

CLINICAL REPORT

Ichthyosis Linearis Circumflexa as the Only Clinical Manifestation of Netherton Syndrome

Liliana GUERRA¹, Paola FORTUGNO², Cristina PEDICELLI³, Cinzia MAZZANTI³, Vittoria PROTO¹, Giovanna ZAMBRUNO¹ and Daniele CASTIGLIA¹

¹Laboratory of Molecular and Cell Biology, ²Dermatology Division, Istituto Dermatologico dell'Immacolata, IDI-IRCCS, and ³Dermatology Unit, Bambino Gesù Children's Hospital-IRCCS, Rome, Italy

Ichthyosis linearis circumflexa (ILC) presents as serpiginous and migratory erythematous patches with double-edged scales. ILC is rarely an isolated skin manifestation, but most commonly a part of Netherton syndrome (NS). NS is caused by *SPINK5* mutations, which lead to absent or sometimes reduced expression of the serine protease inhibitor LEKTI. NS is characterised by congenital ichthyosiform erythroderma, trichorrhexis invaginata (TI) and atopy. We report 2 children who presented since the first months of life cheek erythema followed by the appearance of sparse ILC lesions on the face, trunk and proximal extremities. Erythroderma at birth, TI and atopy were absent. LEKTI immunoreactivity was reduced in patient epidermis, and serine protease activity was modestly increased, while desmoglein-1 expression remained unaffected. *SPINK5* mutation and expression analysis in patient keratinocytes revealed compound heterozygous splicing variants, which allowed residual LEKTI secretion. Our results show that ILC can be the only clinical manifestation of NS. Key words: ichthyosiform disorder; keratinisation; mutational screening; *SPINK5*.

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Daniele Castiglia, PhD, Laboratory of Molecular and Cell Biology, Istituto Dermatologico dell'Immacolata (IDI)-IRCCS, via dei Monti di Creta 104, IT-00167 Rome, Italy. E-mail: d.castiglia@idi.it

Ichthyosis linearis circumflexa (ILC) is a rare dermatological disorder of keratinisation which may be inherited as an autosomal recessive trait (1–3). It is characterised by migratory, annular and polycyclic erythematous patches with double-edged scales at the periphery of the lesions. ILC has rarely been reported as an isolated clinical manifestation (2, 4–9). Most frequently, ILC participates of the classical triad of congenital ichthyosiform erythroderma, trichorrhexis invaginata (TI), and atopic manifestations that characterises Netherton syndrome (NS), a rare autosomal recessive syndromic ichthyosis due to loss-of-function mutations in *SPINK5* (10, 11). The *SPINK5* product, LEKTI (lymphoepithelial Kazal-type

inhibitor), is a 15-domain (D1–D15) inhibitor of trypsin-like and chymotrypsin-like serine proteases expressed in the epidermis. LEKTI is proteolytically processed in multiple bioactive fragments with different inhibitory functions and released in the extracellular space between the granular and horny layer. Its deficiency results in elevated protease activities and increased degradation of corneodesmosomal cadherins, in turn leading to stratum corneum premature detachment and highly defective skin barrier (10, 12). Fragments D6–D9, D7–D9 and D8–D9 have the strongest inhibition towards kallikreins (KLK) 5 and 14 within the stratum corneum (13, 14). Most of the *SPINK5* truncating mutations identified in NS cause undetectable LEKTI in epidermis. However, LEKTI absence is associated with inter- and intra-familial phenotypic variability, indicating the existence of disease modifiers (10). In addition, genotype–phenotype correlation studies have suggested that *SPINK5* mutations interrupting the coding frame near the C-terminus may allow for retention of functional LEKTI fragments, leading to less severe phenotypes (12, 13).

Here, we report the clinical and molecular characterisation of 2 siblings who developed ILC in the first months of life in the absence of any other skin and hair manifestation.

METHODS

Patients

Following written informed consent, skin biopsies and hair samples were collected from both children for immunopathological studies, keratinocyte cultures and hair analysis. Blood samples were taken for genetic analysis. The study was conducted in compliance with the Declaration of Helsinki principles.

Mutation and reverse transcriptase (RT)-PCR analyses

Genomic DNA extracted from blood was used for amplification and bidirectional sequence of *SPINK5* exons and flanking intronic borders (GenBank AJ228139.2), as earlier described (15). Total RNA was extracted from *in vitro* differentiated keratinocytes derived from both the patient and a healthy subject using TRIzol™ reagent (Invitrogen, Carlsbad, CA, USA). Complementary DNA was synthesised using Superscript™ III RT (Invitrogen) as described (16) and amplified with the following primers: (F) 5'- acctgctccatgtgtgaggt and (R) 5'- tctgttgaaagaaggctcac encompassing exons 13–15, and

(F) 5'-aatgctgcaggacatgggttc and (R) 5'-ggcttctaaactcatcacatg encompassing exons 22–25.

Immunodetection of LEKTI and desmoglein-1

Immunohistochemistry was performed on skin sections from formalin-fixed paraffin-embedded biopsies using an anti-LEKTI polyclonal antibody directed to the D13D15 C-terminal region, as described (17).

To perform western blot analysis, a skin biopsy was processed for keratinocyte cultures (17). Keratinocytes were maintained in low calcium KGM (Invitrogen) until confluent, and then, terminal differentiation was induced by raising the calcium concentration to 1.2 mM for 5 days. For LEKTI fragments analysis, conditioned media (400 ml) from differentiated control and patient keratinocytes were separated on a 12% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to a PVDF membrane (Amersham Biosciences, Inc., Piscataway, NJ, USA). Membranes were incubated with anti-LEKTI-D7D12 polyclonal antibody (14). Ponceau S staining (Sigma Aldrich, St Louis, MO, USA) was used to check for protein loading among samples.

For immunofluorescence analysis, frozen skin sections (5 µm thickness) were air-dried for 15 min and fixed in ice-cold methanol:acetone 1:1 for 10 min before the staining procedure. After 1 h preincubation with 1% BSA, the sections were incubated with an anti-desmoglein-1 polyclonal antibody (H-290, Santa Cruz Biotechnology, CA, USA) 1 h at room temperature. Specimens were subsequently incubated with Alexa Fluor® 488 anti-rabbit IgG (Invitrogen) 1 h at room temperature and mounted with Vectashield mounting medium with DAPI (Vector Laboratories, Burlingame, CA, USA). Slides were evaluated using a Zeiss Axioskop 2 (Carl Zeiss, Jena, Germany) and photographed using the Zeiss AxioVision 4 microscope software.

In situ zymography

For *in situ* zymography, frozen skin sections (5 µm thickness) were rinsed with a washing solution (2% Tween 20 in PBS) and incubated at 37°C overnight with 100 µl of BODIPY® FL casein (Invitrogen) (10 µg/ml) to assess total protease activity. All sections were rinsed with PBS solution and visualised as above. Sections from control skin, ILC and NS patients were photographed at equal time points and exposure time. Images were captured and analysed with the ImageJ software (<http://rsb.info.nih.gov/ij/>). The intensity of the fluorescence signals was coded as colour gradient, ranging from 0 (dark) to 255 (white).

RESULTS

Clinical features and laboratory findings

A 29-month-old boy was the first child of healthy non-consanguineous Caucasian parents. The mother reported the appearance of erythematous and scaly lesions on the cheeks in the first month of life. The lesions were intensely itchy and rapidly spread to the trunk and proximal extremities. On examination, several erythematous patches with double-edged scaling borders were observed

on the trunk, buttocks and proximal extremities (Fig. 1a, c). The 1-year-old younger sister also had a similar history of erythematous scaly and itchy lesions, which presented 3 months after birth (Fig. 1b). These findings were consistent with ILC.

In both children, the skin lesions modestly improved following topical steroid application. Both patients showed normal-looking hair, eyebrows and eyelashes (Fig. 1d), and light microscopy examination revealed no abnormalities of the hair shaft. Growth and development of the 2 siblings were normal and they did not present any atopy manifestation. Laboratory results, including total serum IgE, were unremarkable. During the one year follow-up, the skin disease showed a spontaneously remitting and relapsing course with almost complete resolution in summertime.

Histological examination of a skin biopsy showed acanthosis of the epidermis with elongated rete ridges and a reduced granular layer. Immunohistochemical analysis of LEKTI expression showed a positive staining of the granular and upper spinous layers and hair infundibulum in patient skin (Fig. 2b), although the signal was weaker than in control skin (Fig. 2a). Because of LEKTI decreased expression, *SPINK5* sequence analysis was performed and revealed compound heterozygosity for the c.1302+4A>T (intron 14) and c.2240+1G>A (intron 23) splice site mutations, which



Fig. 1. Clinical features of ichthyosis linearis circumflexa in the 2 siblings. Typical annular, erythematous patches in the 29-month-old boy (a) and his 1-year-old sister (b). Closer view showing the double-edged scale at the periphery of the lesions on the trunk of the proband at the age of 30 months. The reddish colour is due to application of 2% eosin (c). Note the normal-looking appearance of hair and eyebrows in the sister, in addition to cheek erythema and scaling (d).

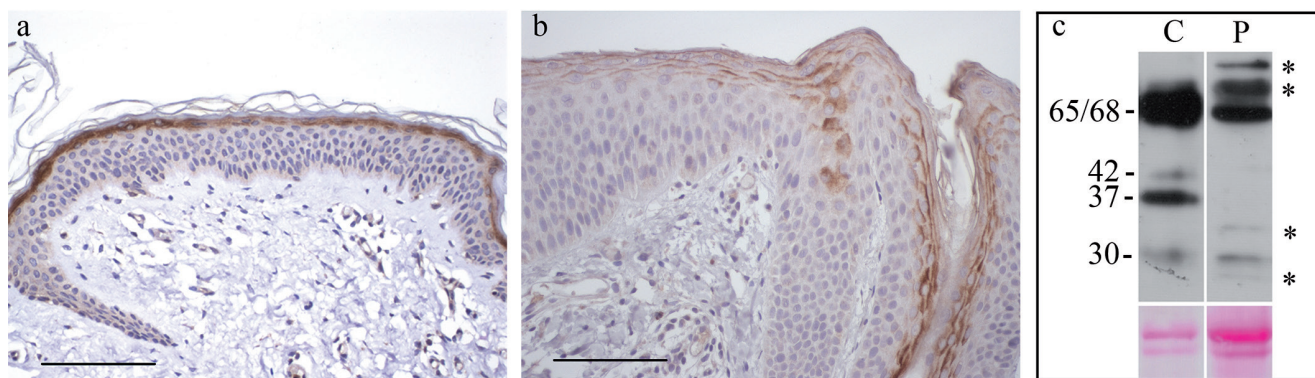


Fig. 2. Patient skin and cultured keratinocytes express reduced amounts of LEKTI protein. Skin immunohistochemistry with anti LEKTI D14-D15 polyclonal antibody shows a positive staining in the granular layer of patient epidermis (b), although the signal is weaker than that observed in control skin (a). Note LEKTI positive staining also in the infundibulum of hair follicle (b). Scale bar: 100 μ m. Immunoblot analysis of LEKTI expression in *in vitro* differentiated keratinocytes using the anti-D7D12 polyclonal antibody (c). LEKTI bioactive fragments D10D15 (65/68 kDa band), D10D13 (42 kDa), D6D9 (37 kDa), and D7D9 (30 kDa) were detected in the keratinocyte-conditioned medium of a normal individual (C). The 65/68 and 30 kDa fragments are observed in the patient's sample (P), although the intensity of each band is extremely reduced. Additional unprocessed and/or partially processed fragments are also visible (asterisks). Protein loading is assessed by Ponceau-S staining of the blotted membrane (bottom panel). Note that patient sample is overloaded to allow better visualisation of the bands.

were inherited from the mother and the father, respectively (Fig. S1a, b, left panels¹). In addition, both siblings were heterozygous for the p.Glu420Lys (c.1258G>A) polymorphism in exon 14. Allele segregation analysis showed that the c.1258G>A and c.1302+4A>T variants were in linkage on the maternal allele (Fig. S1c¹).

SPINK5 mRNA analysis and LEKTI expression in cultured keratinocytes

Mutation consequences were analysed by RT-PCR using mRNA purified from *in vitro* differentiated patient cultured keratinocytes (Fig. S1a, b right panels¹). We confirmed that the c.2240+1G>A mutation raises the usage of a cryptic donor splice site (CT/gtacgt) within exon 23, 59 bp upstream the mutant donor site, resulting in shorter transcripts that terminate translation in exon 24 (18). RT-PCR across the site of c.1302+4A>T mutation also resulted in 2 cDNA bands. Direct DNA sequencing of the shorter fragment demonstrated that it contains an improper exon 14/exon15 junction and a premature termination codon (PTC) in exon 15. These occurred due to the activation of a cryptic donor site (AG/gtaaat) situated 47 bp upstream the mutant site. However, sequencing of the full-sized fragment showed normal exon 14/exon 15 junction and a mixture of nucleotides A and G at position c.1258. The presence of normally spliced transcripts that carry the c.1258A variant, which is linked to the c.1302+4A>T, demonstrates that this mutation does not completely abolish splicing of exon 14. Indeed, analysis of patient keratinocyte conditioned medium by western blot using the anti-D7D12 polyclonal anti-

body (Fig. 2c) revealed the secretion of low amounts of physiological LEKTI peptides, the most appreciable being fragments D10D15 (65/68 kDa band) and D7D9 (30 kDa). Additional unprocessed and/or partially processed LEKTI fragments were also visible (Fig. 2c).

LEKTI inhibitory activity

The total protease activity in the epidermis of our ILC proband and a patient with severe NS (compound heterozygous for the c.238dupG and p.Arg217* mutations) was also examined by *in situ* zymography and compared to that of a normal individual. In normal skin, a weak caseinolytic activity was mainly visualised in the granular layer (Fig. 3a) while in NS epidermis its intensity was greatly enhanced in the granular and upper spinous layers (Fig. 3c). The epidermis of our patient showed in-between intensity of the caseinolytic activity (Fig. 3b). Since desmoglein-1 (DSG1) is a target of an excess of serine protease activity on the corneodesmosome, we tested its expression in the skin of our proband, a normal individual and the patient with severe NS. We observed a highly reduced expression of DSG1 in the upper epidermal layers in NS skin (Fig. 3f), while in our patient (Fig. 3e) the expression was maintained to levels similar to those of a normal control (Fig. 3a).

DISCUSSION

Disease manifestations in our 2 siblings were limited to mild lesions of ILC, whereas congenital erythroderma and hair alterations were absent. Moreover, both children did not show any other NS symptom, such as hypernatraemic dehydration in infancy, recurrent bacterial, fungal and viral infections, growth retardation

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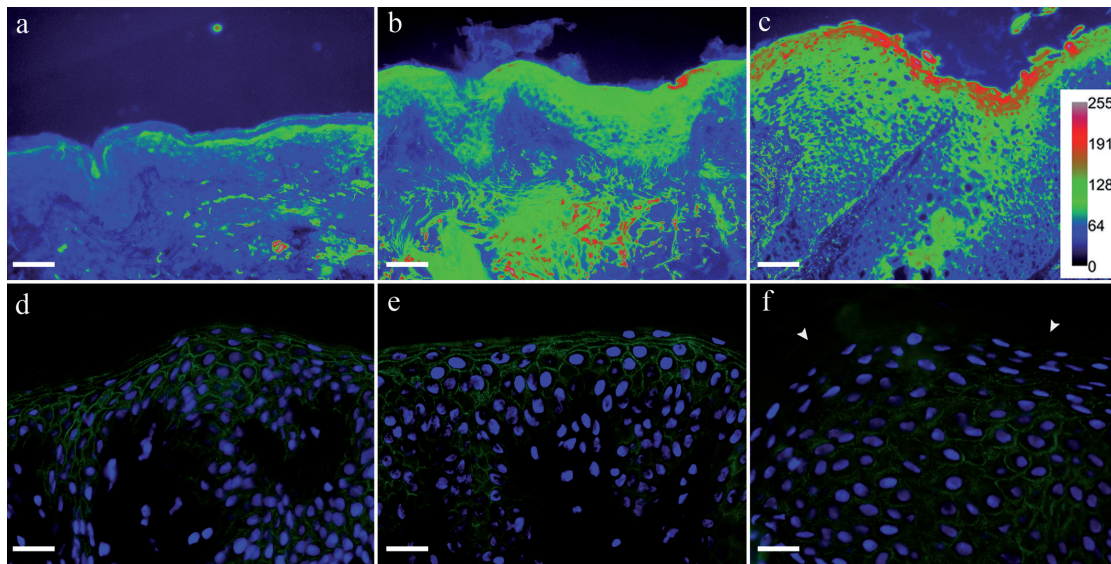


Fig. 3. Patient skin shows a modest increase in protease activity and a normal expression of desmoglein 1 (DSG1). In the skin of a normal individual (a), a weak caseinolytic activity is mainly visualised in the granular layer, while in the epidermis of a severe NS patient (c) its intensity is greatly enhanced in the granular layer and the uppermost layers of the spinous compartment. The epidermis of the ILC patient (b) shows in-between intensity. Immunofluorescence for DSG1 shows a pericellular staining of similar intensity in normal and ILC skin (d, e), and highly reduced staining in particular in the uppermost epidermal layers (arrowheads) in a severe NS patient. Scale bars, 10 μm (a-c) and 50 μm (d-f).

or short stature. Finally, neither atopy manifestations nor elevated IgE levels were present, at least during the still short patient follow-up.

There are several reports in literature of patients suffering from ILC without congenital erythroderma and clinical or microscopic hair anomalies typical of NS (2–9, 19). These cases prompted some authors to consider ILC a distinct entity separated from NS with skin lesions of ILC (20). In 1974, Mevorah and collaborators (20) performed a literature review and proposed to distinguish 2 ILC groups, one represented by patients suffering from ILC with TI and the other by patients with ILC but without hair anomalies. The 2 groups differed significantly when compared for scalp involvement, face lesions and manifestations of atopy or other hypersensitivity, because these symptoms were most frequently associated with TI (20). However, these authors suggested that ILC with TI and ILC without hair changes should be considered as close variants of the same disease in patients showing more or less frequent manifestations of atopy, as atopy may be easily overlooked when only minor symptoms exist. Since this report, 9 additional cases of ILC without any other NS symptom and 3 ILC patients with associated clinical or biological evidence of atopy have been reported (2–9, 19) (Table S1¹). None of these cases have been tested for LEKTI expression or screened for *SPINK5* mutations.

In both our siblings the expression of LEKTI was reduced in the epidermis and hair follicle. Total protease activity was modestly increased in patient epidermis, but it did not affect the expression of DSG1. All together, these findings demonstrate that the residual levels of LEKTI in our siblings are adequate to ensure

a partial maintenance of the skin barrier function and to markedly mitigate phenotype severity. *SPINK5* mutation identification and analysis of mutation consequences in keratinocytes unveiled the molecular basis of patient phenotype. The identified mutations were already known (12, 18), however they had never been found in combination in the same patient. Both mutations affect *SPINK5* pre-mRNA splicing by activating exonic cryptic sites, the usage of which results in shorter transcripts with premature truncation immediately downstream the D6 (c.1302+4A>T) and D10 (c.2240+1G>A) LEKTI domains. The c.2240+1G>A mutation had been described in compound heterozygosity with null mutations (truncating near the N-terminus) in 4 patients with classic severe and/or moderate NS phenotype (12, 18, 21). The c.1302+4A>T transition was reported in combination with a different null mutation and resulted in a milder NS phenotype, which however was associated with TI (12). This “mild” NS patient shares several immunohistochemical parameters with our siblings, including residual LEKTI staining in the uppermost epidermis, modestly increased levels of serine protease activity and maintenance of DSG1 expression. Thus, mutation c.1302+4A>T is predictive for a mild phenotype due to its “leaky” effect on splicing, as demonstrated in the present study by RT-PCR and cDNA sequencing. Full-length transcripts linked to this mutation produce certain levels of wild-type LEKTI polypeptides that are released and processed in the expected pattern, as shown by immunoblotting. Moreover, since the D6D9 (37 kDa) fragment was not detected by immunoblotting, we infer that LEKTI peptides mostly derive from transcripts carrying the c.1258A/p.420Lys

variant, which favours both D6D9 cleavage and accumulation of its by-product D7D9 (30 kDa) (see Fig. 3) (22). In general, fragments generated from the LEKTI region comprised between D6 and D9 have been shown to display the most effective inhibitory effect towards KLK activity (13, 14). Interestingly, the D6–D9 region is spared also by the c.2240+1G>A mutation, which is predicted to generate truncated protein products that retain the D1–D10 region. The peculiar pattern of LEKTI domains released by patient keratinocytes thus likely originates mainly from protein products of the maternal allele and, to a lesser extent, from the paternal allele. We conclude that compound heterozygosity for a “leaky” mutation and a truncating mutation downstream D10 provides sufficient amounts of LEKTI fragments to markedly limit KLKs hyperactivity and determine the isolated and mild cutaneous phenotype of ILC in our patients. However, it is also possible that other genetic and environmental factors might attenuate the consequences of LEKTI defective expression.

In summary, we show for the first time that ILC alone (i.e. without any other NS symptom) results from a rare combination of *SPINK5* mutations. Our results confirm that isolated ILC is part of the NS phenotypic spectrum. They attest at the importance of *SPINK5* molecular analysis to recognise non-classical NS forms and properly counsel the families.

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