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ORIGINAL ARTICLE

A synonymous mutation in *SPINK5* exon 11 causes Netherton syndrome by altering exonic splicing regulatory elements

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Netherton syndrome (NS) is a rare, life-threatening ichthyosiform syndrome caused by recessive loss-of-function mutations in *SPINK5* gene encoding lymphoepithelial Kazal-type-related inhibitor (LEKTI), a serine protease inhibitor expressed in the most differentiated epidermal layers and crucial for skin barrier function. We report the functional characterization of a previously unrecognized synonymous variant, c.891C>T (p.Cys297Cys), identified in the *SPINK5* exon 11 of an NS patient. We demonstrated that the c.891C>T mutation is associated with abnormal pre-mRNA splicing and residual LEKTI expression in the patient's keratinocytes. Subsequent minigene splicing assays and *in silico* predictions confirmed the direct role of the synonymous mutation in inhibiting exon 11 inclusion by a mechanism that involves the activity of exonic regulatory sequences, namely splicing enhancer and silencer. However, this deleterious effect was not complete and a residual amount of normal mRNA and LEKTI protein could be detected, correlating with the relatively mild patient's phenotype. Our study represents the first identification of a disease-causing *SPINK5* mutation that alters splicing without affecting canonical splice sites.
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INTRODUCTION

Netherton syndrome (NS, MIM 256500) is a rare autosomal recessive genodermatosis mainly characterized by the triad of ichthyosiform erythroderma, atopy manifestations and a pathognomonic hair shaft alteration known as trichorrhexis invaginata.[1] The skin disorder begins at birth or shortly after in the form of a generalized exfoliative erythroderma, which can persist through life or evolve in childhood into a milder ichthyosiform condition characterized by migratory, serpiginous and erythematous plaques with distinctive double-edged scales. A relatively high postnatal mortality may mostly result from bacterial infections of the skin and upper/lower airways and electrolyte imbalances.[2]

Netherton syndrome is caused by loss-of-function mutations in the *SPINK5* gene encoding lymphoepithelial Kazal-type -related inhibitor (LEKTI), a 15-domain serine proteases inhibitor with a crucial role in regulating skin desquamation and maintaining epidermal barrier function.[3,4] Differentiated keratinocytes synthesize three different LEKTI precursors, which are rapidly processed and then secreted into several shorter bioactive fragments.[5,6] The *SPINK5* mutation database in NS comprises 62 different mutations,[7–10] and the genotype–phenotype correlation suggests that downstream mutations may partly allow for residual expression of functional LEKTI fragments, which results in less severe phenotypes.[11,12]

Here we report on functional characterization of the c.891C>T variant identified in the *SPINK5* gene in an Italian patient with NS. We use patient keratinocytes, minigene constructs and bioinformatic predictors to demonstrate that this synonymous change (p.Cys 297Cys), despite being distant from the acceptor splice site, induces an incomplete exon skipping by affecting a mechanism underlying splicing regulation.

MATERIALS AND METHODS

Case report and LEKTI analysis

The proband was a 4-year-old girl who was born at term from healthy non-consanguineous parents of Sicilian origin. During the first days of life, she developed erythematous, desquamating lesions at first localized around the mouth and in flexural areas. The lesions were intensely pruritic, presented an eczematous component, and rapidly spread to affect almost all the body surface, requiring hospitalization at 4 months of age. During hospitalization, combined therapy with steroids and antibiotics resulted in a marked improvement of the cutaneous condition, which, however, rapidly relapsed after treatment interruption. She suffered from allergic rhinitis and mild asthma, and growth delay (weight always below the 3rd centile). At 18 months of age, she presented a single episode of urticaria and angioedema requiring hospitalization. During the second year of life, the skin manifestations spontaneously improved, becoming more localized and less inflammatory. The patient also presented an almost complete alopecia till the age of 3 years. On examination,

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eczematous-like lesions and desquamating patches disseminated over the trunk and limbs were observed. Scalp hair was thin, short and brittle, and eyelashes and eyebrows sparse (Figure 1a). Laboratory examination showed elevated IgE levels (1,806 IU ml^{-1}). Light microscopy examination of hair showed trichorrhexis invaginata features, evocative of NS.

A skin biopsy was obtained from the patient after written informed consent and entirely processed for keratinocyte culture, as previously described.[4] Keratinocytes were maintained in low calcium KGM (Invitrogen, Carlsbad, CA, USA) up to confluence, and then, terminal differentiation was induced by raising the calcium concentration to 1.2 mM for 5 days. To evaluate the LEKTI protein expression level, secreted proteins were concentrated from conditioned medium of differentiated keratinocytes by acetone precipitation, separated on 12% SDS-polyacrylamide gel electrophoresis and analyzed by immunoblotting, using the anti-D7D12 polyclonal antibody that recognizes the central region of LEKTI.[13] All the expected LEKTI bioactive fragments were detected in the patient's keratinocyte-conditioned medium, although their expression was strongly reduced compared with the control, confirming NS diagnosis (Figure 1b).

SPINK5 genotyping and reverse-transcriptase PCR analysis in patient's keratinocytes

After written informed consent, genomic DNA extracted from blood was used for amplification and bidirectional sequence of all the 33 *SPINK5* exons and flanking intronic borders (GenBank AJ228139.2), as described.[14] Total RNA was extracted using TRIzol reagent (Invitrogen) from *in vitro* differentiated keratinocytes derived from both the patient and a normal subject. The cDNA was synthesized using Superscript III RT (Invitrogen), as described,[15] and amplified with the following primers: (F) 5′-TCAGAGGAAAACAGTAAAAC AG-3′ (exon 10) and (R) 5′-CTGGTGCAAGCAAGTTTTCC-3′ (exon 13).

SPINK5 minigene construction and splicing assay

To construct recombinant minigenes containing either the wild-type or mutant sequence, a 713-bp fragment of the *SPINK5* gene, encompassing 355 bp of intron 10, exon 11 (128 bp) and 230 bp of intron 11, was amplified by PCR from the constitutive DNA of the affected patient. PCR was performed with the use of Expand High Fidelity PCR System (Roche Molecular Diagnostics, Mannheim, Germany) and primers (F) 5′-CCGCTCGAGAAGTTCCCTG AACTTCCATG-3′ and (R) 5′-CGGGATCCTTGTCAGGACATTCCAGTGC-3′, which introduced 5′-*Xho*I and *Bam*HI linkers. The PCR product was then digested and cloned into the corresponding restriction sites of the exon trapping expression vector pSPL3 (Invitrogen). Subcloned alleles were sequenced to select wild-type and mutant minigenes, and check that no PCR-derived errors were introduced.

As the patient was also heterozygous for a single-nucleotide polymorphism (SNP) in exon 11, an additional minigene, which links the major SNP nucleotide to the synonymous mutation identified in exon 11, was constructed. To this end, the c.891C-to-T change was introduced in the wild-type minigene using the QuikChange-XLII Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) and appropriate primers. Wild-type and mutant vectors were transfected in HEK293 cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Twenty-four hours after transfection, total RNA was purified, reverse transcribed and amplified with vector-specific primers SD6 (5′-TCTGAGTCACCTGGACAACC-3′) and SA2 (5′-ATCTCAGT GGTATTTGTGAGC-3′).

Bioinformatic analysis

Exonic changes were analyzed for possible exonic splicing enhancer (ESE) and silencer (ESS) alterations with ESEfinder 3.0. (http://rulai.cshl.edu/tools/ESE/)[16,17] and Human Splicing Finder 2.4 (http://www.umd.be/HSF/)[18], which integrates multiple software. Splice site scores for *SPINK5* exon 11 were calculated using a program available at http://www.fruitfly.org/.

RESULTS AND DISCUSSION

SPINK5 mutation identification

On the basis of the clinical data and protein analysis, which demonstrated LEKTI deficiency in the proband's keratinocytes, mutational screening of *SPINK5* was initiated. Sequence analysis of the entire gene showed that the affected patient was compound heterozygote for the recurrent frameshift mutation c.153delT[7] in exon 3 of the paternal allele and for the novel variant c.891C>T in exon 11 of the maternal allele (Figure 1c). The patient was also heterozygous for the frequent SNP c.1004C/T in exon 11 (rs34482796, minor allele frequency: T=0.41). The c.891C>T variant is a synonymous substitution (p.Cys297Cys) localized 9 bp from the acceptor splice site. It was absent in the constitutive DNA of 102 ethnically matched normal subjects and, therefore, could represent a genuine pathogenic mutation or a private polymorphism linked to it.

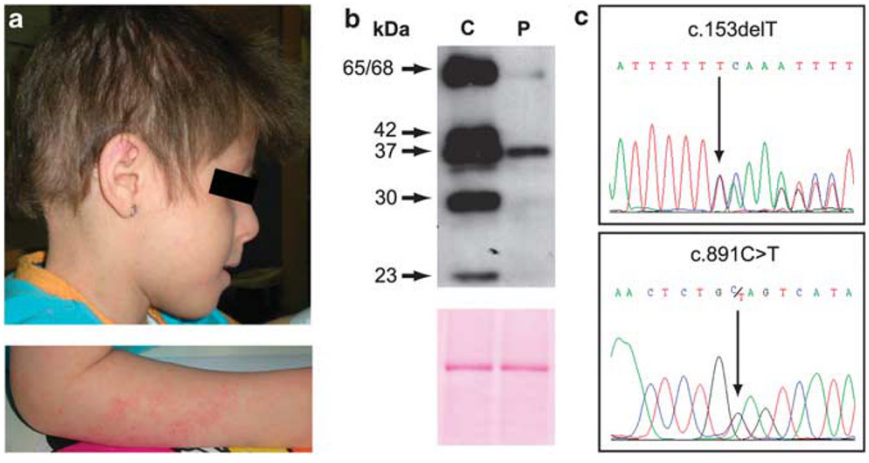

Figure 1 (**a**) Clinical findings of the NS patient. The patient presents short and brittle hair, sparse eyebrows, superficial desquamation with white, thin scales and a mild erythematous component on the neck and submandibular area, and eczematous-like plaques on the upper limb. (**b**) Immunoblot analysis of LEKTI expression in differentiated keratinocyte-conditioned media using the anti-D7D12 polyclonal antibody. LEKTI bioactive fragments D10D15 (65 and 68 kDa band), D10D13 (42 kDa), D6D9 (37 kDa), D7D9 (30 kDa) and D8D9 (23 kDa) are detected in the keratinocyte-conditioned medium of a normal individual (C).[13] The same pattern is observed in the patient's sample (P), although the intensity of each band is extremely reduced. Equal protein loading is assessed by Ponceau-S staining of the blotted membrane (lower panel). (**c**) Sequencing analysis of the compound heterozygous paternal c.153delT and maternal c.891C>T mutations affecting the NS patient. Overlapping sequence traces downstream c.153delT indicate the shift of the reading frame.

Reverse-transcriptase PCR analysis in patient's keratinocytes reveals exon 11 skipping

We then concentrated on the possible consequences of the c.891C>T variant on *SPINK5* pre-mRNA splicing. Reverse-transcriptase PCR analysis of total RNA purified from cultured patient and control keratinocytes was thus performed with primers encompassing exons 10–13. A single product of expected size (341 bp) was detected in the control subject, whereas an additional aberrant fragment with a smaller size (214 bp) was evident in the patient (Figure 2a). Sequencing analysis showed that the 214-bp band identified out-of-frame transcripts showing exon 11 skipping. The predicted LEKTI polypeptide lacks amino acids 295–337 and is truncated in the linker region between domain 4 and 5. Sequencing of the normal-sized cDNA product revealed a prevalent signal for nucleotide C at position c.891. The chromatogram also presented a barely detectable peak for T under the C, suggesting that minimal levels of normal-sized mRNA were expressed from the maternal allele associated with this variation. To further confirm this finding, the normal-sized cDNA fragment was recovered from the gel and digested with *Pst* I, a site that is lost in the allele carrying the c.891C>T transition. As expected, the fragment corresponding to a normal control was totally cut in two smaller products (256 and 85 bp), whereas the band from the patient remained partially undigested (Figure 2b).

Taken together, mRNA studies in the patient's cells indicate that the novel variant is involved in exonic skipping and that this event is not complete. As the c.153delT paternal mutation truncates the protein within the first serine protease inhibitory domain, the residual synthesis of LEKTI fragments detected by immunoblotting (Figure 1b) is directly linked to full-length transcripts from the allele carrying the c.891C>T variant.

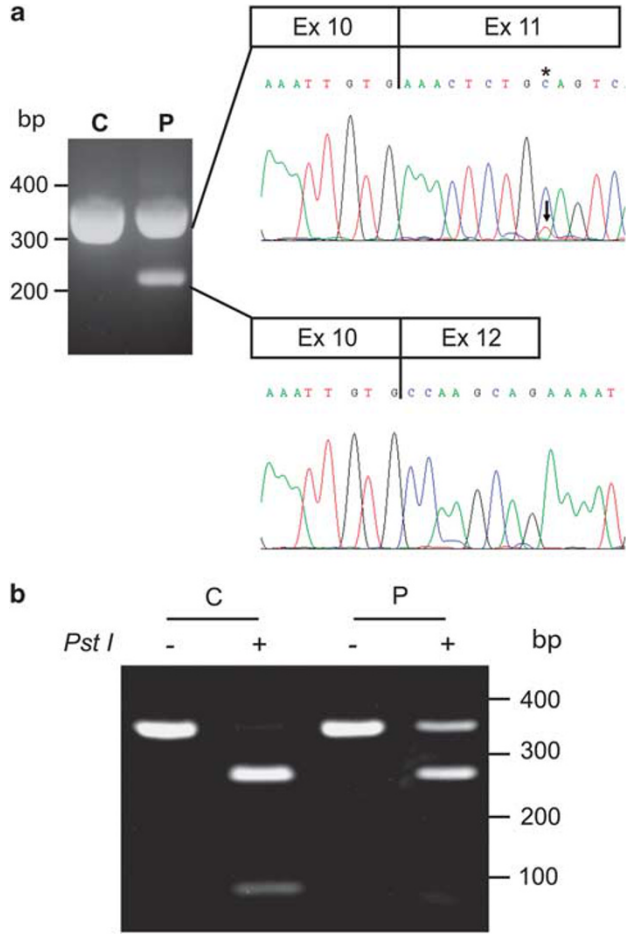

Figure 2 (**a**) Reverse-transcriptase PCR analysis in patient's keratinocytes. Two cDNA fragments of 341 and 214 bp are detected in patient's cells (P). The 214-bp band identifies the out-of-frame skipped transcripts lacking exon 11. The 341-bp fragment corresponds predominantly to full-length mRNA transcribed from the paternal allele bearing the c.153delT mutation, as attested by the high peak for C at position c.891 (indicated by an asterisk in the sequence). Note, however, that also a weak signal for T is detected in the same position (arrow). C, healthy control. (**b**) Allele specific analysis of the 341-bp cDNA fragment by *Pst* I digestion. The band generated from a normal control (C) is completely cut in two shorter products of 256 and 85-bp, whereas the band corresponding to the patient (P) remains partially undigested. Undigested DNA represents full-length transcripts linked to the c.891C>T mutation. Note that the *Pst* I-restricted fragment of 85 bp in the patient's sample is very scant; therefore, it is not visible in the gel.

The c.891C>T variant induces *SPINK5* exon 11 skipping in a minigene splicing assay

To test the possible causative role of the novel variant in preventing exon 11 splicing, we performed a reverse-transcriptase PCR study in HEK293 cells transfected with pSPL3-*SPINK5* minigenes containing either the wild-type or mutant exon 11. As the patient was also heterozygous for the SNP c.1004C/T in exon 11, the wild-type and mutant minigenes carried the C (*SPINK5*-891C/1004C) and T (*SPINK5*-891T/1004T) polymorphic nucleotides, respectively (Figure 3a). As neutral polymorphic variants may modulate the effects of splicing-inactivating mutations,[19] a minigene was also constructed that links the c.891T to c.1004C (*SPINK5*-891T/1004C). Almost all the

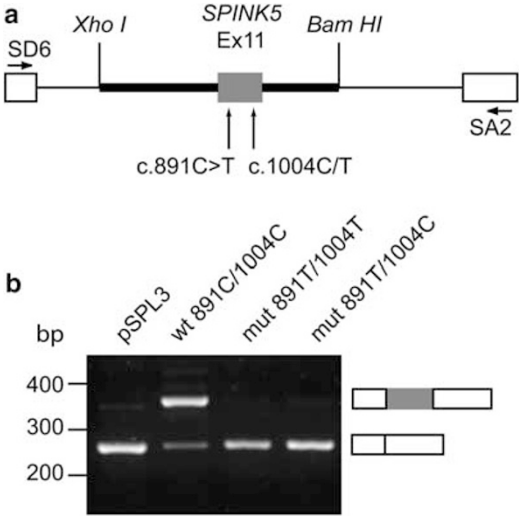

Figure 3 (**a**) Schematic diagram of the pSPL3-SPINK5 hybrid minigenes used for functional splicing assays in HEK293 cells. Three distinct constructs were designed: wt 891C/1004C, mut 891T/1004T and mut 891T/1004C, the last two containing mutation c.891C>T linked to either c.1004T or c.1004C SNP nucleotide, respectively. White boxes indicate vector-specific exons; dark gray box, *SPINK5* exon 11; thick lines, flanking *SPINK5* intronic region; SD6 and SA2, vector-specific primers used for reverse-transcriptase (RT)–PCR. (**b**) Ethidium bromide-stained agarose gel of the RT–PCR products resulting from the minigene transfections. The 389 and 261-bp bands correspond to the transcripts that include or exclude *SPINK5* exon 11, respectively. A scheme is depicted on the right. pSPL3, empty vector. Identity of the PCR products was confirmed by sequencing.

mature RNA expressed by wild-type *SPINK5*-891C/1004C retained exon 11 (Figure 3b, 389-bp band). In contrast, RNA from mutant *SPINK5*-891T minigenes linked to either c.1004C or c.1004T completely lacked exon 11 (Figure 3b, 261-bp band). These data conclusively demonstrated that the c.891C>T variant in *SPINK5* is directly responsible for exon 11 skipping independently from SNP c.1004C/T.

Bioinformatics identification of splicing regulatory sequences in *SPINK5* exon 11

Exonic point mutations away from splice sites may affect pre-mRNA splicing and thus be pathogenic. This phenomenon often involves auxiliary discrete *cis*-acting sequences that bind splicing factors to either positively (ESE) or negatively (ESS) control the choice of splice sites (i.e., exon definition).[20] These motifs are short (6–8 nucleotides) and degenerate, so a human exon of average size will comprise several of such motifs, which may or may not be functional.[16–18] The clinical impact of a splicing-affecting exonic variant is therefore difficult to anticipate. Some criteria can be adopted. Usually, the ESE/ESS variants occur near the ends of exons, where SNP density is reduced, and in exons small in size and with weak splice sites. In addition, they occur in regions where synonymous sites are under greater evolutionary conservation.[21–23] However, these criteria are poorly reliable. The best chance of recognizing a splicing pathology caused by a synonymous mutation relies on bioinformatics analysis using a combination of various predictive algorithms.[24] However, also the bioinformatics approach lacks specificity and, indeed, in our patient, studies at the mRNA levels were at first performed because the consequences of the synonymous change did not fit with the disease phenotype. These studies confirmed a major effect on splicing and thus, retrospectively, attested to the presence of exonic regulatory sequences in the region spanning the mutation site. When examined with the ESEfinder prediction program, which performs search for ESE, exon 11 revealed an ESE motif for SF2/ASF serine/arginine-rich protein across nucleotide c.891C (ctgCagt; score 2.04, with a threshold set to 1.867). This element was disrupted by introduction of c.891C>T mutation (ctgCagt>ctgTagt; score changes from 2.04 to 0, threshold 1.867). Interestingly, Human Splicing Finder analysis predicted also the generation of new ESS motifs in the mutant site. The ESS creation was scored by both the matrix developed by Wang *et al.*[25] (tgCagt>tgTagt) and by an experimental matrix designed for the inhibitory splicing factor hnRNPA1 (Cagtca>Tagtca; score changes from 0 to 74.76, threshold 65.476).[20] Therefore, bioinformatics analysis provides two possible explanations for exon 11 skipping: disruption of a splicing enhancer element for SF2/ASF protein or the creation of an hnRNPA1-dependent splicing silencer motif. Controversial models are not unusual and reflect the nature of ESE and ESS sequences, which are redundant and degenerate, and the complexity of the splicing process, which is finely regulated by an interplay between different regulatory elements.[16–19,24] Mutation c.5080G>T in *BRCA1* provides a precedent. This variant causes exon 18 skipping and was initially thought to disrupt an ESE for SF2/ASF by *in silico* analysis. Later, it was experimentally shown to create an ESS element recognized by hnRNPA1/A2 and DAZAP1 splicing factors.[26,27] In case of *SPINK5* c.891C>T, the generation of an ESS seems more likely than ESE abrogation, because the high strength of exon 11 acceptor and donor sites (score 0.84 and 0.94, respectively) does not apparently require a splicing enhancer.[23,28] Further studies will allow to better decipher the mechanism underlying splicing regulation of *SPINK5* exon 11 and to explore possible therapeutic strategies.[24] Up to know, the c.891C>T variant represents the first example of an ESE/ESS mutation identified in the *SPINK5* gene. In addition to *SPINK5*, several other genes have been targeted by mutations in ESE/ESS motifs, including *BRCA1*,[26] *SMN1/2*,[29,30] *PDHA1*,[31] *GH*,[32] *DMD* [33] and *COL7A1*.[34] Despite these examples and due to the lack of a clear strategy for their identification, mutations known to affect exonic splicing regulatory sequences are currently few in number and likely underestimated.

Genotype–phenotype correlation

The spectrum of the SPINK5 mutations in NS comprises nonsense, frameshift and splice-site mutations, which result in LEKTI truncation and loss of its function.[8] With its 15 inhibitory domains, LEKTI controls the activity of multiple serine proteases, including plasmin, trypsin, subtilisin A, cathepsin P and elastase.[5] Genotype–phenotype correlation in NS is largely incomplete; however, a correlation between the SPINK5 mutation position, LEKTI domain deficiency and disease severity has been suggested.[8,11,12]

In our patient, translation of the aberrant transcript deleted of exon 11 is predicted to result in a truncated protein lacking inhibitory domains 5–15. This, combined with the effect of the c.153delT mutation, would be expected to result in a more severe NS phenotype than the one we actually observed in our patient. However, we also demonstrated that incomplete skipping of exon 11 in the transcript corresponding to the allele linked to c.891C>T mutation results in full-length mRNA encoding for residual LEKTI polypeptides, which could explain the relatively mild features and spontaneously improving disease course of our patient.

NOTE ADDED IN PROOF

While this work was being considered for publication, Lacroix *et al.*[35] reported the identification of mutation c.891C>T in 10 Mediterranean NS families predominantly of Greek origin and sharing a common *SPINK5* haplotype. Our study adds another Mediterranean patient (Sicily) to those by Lacroix *et al.*, thus supporting c.891C>T as the most frequent *SPINK5* mutation in Europe. Geographical and historical contiguity between Southern Italy and Greece strongly suggests that also our patient may have the same *SPINK5* haplotype. The same authors demonstrated that absent staining for LEKTI in the skin associated with a variable phenotype ranging from mild to moderately severe. Differently from Lacroix *et al.*, we detected a residual synthesis of wild-type LEKTI in cultured keratinocytes, resulting from the incomplete skipping of exon 11. We hypothesize that, in NS patients sharing the c.891C>T mutation, phenotypic variability may result, at least in part, from inter-individual, and possibly age-dependent variations, of SF2/ASF and hnRNPA1 cellular levels influencing exon 11 splicing and hence LEKTI synthesis.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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