott *et al*, 1998; Cui *et al*, 1999), suggesting that these enzymes may be crucial for controlling epidermal cell growth and differentiation. Pathogenic mutations in the cathepsin C gene, which encodes a protease expressed in epithelial tissues, have been shown to underlie two genodermatoses, Papillon–Lefevre syndrome (MIM 245000)

Table III. Spectrum of mutations in SPINK5

Family	Mutations	Location	Nucleotide change	Verification	Predicted consequence ^b
1	238insG	Exon 4	GGGCC→GGG <u>G</u> CC	CSGE/sequencing	PTC + 18
	Q46X	Exon 3	CCCAG→CCTAG	+StyI	PTC
2	2017del9	Exon 22	ACTCAGgtgag→Ag	-MslI	Altered splicing
	G103X	Exon 5	AAGGAG→AATGAG	CSGE/sequencing	PTC
3	2579del9	Exon 27	GAAG→GAAG	CSGE/sequencing	PTC + 63
	1888-1 G→A	Intron 20	tagGAG→taGAG	+ MseI	Altered splicing
4	475 -2 A→G	Intron 6	cagGAT→cggGAT	-Eco57I	Altered splicing
	2264insA	Exon 24	AAGGAG→AATGAG	CSGE/sequencing	PTC + 1
5	81+2 T→A	Intron 2	Aggtt→Aggat	CSGE/sequencing	Altered splicing
	81+2 T→A	Intron 2	Aggtt→Aggat	CSGE/sequencing	Altered splicing
6	391insCGTG	Exon 5	TGCA→TGCGTGCA	CSGE/sequencing	PTC + 4
	391insCGTG	Exon 5	TGCA→TGCGTGCA	CSGE/sequencing	PTC + 4
7 ^c	238insG	Exon 4	GGGCC→GGG <u>G</u> CC	CSGE/sequencing	PTC + 18
	238insG	Exon 4	GGGCC→GGG <u>G</u> CC	CSGE/sequencing	PTC + 18
8 ^c	238insG	Exon 4	GGGCC→GGG <u>G</u> CC	CSGE/sequencing	PTC + 18
	238insG	Exon 4	GGGCC→GGG <u>G</u> CC	CSGE/sequencing	PTC + 18
9	392insGTG	Exon 5	GCAC→GCGTGCAC	CSGE/sequencing	PTC = 4
	2264insA	Exon 24	AAAAA→AAAAAT	CSGE/sequencing	PTC + 1
10	81+2 T→A	Intron 2	Aggtt→Aggat	CSGE/sequencing	Altered splicing
	1024ins5	Exon 12	AAG→AGAAAAAAG	CSGE/sequencing	PTC + 29
11	2240+1 G→A	Intron 23	ATTgta→ATTata	CSGE/sequencing	Altered splicing
	R218X	Exon 8	GACGAA→GATGAA	CSGE/sequencing	PTC
12	1151delGA		GAGAAC→GAAC	CSGE	PTC + 19
	?				
13	2212+1 G→A	Intron 22	CAGgtg→CAGatg	-HphI	Altered splicing
	?				
14	R578X	Exon 19	ATG <u>G</u> AC→ATTGAC	+FokI	PTC
	?				
15					
16					
17					
18					
19					

^aNumbers denote amino acid positions of nonsense mutations or cDNA nucleotide position (starting from ATG) for other mutations.

^bPTC + n = premature termination codon occurring n residues downstream to the mutation.

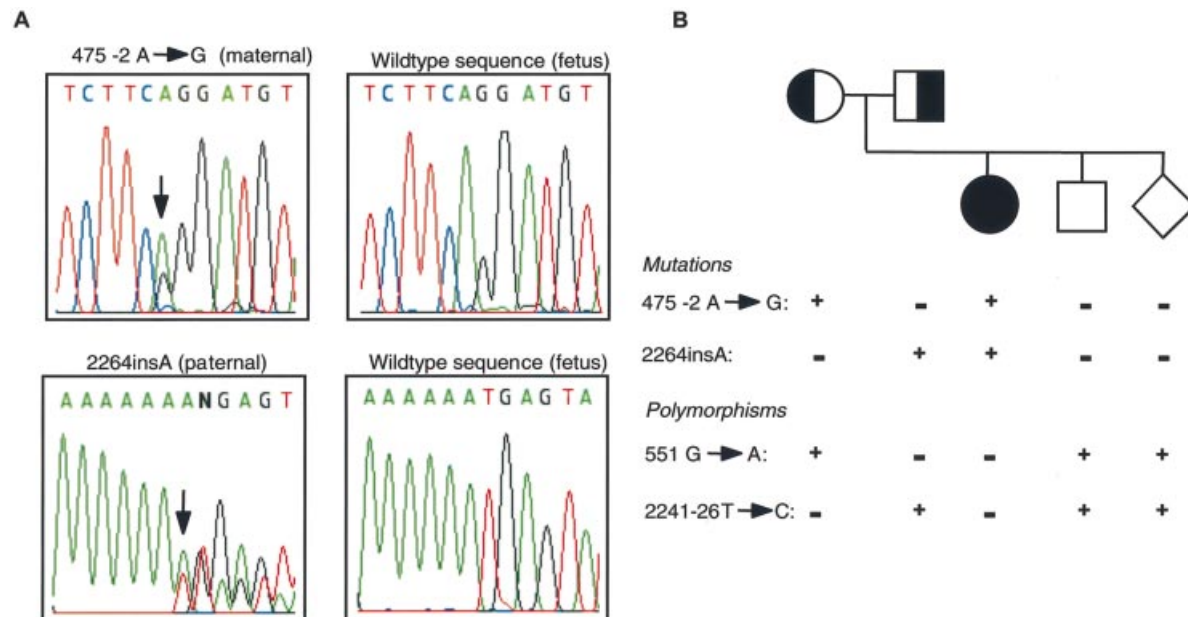


Figure 4. Prenatal diagnosis of NTS. (A) Two mutations were detected in the gDNA of the affected child in family 4, an insertion mutation carried by the father (2264insA) and a splice site mutation carried by the mother (475-2 A→G). Neither mutation was present in the 12 wk old fetus as tested from chorionic villi samples. (B) Summary of mutation analysis in all members of family 4. Note the presence of distinct heterozygous polymorphic markers in both parents. Both polymorphisms were present in the unaffected fetus and a healthy sib, whereas they were absent in the affected child, thus indicating that they must be carried on the normal maternal and paternal alleles.

and Haim-Munk syndrome (MIM 245010) (Hart *et al*, 2000; Toomes *et al*, 1999; Nakano *et al*, 2001). The function of proteases and their inhibitors might be particularly important for proper desquamation, which requires the proteolytic dissolution of intercellular cohesive structures in the stratum corneum (Lundstrom and Egelrud, 1990). Several proteases such as the stratum corneum trypsin-like serine protease (Ekholm *et al*, 2000) and stratum corneum chymotryptic enzyme (Horikoshi *et al*, 1999; Eckholm and Egelrud, 2000) are thought to play a pivotal part during this process, which could potentially be controlled by LEKTI.

We identified 17 distinct SPINK5 mutations in our international cohort of NTS patients. All of the mutations can be predicted to truncate or disrupt the mutant LEKTI protein due to premature termination of translation or generation of altered splice products, respectively. It is conceivable that missense mutations in SPINK5 occur but do not manifest clinically due to the unusual large number of repetitive protease inhibitory domains of LEKTI (15 sites) resulting in functional redundancy (Mägert *et al*, 1999). In contrast, missense mutations of SPINK1, which encodes a protein with a single Kazal domain, were found in both hereditary and sporadic forms of chronic pancreatitis (Witt *et al*, 2000).

Including this report, a total of 26 distinct SPINK5 mutations have been identified to date (Fig 3). Small insertions ($n = 8$) and splice mutations ($n = 8$) account for the majority, whereas small deletions ($n = 5$) and nonsense mutations ($n = 5$) were somewhat less common. Our study confirmed that most pathogenic mutations are private and occur throughout the gene. Of note, however, is that three mutations were identified in two or more unrelated families from different genetic backgrounds (238insG, 81 + 2T→A, 2264insA). As two of these sequence alterations were located within three (238insG) and six (2264insA) mononucleotide repeats, slipped mispairing at the replication fork may account for their repeated occurrence. 238insG in exon 4 and 1888-1G→A in intron 20 were previously reported by Chavanas *et al* (2000b) who also identified mutation 2468insA in exon 26 in three unrelated families. Cumulative data in Fig 3 illustrate that 46% of all 26 identified mutations cluster between exons 2 and 9, accounting for almost half of all disease alleles (40%). Thus, our mutation detection strategy includes an initial analysis of exons 4 and 5, followed by exons 1-3, and finally exons 6-33. The considerable size of the SPINK5 gene indicates the need for a powerful presequencing screening method. We found the sensitivity and efficacy of CSGE not satisfying, but denaturing high performance liquid chromatography, which has reportedly a high sensitivity between 95% and 100% (Ellis *et al*, 2000; Jones *et al*, 1999), may prove of value for future mutation screening of SPINK5.

Regardless of the evidence for locus homogeneity in NTS and despite sequence analysis of the entire coding region, we were unable to detect pathogenic SPINK5 mutations in 34% of the disease chromosomes. Most likely, mutations in untranslated portions of the gene affecting protein expression, including promoter region, regulatory intronic sequences, and distal 3'UTR could account for the disease. It will be challenging to screen these regions because of the large size of SPINK5 introns (on average 1.5 kb) and the great number of nonconsequential sequence variants within these regions (data unpublished). Alternatively, large deletions or rearrangements of SPINK5, which would not be readily detectable with PCR-based screening methods, may occur; however, the presence of several heterozygous sequence polymorphisms in the genomic sequence of SPINK5 in most affected individuals (data not shown) renders this hypothesis less likely.

Genotype-phenotype correlations in our cohort revealed that homozygous frameshift and splicing mutations predicted to result in early truncation of LEKTI were associated with severe disease and in one case (family 8) fatal outcome (Tables I and III); however, an affected individual compound heterozygous for such mutations in exons 3 and 4 displayed a relatively mild phenotype with ILC skin manifestation (family 1, Tables I and III). As both ILC and

CIE-like features have been reported in affected siblings who presumably carry identical mutation(s) (Traupe, 1989), it is likely that other genetic and/or epigenetic factors contribute to the phenotype, which should be considered for genetic counseling of NTS families.

The elucidation of SPINK5 mutations as the proximal cause of NTS has broken the ground for the molecular diagnosis of this complex disease and allows for improved genetic counseling. As attested by our study, it is now possible to perform successfully prenatal diagnosis of NTS as early as in the 10th to 12th gestational week when the disease-causing SPINK5 mutations of the family can be established. The feasibility of prenatal testing will without doubt be of considerable benefit to NTS families with children suffering from a severe or even fatal disease. On the other hand, future molecular testing could strongly facilitate timely diagnosis of NTS in patients with neonatal and infantile erythrodermic ichthyosis or with equivocal clinical features. A systematic investigation of families with NTS or atopic diathesis for SPINK5 mutations may eventually shed light on the long-debated relationship between NTS, atopic eczema, and asthma.

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