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NIPAL4 mutation c.527C>A identified in Romanian patients with autosomal recessive congenital ichthyosis

Mutația c.527C>A în gena NIPAL4 identificată la pacienți români cu ihtioză congenitală autozomal recesivă

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Abstract

Introduction: *Autosomal recessive congenital ichthyosis is a non-syndromic ichthyosis, with a genetic background of mutations in 9 genes. This case series presents clinical and paraclinical particularities of 3 Romanian ARCI patients with NIPAL4 mutation c.527C>A.*

Material and methods: *Three Caucasian patients were investigated, two sisters and an unrelated female patient, aged 47, 49, and 42 respectively. Skin anomalies were recorded and documented photographically; peripheral blood samples were harvested for DNA extraction and gene analysis. Skin biopsies were used for histological assessment, electron microscopy, and evaluation of in situ transglutaminase 1 activity.*

Results: *All patients presented with generalized ichthyosis, palmoplantar keratoderma, normal hair shafts, and significant oral manifestations. Natural evolution was relatively stable in all cases, without phenotype changing. Medical treatment with retinoids in patients 1 and 2 resulted in normalisation of the skin condition.*

Histological samples showed hyperkeratosis, acanthosisand perivascular inflammatory infiltrates in the dermis. Positive findings of transglutaminase 1 in situ activity excluded TGM1 deficiency. Direct sequencing of amplicons revealed one homozygous mutation in exon 4, a c.527C>A missense mutation.

Conclusions: *This is the first report of the hotspot mutation NIPAL4 c.527C>A in Romanian autosomal recessive congenital ichthyosis patients. The phenotype was similar to that reported in the literature, while transglutaminase 1 activity in situ assay detected differences in enzyme distribution between patients bearing the same mutation but different phenotypes. Based on the current data, NIPAL4 mutations are more frequent than TGM1 mutations in Romanian patients with autosomal recessive congenital ichthyosis.*

Keywords: *autosomal recessive congenital ichthyosis;NIPAL4 mutation; transglutaminase 1; electron microscopy.*

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Rezumat

Introducere: *Ihtioza congenitală autozomal recesivă este o ihtioză nonsindromică, cu posibile mutații în 9 gene. Lucrarea prezintă particularitățile clinice și paraclinice a 3 pacienți români cu mutația c.527C>A. a genei NIPAL4.*
Material si metodă: *Studiul a inclus 2 surori și o pacientă neînrudită în vârstă de 47, 49 și respectiv 42 de ani. Modificările tegumentare au fost înregistrate și documentate fotografic; s-au recoltat probe de sânge periferic pentru extracția ADN și analiza genetică. Biopsiile cutanate au fost utilizate pentru evaluări histologice, de microscopie electronică, și evaluarea activității în situ a transglutaminazei 1.*
Rezultate: *Toți pacienții prezentau ihtioză generalizată, keratodermie palmo-plantară, foliculi piloși normali și manifestări orale semnificative. Evoluția naturală a bolii a fost relativ stabilă, fără modificări fenotipice, iar la două paciente tratamentul medicamentos cu retinoizi a produs normalizarea modificărilor cutanate.*
Histologic s-au evidențiat hiperkeratoză, acantoză și infiltrate inflamatorii perivasculare în derm. Rezultatele pozitive ale activității in situ a transglutaminazei 1 au exclus prezența unei mutații a TGM1. Secvențierea directă a ampliconilor a demonstrat o mutație homozigotă în exonul 4, o mutație cu sens greșit c.527C>A.
Concluzii: *Acesta este primul raport asupra mutației "hotspot" c.527C>A a genei NIPAL4 la pacienți români cu ihtioză congenitală autozomal recesivă. Deși fenotipul a fost asemănător celor descrise în literatură, evaluarea activității in situ a transglutaminazei 1 a demonstrat diferențe în distribuția enzimei la pacienți cu aceeași mutație dar fenotip diferit. Datele actuale susțin faptul că mutațiile NIPAL4 sunt mai frecvente decât cele ale TGM1 la pacienții români cu această afecțiune.*

Cuvinte cheie: *ihtioză congenitală autozomal recesivă; mutație NIPAL4; transglutaminază 1; electronomicroscopie.*

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Introduction

Autosomal recessive congenital ichthyosis (ARCI) is part of the rare Mendelian disorders of cornification with a prevalence of 7:1,000,000 (1), and skin damage consisting of severe generalized scaling and hyperkeratosis, greatly impacting quality of life (1, 2). It is considered a non-syndromic ichthyosis, as the genetic defect induces phenotypic expressions confined to the tegument, and includes three major clinical entities: lamellar ichthyosis (LI), congenital erythroderma (CIE), and harlequin ichthyosis, with minor variants thereof (2).

The two main clinical phenotypes: LI with large, dark grey/ brownish thick scales, and CIE with generalized severe whitish scaling and erythema without blister formation (previously non-bullous ichthyosiform erythroderma) have been recognized as distinct autosomal congenital ichthyosis since the 1980s (3). However, because of the high clinical overlap and variability of the phenotypes, they are now considered extremities of the spectrum of a single disease, named ARCI (3).

The genetic background of ARCI consists of mutations in 9 genes: TGM1, encoding transglutaminase 1 (TGase1), an enzyme mandatory for cornified envelope formation, and 8 other genes encoding proteins involved in lipid transport and secretion of lamellar bodies (ABCA12) or the metabolic pathways of fatty acids, triglycerides and ceramides (ALOX12B, ALOXE3, NIPAL4, CYP4F22, LIPN, PNPLA1 and CERS3) (4). Although NIPAL4 (NIPA like domain containing 4) was identified in 2004 (5), the physiological role of the membrane associated protein *ichthyin* – frequently found in skin and other organs – is still not clear. Recently it has been considered a magnesium transporter (6), probably of fatty acid transporter protein 4 (FATP4) in the epidermal lipid processing cascade (7).

A large study found NIPAL4 mutations in 16% of ARCI patients, second only to TGM1 defects (8), with 10 different NIPAL4 mutations described in 39 families of various origins worldwide: c.403C>A, c.425G>T,

c.433C>T, c.464-1G>A, c.527C>A, c.623C>T, c.688G>A, c.709C>G, c.772+1G>A and c.889G>A (5, 9-13).

This report presents clinical particularities of 3 Romanian ARCI patients with NIPAL4 mutation, highlighting histological and ultrastructural findings, and TGase1 activity assay results.

Material and methods

Three Caucasian ARCI patients were investigated: P1 and P2 –sisters, aged 47 and 49 years, respectively – and an unrelated female patient P3, aged 42. None had a history of other skin anomalies or consanguinity in their families. The study was a case series approved by the local Ethics Committee and performed according to the World Medical Association Declaration of Helsinki, revised in 2000, Edinburgh, with informed consent from all subjects.

Specific traits of the skin disease were recorded and documented photographically. Peripheral blood samples (6 ml) were harvested for DNA extraction and gene analysis; skin biopsies (3-4 mm) were used for histological assessment, electron microscopy, and evaluation of *in situ* TGase1 activity. Normal skin and peripheral blood samples from 3 patients without ichthyosis were used as controls.

TGase1 assay in skin samples: The TGase1 *in situ* activity assay was performed as previously described (14). Shock frozen skin biopsies were incubated using biotinylated cadaverine (Molecular Probes, Leiden, the Netherlands), in the presence of Ca2+ and a controlled pH (7.4), promoting covalent incorporation of the biotinylated substrate in the cornified envelope almost exclusively by TGase1. Slides incubated with fluorocrome coupled streptavidin 1:100 (Jackson ImmunoResearch Laboratories Inc., West Groove, PA, USA) and assessed with an Olympus BX41TF fluorescence microscope (U-LH-100HG, Olympus, Japan).

Transmission electron and light microscopy: Skin biopsies were prefixed with 2.7% glutaraldehyde, postfixed with 1% osmium tetroxide, dehydrated in acetone series, and embedded in Epon 812. Ultrathin sections of 70-80 nm obtained on a Bromma 8800 Ultratome III (LKB, Sweden) were taken on copper grids (mesh 200), contrasted with uranyl acetate and lead citrate, and examined using a Jeol JEM 1010 transmission electron microscope (Jeol, Tokyo, Japan), to establish ultrastructural type (I-IV).

For histological assessment, skin samples were prepared and stained with hematoxylin and eosin (HE) according to the usual protocol of the laboratory.

Sequence analysis: Genomic DNA was extracted from whole peripheral blood of patients and controls using the QIAamp DNA blood midi kit (Qiagen, Hilden, Germany). The entire NIPAL4 gene (NM_001099287.1) all exons independently, including all intron-exon boundaries, was amplified by polymerase chain reaction (Touch-Down 65-55°C PCR under the following conditions 95°C 3 min + 10X (95°C 30 s + TD65-55°C 30 s + 72°C 30 s) + 30X (95°C 30 s + 55°C 30 s + 72°C 30 s) + 72°C 7min + 4°C ∞). Specific 20-21 nucleotide forward and reverse sequencing primers were used for each of the 6 exons of the gene (table 1). The Sanger method was used for sequencing, with a 3130 XL Applied Biosystems Genetic Analyser (Applied Biosystems, Foster City, CA, USA).

Results

Clinical assessment: All patients presented generalized ichthyosis, with characteristics described in Table 1. Patients 1 and 2 presented fine generalised whitish scaling on an erythematous background (more intense in P1), with larger scales on the legs. In P3 generalized yellowish lamellar adherent scales and reticulated brownish ichthyosis were present on the trunk, associating very mild erythroderma.

Only P1 had a collodion membrane at birth, while P2 developed CIE soon after birth, and P3 had yellow cracked skin at birth, developing LI later on. Palmoplantar keratoderma was present in all patients (mild form in P3), showing a yellowish hue in P1 and P2 that was more severe in P1; the latter also showed nail clubbing and mild deformity of the hands, and partial func-

Table 1. Clinical and paraclinical characteristics of the patients (CIE = congenital erythroderma, LI = lamellar ichthyosis) and the forward and reverse sequencing primers used for each of the 6 exons of NIPAL4.

Characteristics	Patient 1	Patient 2	Patient 3
Gender	F	F	F
Age	47	49	42
Collodion baby	Yes	No	No
Phenotype	CIE	CIE	LI
Palmar-plantar keratoderma	Present	Present	Present
Hyperlinearity	Absent	Absent	Absent
Ectropion	Present	Present	Absent
Alopecia	Present	Absent	Absent
Ear cartilage hypoplasia	Present	Absent	Absent
Hypohidrosis	Present	Present	Absent
Pruritus	Present	Absent	Absent
Skin sample TGase 1 *in situ* assay	Positive	Positive	Positive
Ultrastructural type (electron microscopy)	I, III	I, III	I, III
NIPAL4 mutation	E4: c.[527C>A]	E4: c.[527C>A]	E4: c.[527C>A]

Exon	Primer	Length (bp)	Forward sequence	Reverse sequence	Analysed region
1	NIPAL4-E1D/R	701	TTTCCCTGGG-GATGGAGCTG	TGCTGTCGTCAAAA TGCCTG	c.-82-116_c.223+280
2	NIPAL4-E2D/R	507	GTGGAGGCACG-GTATATGGG	GGCAGGTGGGATTCC AGATAG	c.224-132_c.463+135
3	NIPAL4-E3D/R	256	GAACCAAGCCT-CAAGGAGCA	AAAAATGCCCTACCC ACCCC	c.464-116_c.520+83
4	NIPAL4-E4D/R	239	GTCTGGAATG-GAGATGGTGCT	GTGGCAGTCGATCCC ACTTG	c.521-61_c.611+87
5	NIPAL4-E5D/R	402	TTGCT-CAGGCCCCAT-TTTCTT	GCCTTGCTAACAAGTA CCAGC	c.612-78_c.772+163
6	NIPAL4-E6D/R	812	CTCCCACTGC-CATGAGTCTG	GCCCATTGGTAACAG TTGCAC	c.773-81_c.1401+102

tional impotence due to painful fissures in lack of continuous local treatment.

Ectropion was noted in P1 and P2, with a more severe form in P1, requiring constant use of artificial tears. All patients had normal hair shafts, but P1 reported cicatricial alopecia starting in adolescence. Both P1 and P2 presented hypohidrosis and complained of an important reduction in heat and effort tolerance, with P1 only sweating on the upper lip and glabella, and P2 reporting facial sweating and reduced axillar and inguinal sweating.

Pruritus was present in P1 and it was related to overheating; this had been more intense in childhood, on exertion, and when in a warm environment, although laboratory findings showed normal IgE levels. Significant oral manifestations were present in all patients, with multiple

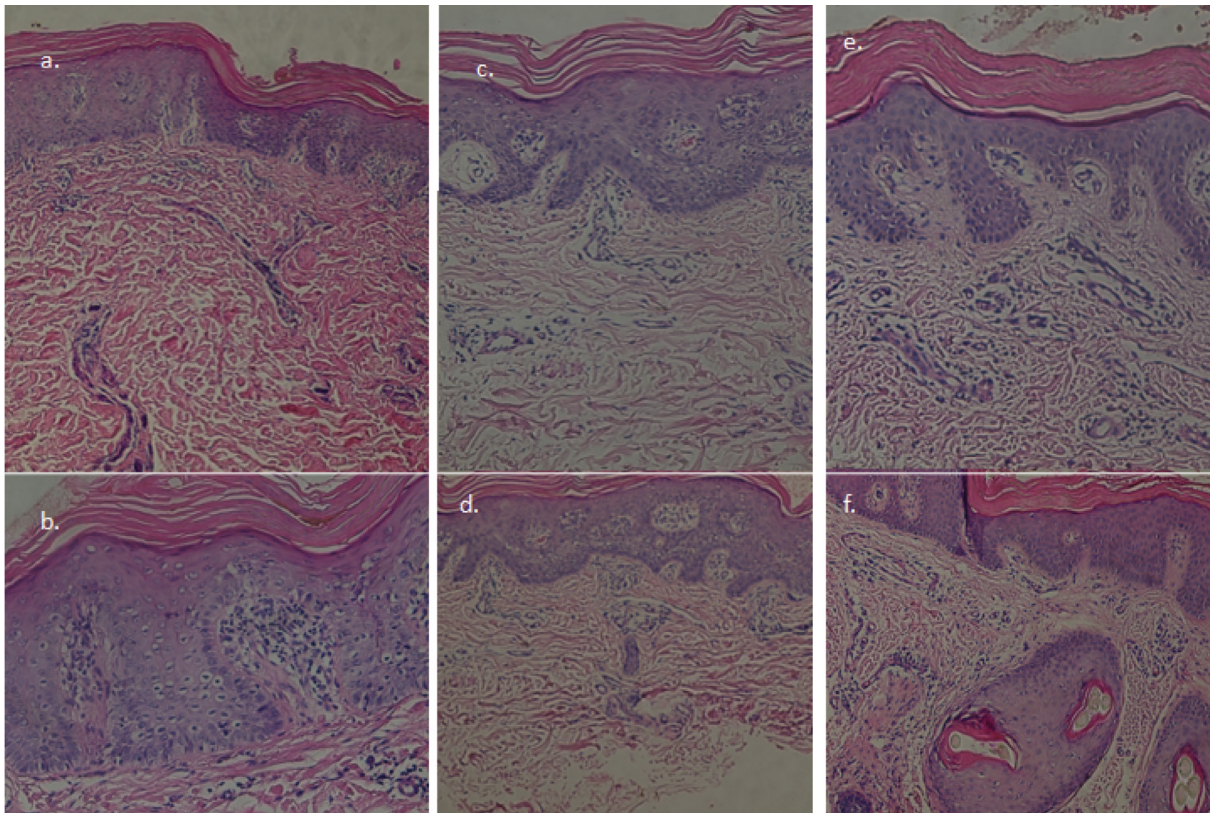

Figure 1. P1 (a, b): hyperkeratosis and acanthosis with follicular plugging; epidermal squamous cell layer thickening, with several layers of polygonal cells with eosinophilic cytoplasm, round, central nuclei, well developed nucleoli; continuous granular layer consisting of flattened cells, with cytoplasm and nucleus masked by basophilic granules of keratohyalin; thickened stratum corneum, consisting of eosinophilic lamellae; irregular dermal papillae; sweat glands and dilated blood vessels in the dermis, with abundant lymphoplasmacytic infiltrate in the dermal papillae and perivascularly. P2 (c, d): similar to P1, with hyperkeratosis, well developed, acanthotic stratum corneum, moderate lymphoplasmacytic infiltrate perivascularly and in the dermal papillae. P3 (e, f): Similar to P1 and P2, but with marked hyperkeratosis, more severe irregularities of the dermal papillae, discontinuous granular layer, very thick, acanthotic stratum corneum, predominantly perivascular moderate chronic inflammatory infiltrate.

caries, artificial crowns, dental bridges, and partially edentulous arches.

Natural evolution was relatively stable in all cases, without phenotype changing. Medical treatment was conducted using retinoids for P1 and P2 - etretinate and acitretin at approximately 0.5mg/ kg body weight, for around 5 years, with very favourable results and only short discontinuous periods. However, medication was subsequently stopped over a period of 15 years, due to complaints of multiple arthralgia. When treatment restarted with 25 mg acitretin/ day, P2 showed a spectacular normalisation of her skin condition in approximately 6 weeks (no scaling or erythema), while P1 still presented mild residual erythroderma, but no scaling and pruritus. Hypohidrosis was partially improved in P1 with supplementary sweating in the lumbar area, while in P2 the symptom completely disappeared. Additionally, regular use of emollients and keratolytics was mandatory for both P1 and P2.

Although P3 had a more severe skin condition in childhood, currently she was undergoing no specific medical treatment for ichthyosis, except for the use of emollients and baths 2-3 times/ week, with significant symptom improvement during summer months.

Histology: Samples showed hyperkeratosis, acanthosis (emphasized with follicular plugging in P3) and the presence of the granular layer in

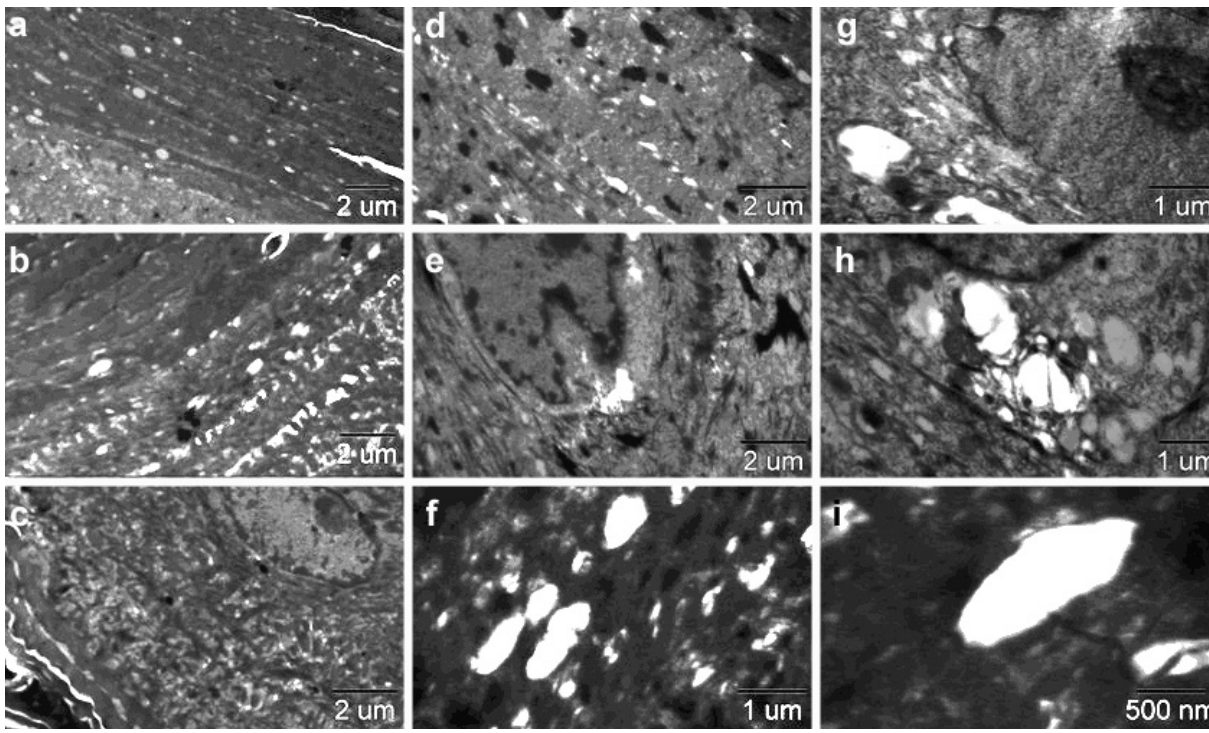

Figure 2. Electronmicrographs of the epidermis showing thickened stratum corneum and corneocytes filled with round-shaped electron transparent structures (lipid droplets) in P1 (a), in contrast to the more compact aspect of stratum corneum in P3 (b) and the normal aspect of the epidermis in a control (c). Stratum granulosum with many electron-dense granules of various shapes and sizes in the keratinocytes in P1 (d), P2 (e) and P3 (f) – probably filaggrin and NIPAL4 aggregates. The main diagnostic feature: numerous large vesicles containing several smaller vesicles in the granular layer of P1 (g), P2 (h) and P3 (f). Detailed view of a large vesicle from P3 (i).

all patients (**Figure 1**). Patients 2 and 3 had a mild inflammatory infiltrate in the dermis around dilated vessels, while P1 had very abundant inflammatory infiltrate. All patients presented sweat glands.

***In situ* TGase1 activity**: TGM1 deficiency was excluded by the positive findings of TGase1 *in situ* activity. For P1 and P2 the fluorescence pattern included 6-7 layers of the stratum granulosum, while in P3 and controls it was present only at the periphery of cells from 2-3 layers of the stratum granulosum.

Electron microscopy: The stratum corneum consisted of over 40 layers of corneocytes, and in patients with CIE (P1 and P2) these were filled with abundant round-shaped electron-transparent structures, probably accumulations of lipid droplets (**Figure 2**), with the aspect of type I congenital ichtyosis. In the LI phenotype (P3), the stratum corneum was more compact, with rare round-shape electron-transparent structures. All cases presented less distinct transition between the stratum corneum and granulosum compared to controls, with many electron-dense granules of various shapes and sizes in the keratinocytes of the stratum granulosum. The granular layer had a vesicular appearance, characteristic for type III congenital ichtyosis: numerous large vesicles containing several smaller vesicles. Peri-nuclear membranes were not clearly observable in the granular layer cells, except for one slide from P3.

Gene sequencing: In all cases, direct sequencing of the amplicons revealed one homozygous variant in exon 4, a c.527C>A missense mutation resulting in Alanine-to-Aspartic acid substitution (p.Ala176Asp) (**Figure 3**).

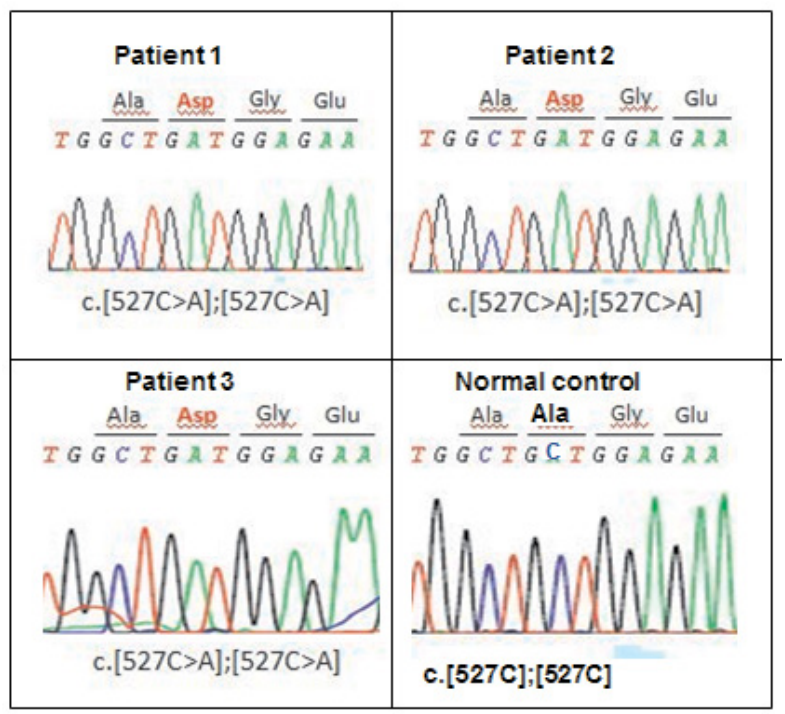

Figure 3. Sequencing of NIPAL4 revealed a homozygous C>A substitution at cDNA position 527 in patients 1, 2 and 3. The normal control is showed in the last position.

Discussions

We present the first identification of the NIPAL4 missense mutation c.527C>A (p.Ala176Asp) in Romanian ARCI patients. This was also the most frequently identified NIPAL4 mutation in ARCI patients of diverse ethnic origins, accounting for approximately 2/3 of NIPAL4 mutated alleles (5, 9, 10), and thus suggesting the existence of a possible hotspot (9, 10). The phenotype of our patients was similar to that reported in the literature for other patients with NIPAL4 mutations (5, 9-13). The mild intra-familial variation of phenotype severity between patients P1 and P2 was opposed to the results of Alavi et al. (12). However, inter-familial phenotype variation was marked, a result similar to literature data (5, 9). Only one of the patients presented a whole series of clinical ARCI traits reported in NIPAL4 mutations (association of collodion membrane at birth, childhood pruritus, alopecia, hypohydrosis, ectropion, nail clubbing, and reticular brownish hyperkeratosis) while yellowish palmoplantar keratoderma without hyperlinearity was constantly present in all cases. Fischer et al. consider palmoplantar findings such as keratoderma and hyperliniarity, as important clues for diagnostic orientation in ARCI patients (8). Of these, palmoplantar keratoderma is a constant feature of ARCI patients with NIPAL4 mutations, while the yellowish hue and absence of hyperlinearity are considered highly suggestive of the presence of a NIPAL4 mutation (12).

Anhidrosis or hypohidrosis is one of the most troublesome problems for ARCI patients, as it might associate heat intolerance, fatigue and erythroderma aggravation when in a hot environment (8).Two of our patients (P1 and P2) presented this symptom, which seems to be a frequent find in patients with NIPAL4 mutations, or mutations of TGM 1, ALOX12B, and ALOXE3, and in some cases requires retinoid therapy (15-17). Given that anhidrosis is frequently seen in mild cases of ichthyosis, where it might show a tendency for self-improving, it is considered that gland suppression is not related to the degree of disease severity (18).

Pruritus in ARCI is rare, especially the more severe manifestation in childhood; when present, pruritus is sometimes considered an overlap with atopic dermatitis (10). However, P1 reported having severe childhood pruritus, in spite of previously detected normal levels of IgE and no filaggrin defects found on ultrastructural analysis of skin biopsies. In this case, pruritus might be caused by the irritant effect of entrapped sweat in the epidermis, given that children are more prone to thermo-regulatory imbalances when exposed to higher temperatures. The gradual decrease of pruritus intensity and frequency over time is probably explained by the patient's better adapting of her activities and clothing to environmental conditions.

Usually, ARCI patients with NIPAL4 mutations respond particularly well to retinoid treatment (19) – this was the case in P1 and P2, who received the same dosage of a second generation retinoid with excellent results: skin condition was fully restored in P2 (general aspect and sweating capacity became similar to the healthy elder sister), while in P1 scaling was normalized, pruritus decreased, supplementary sweating appeared on the lower back, with only mild erythroderma. Incomplete treatment response in P1 might be explained by the severity of the disease (collodion baby, most severe phenotype as adult) or perhaps an inadequate dosage of retinoid.

An interesting aspect in NIPAL4 mutated ARCI patients is the oral involvement, which we described in the 2 patients previously reported (11). Extensive teeth destruction and caries were present in the 3 patients from the current study, with subsequent partial edentation, possibly due to the implication of NIPAL4 in the regulation

of magnesium levels in enamel, an interesting hypothesis for future studies (20).

Although all 3 patients were bearing the same mutation, histological, ultrastructural and TGase1 activity particularities were similar in patients with the same phenotype (P1, P2), but differed from those of P3. Histological examination did not offer clues for orienting genetic diagnosis, as the main feature was the presence of a granular layer. Based on TGase1 *in situ* activity assay, a TGM1 defect was excluded in all cases. However, there were differences in the amount of fluorescence found, with and enhanced activity identified in erythrodermic patients. This might be a compensatory mechanism for the malfunctions of the stratum corneum, as suggested by Li et al. (21) who found increased immunofluorescence staining for TGase1 in skin specimens from NIPAL4 mutated patients compared to controls.

Skin samples from CIE patients (P1 and P2) showed a non-specific pattern of electron-transparent structures classified as type I – also common in other genetic defects of CIE patients (TGM 1, lipoxygenase, etc.) (22) –, and different from the findings in the LI phenotype (P3). The granular layer was similar in all patients, with multiple electron-dense granules of various shapes and sizes in the keratinocytes, considered aggregates of filaggrin/ ichtyin. Ichthyin has been shown to have a tendency towards aggregation and accumulation in the cytoplasm of granular cells in cases of missense mutation (23). The main common abnormal feature of the granular layer in NIPAL4 mutated patients is the presence of numerous large vesicles containing smaller vesicles, probably representing defective lamellar bodies. It has been suggested that ichthyin might influence the formation and transport of lamellar bodies and is therefore present in the majority of patients with NIPAL4 mutations (9). Another important characteristic of type III congenital ichtyiosis, significantly correlated with NIPAL4 mutations (9), is the presence of perinuclear membranes in granular layer cells; this was not clear in our samples except for one slide of P3, although over 100 slides were analysed for each patient.

The importance of the study is given by the fact that it increases the number of NIPAL4 mutations identified in Romanian ARCI patients. The 3 patients presented here were also part of a study on TGase1 deficiency that included 18 Romanian ARCI patients (24), of which we previously reported 2 other patients with a different new NIPAL4 mutation (11). Thus the total number of cases identified with NIPAL4 mutations was 5 out of 18; furthermore, only 3 cases of TGM1 mutations were found. Although sequencing for the rest of the patients is still in progress, our results so far indicate that NIPAL4 mutations appear to be more frequent than TGM1 mutations in Romanian ARCI patients. In light the of this information, we consider NIPAL4 mutations as a major cause of ARCI in the Romanian population. This is in contrast with the low frequency/ absence of NIPAL4 mutations reported in ARCI patients from Israel and Spain, as well as with the results of a larger study conducted on various populations which found fewer NIPAL4 mutations compared to those of TGM1 (8, 13, 25).

The limitations of the study are mainly due to the low number of patients identified with the specific NIPAL4 mutation, hindering proper statistical analysis of clinical and imagistic features.

Conclusions

This is the first report of the hotspot mutation NIPAL4 c.527C>A in Romanian ARCI patients. Although we identified no supplementary clues for NIPAL4 mutations in these patients compared to those presented in the literature, we did detect differences in TGase1 enzyme distribution and

electron microscopic characteristics between patients bearing the same mutation. Interestingly, both patterns were similar for the two patients with the CIE phenotype. This relationship between clinical, histological, ultrastructural, and genetic features is detailed for the first time in this category of patients.

Our results support previous data on Romanian ARCI patients, highlighting the importance of NIPAL4 sequencing in these cases, as NIPAL4 mutation probably represents a major cause of ARCI in Romanian patients.

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List of abbreviations

ARCI	= autosomal recessive congenital ichthyosis
CIE	= congenital erythroderma
FATP4	= fatty acid transporter protein
HE	= hematoxylin and eosin
LI	= lamellar ichthyosis
TGase1	= transglutaminase 1

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