







RESEARCH ARTICLE

Genetical, clinical, and functional analysis of a large international cohort of patients with autosomal recessive congenital ichthyosis due to mutations in *NIPAL4*

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Abstract

Autosomal recessive congenital ichthyosis (ARCI) belongs to a heterogeneous group of disorders of keratinization. To date, 10 genes have been identified to be causative for ARCI. *NIPAL4* (*Nipa-Like Domain-Containing 4*) is the second most commonly mutated gene in ARCI. In this study, we present a large cohort of 101 families affected with ARCI carrying mutations in *NIPAL4*. We identified 16 novel mutations and increase the total number of pathogenic mutations in *NIPAL4* to 34. Ultrastructural analysis of biopsies from six patients showed morphological abnormalities consistent with an ARCI EM type III. One

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patient with a homozygous splice site mutation, which leads to a loss of *NIPAL4* mRNA, showed additional ultrastructural aberrations together with a more severe clinical phenotype. Our study gives insights into the frequency of mutations, a potential hot spot for mutations, and genotype–phenotype correlations.

KEYWORDS

ARCI, ARCI EM type III, collodion baby, ichthyosis, *NIPAL4*

1 | INTRODUCTION

Autosomal recessive congenital ichthyosis (ARCI) belongs to a heterogeneous group of rare genetic skin disorders and is characterized by abnormal scaling of the skin over the whole body (Fischer, 2009). Patients with ARCI are born as collodion babies or with congenital ichthyosiform erythroderma (CIE) and later develop lamellar ichthyosis (LI) with coarse brown scales or CIE with fine white scales (Oji et al., 2010). In cases of harlequin ichthyosis (HI), a rare, severe form of ARCI, perinatal mortality is still about 50% (Traupe, Fischer, & Oji, 2014). To date mutations in 10 genes have been identified to cause ARCI: *TGM1* (MIM# 190195), *ALOX12B* (MIM# 603741), *ALOXE3* (MIM# 607206), *NIPAL4/ICHTHYIN* (MIM# 609383), *CYP4F22* (MIM# 611495), *ABCA12* (MIM# 607800), *PNPLA1* (MIM# 612121), *CERS3* (MIM# 615276), *SDR9C7* (MIM# 609769, Shigehara et al., 2016), and *SULT2B1* (MIM# 604125, Heinz et al., 2017). Mutations in *FATP4* (*SLC27A4*; MIM# 608649) cause ichthyosis prematurity syndrome, which constitutes another congenital ichthyosis, which is classified as a syndromal form of ARCI, but leads *de facto* to nonsyndromic ichthyosis (Klar et al., 2009).

Nipa-Like Domain-Containing 4 (*NIPAL4*) is the second most commonly mutated gene in ARCI after *TGM1* (11–16% in *NIPAL4*, Fischer, 2009; Vahlquist, Fischer, & Törmä, 2018) and is predicted to encode a transmembrane protein with nine transmembrane domains (Lefèvre et al., 2004). Since its first identification by Lefèvre et al., in 2004, 18 disease-causing mutations have been reported in *NIPAL4* including the highly recurrent mutation c.527C>A, p.(Ala176Asp; Balci et al., 2017; Bučková et al., 2016; Dahlqvist et al., 2007; Kusakabe et al., 2017; Lefèvre et al., 2004; Maier, Mazereeuw-Hautier, Tiliņa, Cosgarea, & Jonca, 2016; Palamar et al., 2015; Scott et al., 2013; Wajid, Kurban, Shimomura, & Christiano, 2010). Clinical phenotypes in this molecular genetically characterized subgroup of ARCI are heterogeneous and show interfamilial, intrafamilial, and intraindividual variability (Alavi et al., 2012; Vahlquist et al., 2018). In both LI and CIE, the presence or absence of a collodion membrane at birth, ectropion and anhidrosis have been reported in patients with mutations in *NIPAL4* (Alavi et al., 2012; Dahlqvist et al., 2007; Lefèvre et al., 2004).

All ARCI genes encode proteins that are involved in the formation of the cornified lipid envelope (CLE) and many of them, including *NIPAL4*, in the synthesis of fatty acids and ceramides (Hirabayashi, Murakami, & Kihara, 2019; Mauldin et al., 2018). There is increasing evidence that *NIPAL4* works as an Mg²⁺ transporter for *FATP4*, an acyl-CoA synthetase, which is considered important for the generation of ω-

hydroxy ultra-long chain fatty acid-CoA which is involved in the epidermal lipid metabolism (Li, Vahlquist, & Törmä, 2013; Mauldin et al., 2018). The resulting accumulation of long-chain free fatty acids (FFAs) in *NIPAL4* deficient skin is assumed to trigger membrane stripping and disruption of organelle membranes (Mauldin et al., 2018), which disturbs overall metabolic and intracellular membrane component processing and eventually the transport of components for the formation of lamellar bilayers to the extracellular space by lamellar bodies. This may explain specific epidermal ultrastructural abnormalities identified in patients with ARCI having mutations in *NIPAL4*, formerly classified as ARCI EM type III, which is characterized by deposition of complex or empty vesicles and perinuclear elongated membranes in granular layer cells (Arnold, Anton-Lamprecht, Melz-Rothfuss, & Hartschuh, 1988; Dahlqvist et al., 2007). In addition to these cytotoxic events by putatively accumulating FFAs, *NIPAL4* deficiency should lead to a lack of sphingolipid end products, resulting in a disturbed formation of the CLE. These two mechanisms may explain the impairment of the epidermal permeability barrier in patients with ARCI and thereby cause the observed clinical phenotype (Mauldin et al., 2018).

2 | METHODS

In our large collection of over 1,000 families with ichthyosis, 101 families with mutations in *NIPAL4* were identified by Sanger sequencing or NGS methods. At least one affected member of each family was analyzed and informed consent was obtained from all patients. This study was conducted according to the Declaration of Helsinki principles.

Mutation screening was performed on genomic DNA isolated from peripheral blood lymphocytes. We applied standard methods for polymerase chain reaction (PCR) amplification, Sanger sequencing, and next-generation sequencing (NGS) to initially identify potential pathogenic variants. In the case of PCR amplification, exon-specific primer pairs were used to amplify all coding exons and flanking intronic sequences of *NIPAL4* (reference NM_001099287.1, GRCh37.p13). Sequences of primers can be provided on request. Automated DNA sequence analysis was performed using an ABI DNA Sequencer (Applied Biosystems, Foster City). In 17 patients, we applied NGS methods through multigene panel testing. DNA sequences were enriched by a HaloPlex Custom Kit (Agilent Technologies, Inc. Santa Clara, CA) and sequence analysis was performed using the Illumina MiSeq instrument (Illumina, San Diego, CA). Resulting data were analyzed using an in-house bioinformatics

pipeline and the commercial software SeqNext (JSI Medical Systems). All mutations were validated by sequencing of an independent PCR product.

The following databases were used in this study: The Exome Aggregation Consortium (ExAC; <http://exac.broadinstitute.org/>; version 0.3.1, Lek et al., 2016), The Genome Aggregation Database (gnomAD; <http://gnomad.broadinstitute.org/>; version v2.0.2), Database of Single-Nucleotide Polymorphisms (dbSNP; <http://www.ncbi.nlm.nih.gov/projects/SNP/>; version build 151), HGMD Professional (<http://www.biobase-international.com/product/hgmd>; version 2018.3), and PubMed (<http://www.ncbi.nlm.nih.gov/pubmed/>).

For in silico analysis of the effect of mutations we applied the following bioinformatics tools and used *NIPAL4* NM_001099287.1 as reference: Mutation Taster (<http://www.mutationtaster.org/>; Schwarz, Cooper, Schuelke, & Seelow, 2014), PolyPhen-2 (<http://genetics.bwh.harvard.edu/pph2/>, Adzhubei et al., 2010); SIFT (<http://sift.jcvi.org/>; Ng & Henikoff, 2001), Provean (<http://provean.jcvi.org/index.php>; Choi & Chan, 2015), fathmm (<http://fathmm.biocompute.org.uk/>; Shihab et al., 2014), Human Splicing Finder version 3.0 (<http://www.umd.be/HSF3/>; Desmet et al., 2009), NetGene2 (<http://www.cbs.dtu.dk/services/NetGene2/>; Hebsgaard et al., 1996), and NNSplice version 0.9 (<http://www.fruitfly.org/>; Reese, Eeckman, Kulp, & Haussler, 1997). The results of the prediction tools and databases for all *NIPAL4* mutations found in our cohort are listed in Table S1.

2.1 | Reverse transcription PCR (RT-PCR)

Keratinocytes from patient P79 and control cell line (NHEK – normal human epidermal keratinocytes) were collected when cells reached confluency. Total RNA was extracted using the Roti-Quick kit (Carl Roth, Karlsruhe, Germany). The quality of RNA was analyzed by the NanoDrop spectrophotometer (PEQLAB, Erlangen, Germany) and 500 ng was reverse-transcribed using SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad) and oligo(dT)15 primers (Thermo Fisher Scientific, Waltham) according to the manufacturer's protocol. For the amplification of the *NIPAL4* transcript, Taq Polymerase (Qiagen, Venlo, Netherlands) and the following primers were used: 5'-CGGGGACTCTCCCACTCT-3' (forward primer *NIPAL4* exon 1) and 5'-CTTTGGAGCCTACGCATTTG-3' (reverse primer *NIPAL4* exon 4). PCR products were measured by electrophoresis on a 1.2% agarose gel.

Total RNA was isolated from peripheral blood lymphocytes from P76 and a healthy individual via the QIAamp RNA Blood Mini Kit (Qiagen, Venlo, Netherlands) according to the manufacturer's protocol. RNA concentrations and quality were assessed via NanoDrop spectrophotometer (PEQLAB). A total amount of 500 ng RNA was reverse-transcribed into complementary DNA (cDNA) using SuperScript II Reverse Transcriptase (Invitrogen) and oligo(dT) 15 primers (Thermo Fisher Scientific) according to the manufacturer's protocol. *NIPAL4* and *GAPDH* mRNA transcripts were amplified using Taq Polymerase and the following primers:

5'-CGGGGACTCTCCCACTCT-3' (forward primer *NIPAL4* exon 1) and 5'-CTTTGGAGCCTACGCATTTG-3' (reverse primer *NIPAL4* exon 4), 5'-GACAGTCAGCCGCATCTTCT-3' (forward primer *GAPDH*) and 5'-TTAAAAGCAGCCCTGGTGAC-3' (reverse primer *GAPDH*).

In a first PCR a touchdown gradient from 63°C with a decrease of 0.5°C per cycle was used for the first 15 cycles followed by another 15 cycles at 55°C. For *NIPAL4* mRNA, nested RT-PCR was performed using 5 µl of the initial PCR product under the same thermocycling conditions. The resulting RT-PCR products were analyzed by electrophoresis on a 1.5% agarose gel.

2.2 | Histological analysis of skin sections

Biopsies from patients P79, P101, and a healthy individual were fixed in 4% formaldehyde, gradually dehydrated, and embedded in paraffin (Paraplast Plus, McCormick; formalin-fixed paraffin-embedded FFPE). Sections with a thickness of 7 µm were cut, gradually dewaxed, and incubated with hematoxylin (C16H14O6, Carl Roth) for 5 min and counterstained with 0.25% eosin Y (C20H6Br14Na2O5, Carl Roth) for 3 min. Sections were dehydrated and mounted in Roti-Histokitt medium (Carl Roth). For microscopy, a Carl Zeiss Axioskop 40 light microscope equipped with an AxioCam MRC5 camera (software AxioVision Rel. 4.6) was used.

2.3 | Electron microscopy

Skin biopsies of six patients (P72, P73, P76, P80, P91, and P93) from the back were fixed for at least 2 hr at room temperature in 3% glutaraldehyde solution in 0.1 M cacodylate buffer pH 7.4, cut into 1 mm³ pieces, washed in buffer, postfixed for 1 hr at 4°C in 1% aqueous osmium tetroxide, rinsed in water, dehydrated through graded ethanol solutions, transferred into propylene oxide, and embedded in epoxy resin (glycidether 100). Semithin and ultrathin sections were cut with an ultramicrotome (Reichert Ultracut E). Semithin sections of 1 µm were stained with methylene blue. About 60–80 nm ultrathin sections were treated with uranyl acetate and lead citrate and examined with an electron microscope JEM 1400 equipped with a 2K TVIPS CCD Camera TemCam F216. Skin biopsies from individuals not suffering from ichthyosis were taken as controls.

3 | RESULTS

Patients with ARCI from 101 families carrying mutations in *NIPAL4* were identified by Sanger sequencing or by next-generation sequencing methods (Table 1). Twenty of these pedigrees have already been published in Lefèvre et al. (2004), and Pigg et al. (2016). Altogether this large cohort provides insights into the distribution of mutations within *NIPAL4*, the frequency of their occurrence and potential genotype–phenotype correlations. Furthermore, 16 novel mutations were identified: eight missense mutations (c.407T>A, p.(Val136Asp); c.470G>A, p.(Gly157Glu); c.534A>C, p.(Glu178Asp); c.695C>G, p.(Tyr232Arg); c.836C>T, p.(Pro279Leu); c.844G>A, p.(Gly282Arg);

TABLE 1 Mutations in *NIPAL4* in patients from 101 families with autosomal recessive congenital ichthyosis

| Pedigree | Year of birth | Sex | Origin | Consanguineous parents | Mutations in <i>NIPAL4</i> (NM_001099287) | Consequences of mutation (Amino acid level) |
|----------|---------------|-----|----------|------------------------|---|---|
| 1 | 1965 | M | France | N | c.527C>A / c.527C>A | p.(Ala176Asp) / p.(Ala176Asp) |
| 2 | 1950 | M | France | Y | c.527C>A / c.527C>A | p.(Ala176Asp) / p.(Ala176Asp) |
| 3 | 1994 | M | France | N | c.527C>A / c.527C>A | p.(Ala176Asp) / p.(Ala176Asp) |
| 4 | 1985 | F | France | N | c.527C>A / c.612-3del | p.(Ala176Asp) / p.(?) |
| 5 | 1975 | M | France | N | c.527C>A / c.527C>A | p.(Ala176Asp) / p.(Ala176Asp) |
| 6 | 1963 | F | France | Y | c.527C>A / c.527C>A | p.(Ala176Asp) / p.(Ala176Asp) |
| 7 | 1986 | F | France | N/A | c.527C>A / c.527C>A | p.(Ala176Asp) / p.(Ala176Asp) |
| 8 | 1943 | F | France | N/A | c.527C>A / c.527C>A | p.(Ala176Asp) / p.(Ala176Asp) |
| 9* | 1993 | M | Morocco | Y | c.527C>A / c.527C>A | p.(Ala176Asp) / p.(Ala176Asp) |
| 10* | 1958 | F | Morocco | Y | c.623C>T / c.623C>T | p.(Ser208Phe) / p.(Ser208Phe) |
| 11 | 1983 | F | France | N | c.527C>A / c.527C>A | p.(Ala176Asp) / p.(Ala176Asp) |
| 12 | 1982 | F | France | N | c.527C>A / c.527C>A | p.(Ala176Asp) / p.(Ala176Asp) |
| 13 | 1984 | F | France | N | c.527C>A / c.527C>A | p.(Ala176Asp) / p.(Ala176Asp) |
| 14 | 1977 | F | France | N | c.527C>A / c.709del | p.(Ala176Asp) / p.(His237Metfs*18) |
| 15* | 1935 | F | Colombia | Y | c.527C>A / c.527C>A | p.(Ala176Asp) / p.(Ala176Asp) |
| 16 | 1960 | F | France | N | c.527C>A / c.223+5G>C | p.(Ala176Asp) / p.(?) |
| 17 | 1980 | F | Portugal | N | c.527C>A / c.527C>A | p.(Ala176Asp) / p.(Ala176Asp) |
| 18 | 1977 | M | Algeria | Y | c.889G>A / c.889G>A | p.(Gly297Arg) / p.(Gly297Arg) |
| 19 | 1963 | M | Italia | N | c.772+1G>A / c.772+1G>A | p.(?) / p.(?) |
| 20 | 1970 | F | Maghreb | Y | c.283del / c.283del | p.(Leu95Serfs*68) / p.(Leu95Serfs*68) |
| 21* | 1990 | M | Algeria | Y | c.433C>T / c.433C>T | p.(Ala145*) / p.(Ala145*) |
| 22* | 1981 | F | Algeria | Y | c.527C>A / c.527C>A | p.(Ala176Asp) / p.(Ala176Asp) |
| 23 | 1969 | F | France | N | c.527C>A / c.939C>G | p.(Ala176Asp) / p.(Asn313Lys) |
| 24* | 1982 | F | Algeria | Y | c.433C>T / c.433C>T | p.(Ala145*) / p.(Ala145*) |
| 25 | 1971 | F | France | N | c.527C>A / c.463+5G>A | p.(Ala176Asp) / p.(?) |
| 26* | N/A | F | Turkey | Y | c.527C>A / c.527C>A | p.(Ala176Asp) / p.(Ala176Asp) |
| 27* | N/A | M | Turkey | Y | c.527C>A / c.527C>A | p.(Ala176Asp) / p.(Ala176Asp) |
| 28* | 1981 | F | Syria | Y | c.709C>G / c.709C>G | p.(His237Asp) / p.(His237Asp) |
| 29 | 1973 | M | France | Y | c.527C>A / c.527C>A | p.(Ala176Asp) / p.(Ala176Asp) |
| 30* | 1991 | M | Algeria | Y | c.425G>T / c.425G>T | p.(Gly142Val) / p.(Gly142Val) |
| 31* | N/A | M | Turkey | Y | c.889G>A / c.889G>A | p.(Gly297Arg) / p.(Gly297Arg) |
| 32* | 1998 | F | Algeria | Y | c.433C>T / c.433C>T | p.(Ala145*) / p.(Ala145*) |
| 33 | 1960 | M | Algeria | Y | c.527C>A / c.527C>A | p.(Ala176Asp) / p.(Ala176Asp) |
| 34 | 1977 | M | Algeria | Y | c.433C>T / c.433C>T | p.(Ala145*) / p.(Ala145*) |
| 35* | N/A | F | Turkey | Y | c.527C>A / c.527C>A | p.(Ala176Asp) / p.(Ala176Asp) |
| 36 | 1980 | F | Algeria | Y | c.527C>A / c.527C>A | p.(Ala176Asp) / p.(Ala176Asp) |
| 37 | 1998 | M | Tunisia | Y | c.2T>C / c.2T>C | p.(Met1?) / p.(Met1?) |
| 38 | 1969 | M | Tunisia | Y | c.534A>C / c.534A>C | p.(Glu178Asp) / p.(Glu178Asp) |
| 39 | 1985 | M | Morocco | Y | c.555C>G / c.555C>G | p.(Tyr185*) / p.(Tyr185*) |
| 40 | 1964 | M | Morocco | Y | c.555C>G / c.555C>G | p.(Tyr185*) / p.(Tyr185*) |
| 41 | 1992 | F | Tunisia | Y | c.223+5G>C / c.223+5G>C | p.(?) / p.(?) |
| 42 | 1961 | F | Tunisia | Y | c.534A>C / c.534A>C | p.(Glu178Asp) / p.(Glu178Asp) |
| 43 | 1986 | M | Tunisia | Y | c.534A>C / c.534A>C | p.(Glu178Asp) / p.(Glu178Asp) |
| 44 | 1965 | M | Tunisia | Y | c.534A>C / c.534A>C | p.(Glu178Asp) / p.(Glu178Asp) |

(Continues)

TABLE 1 (Continued)

| Pedigree | Year of birth | Sex | Origin | Consanguineous parents | Mutations in NIPAL4 (NM_001099287) | Consequences of mutation (Amino acid level) |
|----------|---------------|-----|-------------|------------------------|------------------------------------|---|
| 45 | 1958 | F | Tunisia | Y | c.534A>C / c.534A>C | p.(Glu178Asp) / p.(Glu178Asp) |
| 46 | 1921 | M | Tunisia | Y | c.534A>C / c.534A>C | p.(Glu178Asp) / p.(Glu178Asp) |
| 47 | 1980 | F | France | N/A | c.527C>A / c.463+5G>A | p.(Ala176Asp) / p.(?) |
| 48 | N/A | F | Italy | N/A | c.527C>A / c.889G>A | p.(Ala176Asp) / p.(Gly297Arg) |
| 49 | N/A | N/A | N/A | N/A | c.527C>A / c.527C>A | p.(Ala176Asp) / p.(Ala176Asp) |
| 50 | 1944 | F | France | N/A | c.527C>A / c.527C>A | p.(Ala176Asp) / p.(Ala176Asp) |
| 51 | 1983 | F | Maghreb | Y | c.1014C>A / c.1014C>A | p.(Ser338Arg) / p.(Ser338Arg) |
| 52 | 1992 | M | France | Y | c.889G>A / c.407T>A | p.(Gly297Arg) / p.(Val136Asp) |
| 53 | 1943 | F | France | N/A | c.527C>A / c.527C>A | p.(Ala176Asp) / p.(Ala176Asp) |
| 54 | 1995 | M | Maghreb | Y | c.611+1G>A / c.611+1G>A | p.(?) / p.(?) |
| 55 | 1990 | M | France | N | c.527C>A / c.527C>A | p.(Ala176Asp) / p.(Ala176Asp) |
| 56 | 1958 | M | France | N | c.527C>A / c.527C>A | p.(Ala176Asp) / p.(Ala176Asp) |
| 57 | 1962 | F | France | N | c.527C>A / c.527C>A | p.(Ala176Asp) / p.(Ala176Asp) |
| 58 | 1978 | F | France | N | c.527C>A / c.527C>A | p.(Ala176Asp) / p.(Ala176Asp) |
| 59 | 1981 | M | Switzerland | N | c.527C>A / c.527C>A | p.(Ala176Asp) / p.(Ala176Asp) |
| 60 | 1985 | N/A | Switzerland | N | c.527C>A / c.527C>A | p.(Ala176Asp) / p.(Ala176Asp) |
| 61 | 2000 | M | Switzerland | N/A | c.889G>A / c.889G>A | p.(Gly297Arg) / p.(Gly297Arg) |
| 62 | 1977 | N/A | Switzerland | N/A | c.527C>A / c.939C>G | p.(Ala176Asp) / p.(Asn313Lys) |
| 63 | 1964 | M | Switzerland | N/A | c.527C>A / c.527C>A | p.(Ala176Asp) / p.(Ala176Asp) |
| 64** | N/A | N/A | Sweden | N/A | c.527C>A / c.889G>A | p.(Ala176Asp) / p.(Gly297Arg) |
| 65** | N/A | N/A | Denmark | N | c.527C>A / c.527C>A | p.(Ala176Asp) / p.(Ala176Asp) |
| 66** | N/A | N/A | Denmark | N | c.527C>A / c.527C>A | p.(Ala176Asp) / p.(Ala176Asp) |
| 67** | N/A | N/A | Denmark | N | c.527C>A / c.527C>A | p.(Ala176Asp) / p.(Ala176Asp) |
| 68** | N/A | N/A | Denmark | N | c.527C>A / c.527C>A | p.(Ala176Asp) / p.(Ala176Asp) |
| 69** | N/A | N/A | Denmark | N | c.527C>A / c.527C>A | p.(Ala176Asp) / p.(Ala176Asp) |
| 70** | N/A | N/A | Denmark | N | c.527C>A / c.527C>A | p.(Ala176Asp) / p.(Ala176Asp) |
| 71 | N/A | N/A | Germany | N/A | c.527C>A / c.527C>A | p.(Ala176Asp) / p.(Ala176Asp) |
| 72 | 2006 | M | Morocco | N/A | c.695C>G / c.695C>G | p.(Tyr232Arg) / p.(Tyr232Arg) |
| 73 | 1992 | F | Germany | N | c.527C>A / c.844G>A | p.(Ala176Asp) / p.(Gly282Arg) |
| 74 | 2004 | M | N/A | N | c.527C>A / c.688G>A | p.(Ala176Asp) / p.(Gly230Arg) |
| 75 | 2010 | F | Germany | N | c.527C>A / c.527C>A | p.(Ala176Asp) / p.(Ala176Asp) |
| 76 | 2005 | F | Germany | N | c.223+5G>C / c.223+5G>C | p.(?) / p.(?) |
| 77 | 1967 | F | Germany | N | c.527C>A / c.527C>A | p.(Ala176Asp) / p.(Ala176Asp) |
| 78 | 1980 | M | Italy | N/A | c.772+1G>A / c.772+1G>A | p.(?) / p.(?) |
| 79 | 2000 | F | France | N | c.527C>A / c.463+5G>A | p.(Ala176Asp) / p.(?) |
| 80 | 1991 | F | Germany | N | c.527C>A / c.527C>A | p.(Ala176Asp) / p.(Ala176Asp) |
| 81 | 2007 | F | Ireland | N | c.527C>A / c.527C>A | p.(Ala176Asp) / p.(Ala176Asp) |
| 82 | 2007 | M | Ireland | N | c.527C>A / c.527C>A | p.(Ala176Asp) / p.(Ala176Asp) |
| 83 | 2013 | M | Germany | N | c.527C>A / c.463+5G>A | p.(Ala176Asp) / p.(?) |
| 84 | 1985 | F | Germany | N | c.527C>A / c.527C>A | p.(Ala176Asp) / p.(Ala176Asp) |
| 85 | 2001 | F | N/A | N/A | c.527C>A / c.527C>A | p.(Ala176Asp) / p.(Ala176Asp) |
| 86 | 1994 | F | Germany | N | c.527C>A / c.433C>T | p.(Ala176Asp) / p.(Ala145*) |
| 87 | 1959 | F | Germany | N | c.527C>A / c.527C>A | p.(Ala176Asp) / p.(Ala176Asp) |
| 88 | 2008 | F | N/A | N/A | c.527C>A / c.1268del | p.(Ala176Asp) / p.(Asn423Thrfs*25) |

(Continues)

TABLE 1 (Continued)

| Pedigree | Year of birth | Sex | Origin | Consanguineous parents | Mutations in <i>NIPAL4</i> (NM_001099287) | Consequences of mutation (Amino acid level) |
|----------|---------------|-----|---------|------------------------|---|---|
| 89 | 1991 | F | France | N/A | c.527C>A / c.527C>A | p.(Ala176Asp) / p.(Ala176Asp) |
| 90 | 2008 | F | France | N/A | c.527C>A / c.836C>T | p.(Ala176Asp) / p.(Pro279Leu) |
| 91 | 1973 | F | Germany | N/A | c.527C>A / c.527C>A | p.(Ala176Asp) / p.(Ala176Asp) |
| 92 | 2008 | F | Ireland | N/A | c.527C>A / c.433C>T | p.(Ala176Asp) / p.(Ala145*) |
| 93 | 2004 | F | Germany | N/A | c.527C>A / c.527C>A | p.(Ala176Asp) / p.(Ala176Asp) |
| 94 | 1950 | M | India | Y | c.889G>A / c.889G>A | p.(Gly297Arg) / p.(Gly297Arg) |
| 95 | 1969 | F | France | Y | c.527C>A / c.527C>A | p.(Ala176Asp) / p.(Ala176Asp) |
| 96 | 1998 | M | N/A | N/A | c.527C>A / c.527C>A | p.(Ala176Asp) / p.(Ala176Asp) |
| 97 | 2016 | F | N/A | N/A | c.527C>A / c.463+5G>A | p.(Ala176Asp) / p.(?) |
| 98 | 1996 | M | Syria | N/A | c.470G>A / c.470G>A | p.(Gly157Glu) / p.(Gly157Glu) |
| 99 | 1968 | F | Germany | N/A | c.527C>A / c.527C>A | p.(Ala176Asp) / p.(Ala176Asp) |
| 100 | 1987 | F | Turkey | N | c.527C>A / c.527C>A | p.(Ala176Asp) / p.(Ala176Asp) |
| 101 | 1978 | F | France | N/A | c.527C>A / c.527C>A | p.(Ala176Asp) / p.(Ala176Asp) |

*Published in Lefèvre et al. (2004).

**Published in Pigg et al. (2016).

Novel mutations are indicated in bold letters; M, male, F, female, N, no, Y, yes, and N/A, no information on this feature.

c.939C>G, p.(Asn313Lys); and c.1014C>A, p.(Ser338Arg)); four nonsense mutations (c.283del, p.(Leu95Serfs*68); c.555C>G, p.(Tyr185*); c.709del, p.(His237Metfs*18); and c.1268del, p.(Asn423Thrfs*25)), and four potential splice site mutations (c.223+5G>C, **c.463+5G>A**, c.611+1G>A, and c.612-3del; Figure 1a, in bold). This increases the total number of pathogenic *NIPAL4* mutations to 34, which are shown in Figure 1a. Known mutations were obtained by HGMD Professional. All mutations reported by our study were submitted to the database ClinVar (<https://www.ncbi.nlm.nih.gov/clinvar/>).

3.1 | Spectrum of mutations in *NIPAL4*

Mutations in *NIPAL4* are distributed over the entire coding sequence and adjacent intronic sequences which are required for correct splicing. The major types of mutations are missense mutations (20 out of 34, corresponding to 59%), while frameshift and nonsense mutations together account for 23.5% (8 out of 34) and splice site mutations represent 17.5% (6 out of 34) of all mutations.

Due to the large number of affected patients in our cohort, the frequency of appearance of mutations could be estimated. In this context, missense mutation c.527C>A, p.(Ala176Asp) was identified either on a homozygous or a compound heterozygous background in 70% of all patients (Figure 1b). This highly recurrent mutation probably represents a mutational hot spot at position c.527 of the *NIPAL4* gene. Another mutation which appeared several times in our cohort (6% of all patients) was the novel missense mutation c.534A>C, p.(Glu178Asp). Since it was identified in six consanguineous families from Tunisia, it probably represents a founder effect in this region and not a hot spot for mutations. Other frequent mutations are missense mutation c.889G>A, p.(Gly297Arg), which was found in seven consanguineous and nonconsanguineous families with an Indian,

Caucasian, or North African origin (7% of all patients) and the nonsense mutation c.433C>T, p.(Ala145*), which was detected in four consanguineous families from Algeria and in two nonconsanguineous families from Germany and Ireland (6% of all patients).

3.2 | Novel splice site variants lead to different splicing defects

To understand and to verify the effect of splice site variants detected in patients upon sequencing of *NIPAL4*, mRNA derived from keratinocytes of P79 (c.463+5G>A, c.527C>A) was isolated and *NIPAL4* mRNA was analyzed by RT-PCR and subsequent gel electrophoresis (Figure 2a). This showed an additional *NIPAL4* mRNA product that was 91 bp longer which was subjected to Sanger sequencing (Figure 2b). Sequencing also showed that the two mutations are located *in trans*, that is, on different alleles (data not shown), confirming their heterozygous state and further supporting the pathogenicity of the novel mutation c.463+5G>A. In summary, the results indicate that c.463+5G>A inhibits correct splicing.

The novel splice site mutation c.223+5G>C was found on a compound heterozygous background in one patient (P16) and on a homozygous background in two patients (P41 and P76). In homozygous patients an unbiased functional analysis of the effect of this variation was possible. Therefore, *NIPAL4* mRNA was analyzed in P76 and a healthy individual via RT-PCR and subsequent agarose gel electrophoresis (Figure 2c). This revealed a strong deficiency of *NIPAL4* mRNA in P76, whereas control mRNA (*GAPDH*) was detectable in comparable amounts in P76 and in a healthy control. Consequently, mutation c.223+5G>C prevents the correct splicing of *NIPAL4* and most likely results in a functional “knock-out” of *NIPAL4* via nonsense mediated decay.

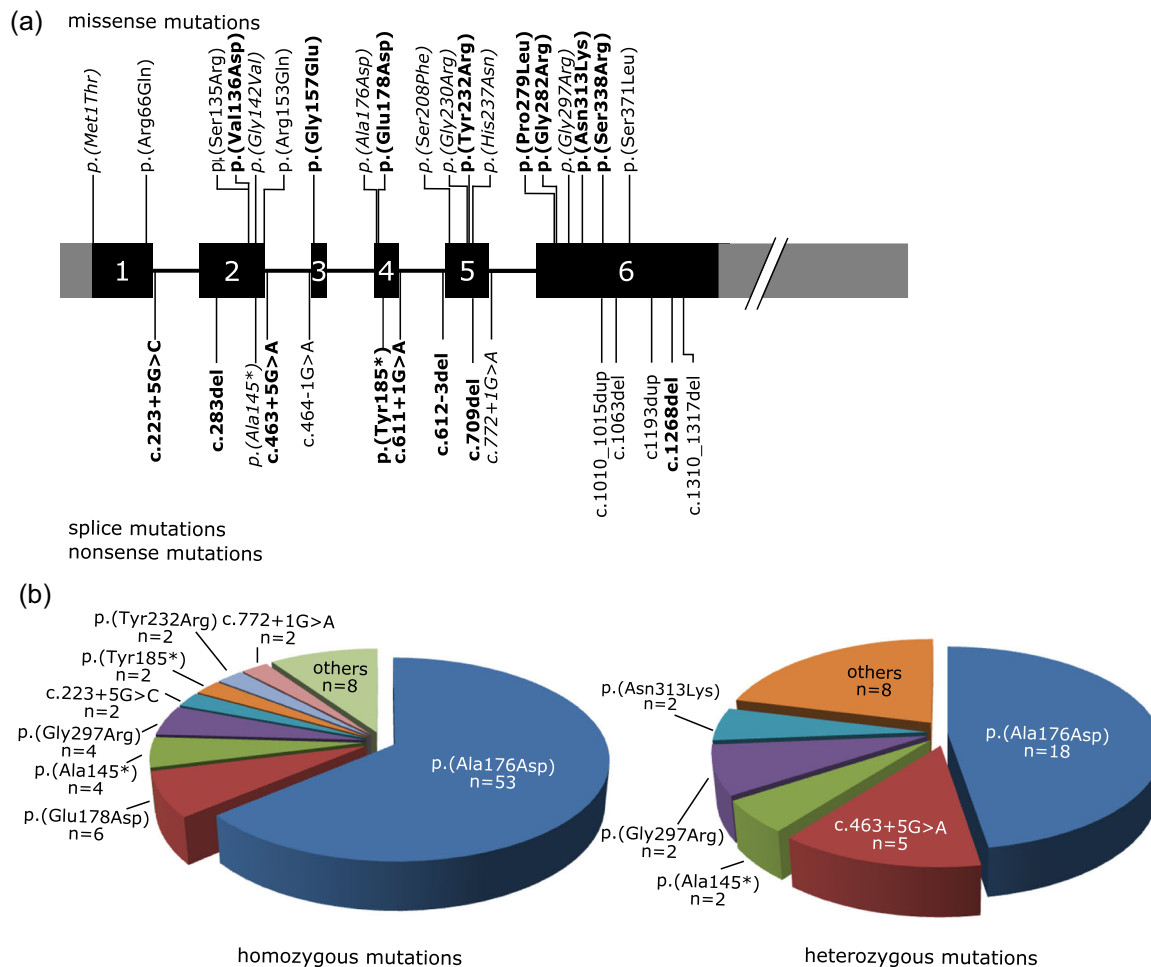


FIGURE 1 *NIPAL4* mutation spectrum. (a) Diagram of *NIPAL4* gene structure and location of pathogenic variants. Novel mutations are indicated in bold letters, known mutations detected in this study are in italic letters, other known mutations not found in this study are in nonbold and nonitalic letters. (b) Distribution of mutations in *NIPAL4* in our cohort of 101 patients with autosomal recessive congenital ichthyosis. Missense mutation p.(Ala176Asp) accounts for the majority of homozygous and heterozygous mutations. Mutations, which are combined under the term “others,” were only detected once. These were in a homozygous state: p.(Met1Thr), c.283del, p.(Gly142Val), p.(Gly157Glu), c.611+1G>A, p.(Ser208Phe), p.(His237Asn), and p.(Ser338Arg). In a heterozygous state: c.223+5G>C, p.(Val136Asp), c.612-3del, p.(Gly230Arg), c.709del, p.(Pro279Leu), p.(Gly282Arg), and c.1268del

3.3 | Genotype–phenotype correlations in ARCI patients with mutations in *NIPAL4*

Retrospective assessment of clinical features of patients with mutations in *NIPAL4*, when available, reflected diverse clinical phenotypes as reported for other mutations in other genes associated with ARCI. In a part of our cohort we revealed more detailed information about the phenotype of our patients (Table 2). In these cases, ichthyosis phenotypes were frequently accompanied by an erythema, a palmo-plantar keratoderma (PPK, Figure 3i,m–o) and anhidrosis. In some cases ectropion, eclabium or nail abnormalities were also present. All features were observed in varying degrees of severity with a tendency to an average moderate phenotype. Predilection sites of scales were neck (Figure 3a,d,g,i) and skin folds, while the face was unaffected in most cases (not shown). Patients were treated either with topical application of creams (urea, lactic acid-based, and moisturizing products) or with systemic administration of retinoids.

We examined a potential correlation of nonsense and splice site mutations with a more severe phenotype. Seven patients of our cohort were reported to suffer from severe LI (P21, P24, P26, P32, P75, P76, and P86). Three of them carried the homozygous stop mutation c.433C>T (P21, P24, and P32), one was homozygous for the splice site mutation c.223+5G>C (P76), two carried the recurrent homozygous missense mutation c.527C>A, p.(Ala176Asp; P26 and P75) and one was compound heterozygous for c.527C>A, p.(Ala176Asp) and c.433C>T, p.(Ala145*; P86). This difference in severity is shown by the example of P32 (c.433C>T, p.(Ala145*) homozygous) in comparison to patients with other mutations in *NIPAL4* (Figure 3). However, it should be noted that in the case of the recurrent homozygous mutation c.527C>A, p.(Ala176Asp), both severe (P26 and P75) and more moderate ichthyosis phenotypes (P22, Figure 3d–f) were observed. However, our clinical data are not sufficient to verify a correlation of nonsense and splice site mutations to a more severe phenotype. In this point, further studies are necessary.

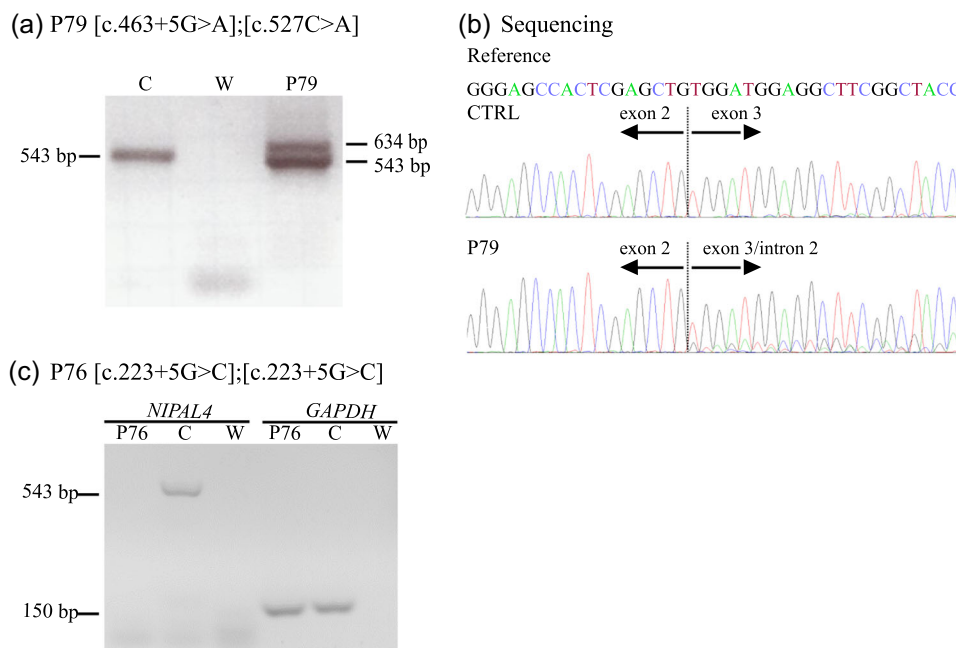


FIGURE 2 In vitro analysis of the effect of novel splice variants c.223+5G>C and c.463+5G>A on *NIPAL4* mRNA. (a) Reverse transcription (RT)-PCR showed an additional *NIPAL4* mRNA transcript in P79. (b) Sequencing of the RT-PCR products of P79 confirmed the presence of the additional mRNA transcript, whose sequence contained parts of intron 2. (c) RT-PCR of mRNA from P76, who carried the homozygous splice variant c.223+5G>A, revealed a strong reduction of *NIPAL4* mRNA. RT-PCR of *GAPDH* was used as a positive control for mRNA integrity and cDNA synthesis. C, tissue matched control; mRNA, messenger RNA; W, water control

3.4 | ARCI patients with mutations in *NIPAL4* show abnormal skin morphology with hyperkeratosis and ultrastructural characteristics of ARCI EM type III

Histological skin biopsies from P79 (*NIPAL4* c.527C>A/c.463+5G>A; Figure 4b) and P101 (*NIPAL4* c.527C>A/c.527C>A; Figure 4d) showed a hyperkeratosis typical of ARCI in comparison with control skin (Figure 4a +c). In addition, skin sections of both patients revealed a slight epidermal hyperplasia and granular layer cells with large nuclei (asterisks in Figure 4b+d). Hyperkeratosis was more pronounced in P79 than in P101, whereas P101 additionally showed a parakeratosis within the cornified layers (arrows in Figure 4d).

Ultrastructural analysis of skin biopsies from six patients (P72, P73, P76, P80, P91, and P93) was performed to obtain more detailed insights into structural alterations within the epidermis caused by mutations in *NIPAL4*. This may help to better understand the functional role of *NIPAL4* in the pathogenesis of ARCI.

Four of the six biopsies presented with a typical morphological phenotype of this type of ichthyosis, such as vesicular complexes and membrane-bound vesicles within the upper granular layers, some of which were empty and some of which contained small vesicles (Figure 4e, Table S2) and perinuclear membrane material. P72, who carried the novel missense mutation c.695C>G, p.(Tyr232Arg), exhibited specific features with more spacious regions of deposits (Figure 4f,g), whereas P76 (homozygous splice site mutation with loss of mRNA), who clinically displayed an unusual severe inflammatory ichthyosis, was also peculiar on the morphological level, with less

hyperkeratosis but parakeratosis and incomplete keratinization associated with eczematous processes, superposed on the ichthyotic phenotype. Nevertheless, vesicular complexes and membrane stripping were discovered by thorough examination (Figure 4h).

4 | DISCUSSION

This study summarizes mutation analysis and clinical data from 101 families with ARCI due to mutations in *NIPAL4*, and was complemented by functional studies on the molecular and ultrastructural level.

Mutation analysis in this cohort expands the current mutation spectrum in *NIPAL4* by adding 16 novel pathogenic variants. Among these, there was a potential splice site variant c.612-3del found in a heterozygous state together with c.527C>A, which affects the highly conserved 3' splicing consensus motif YAG/RNNN (Lewandowska, 2013). In this variant the pyrimidine residue (Y; here C) of the consensus motif is deleted and replaced by a purine residue (here G). In silico analysis with different splice site prediction tools points towards a pathogenic effect of c.612-3del as it leads to a reduction or loss of recognition of the intrinsic splicing acceptor site.

The novel mutation c.463+5G>A interfered with the recognition of the adjacent donor splice site and therefore a part of intron 2 was detected in patient-derived cDNA. The position c.463+5 is a highly conserved position in human splice regions (Zhang, 1998). In silico translation of the altered mRNA sequence predicts a frameshift at amino

TABLE 2 Phenotypic features of patients from 101 families with autosomal recessive congenital ichthyosis and mutations in NIPAL4

| No. | Diagnosis | Reticulate | | Yellowish | | Erythema | Ectropion | Eclabium | Hair | Ears | Nails | Moucosae | Heat intolerance | Treatment |
|-----|----------------------|------------|-------------|-----------|-----|----------|-----------|----------|------|--------|----------|----------|------------------|------------|
| | | CB | Scale color | PPK | PPK | | | | | | | | | |
| 1 | LI | N/A | N/A | N/A | Y | N | N | N | N/A | N | N | N/A | N/A | Acitretine |
| 2 | LI | Y | Dark* | Y | Y | N | Y | N | N | N | N | N | N/A | Cream |
| 3 | Moderate LI | Y | N/A | N/A | N | N | N | N | N | Y | N | N | N/A | N/A |
| 4 | LI | Y | Brown | N/A | Y | N/A | N | N | N | N | N | N/A | N/A | N/A |
| 5 | LI | N | Brown | Y | N | N/A | Y | Y | N | N | N | N | N | Cream |
| 6 | LI | N | N/A* | Y | Y | N | N/A | N | N | N | Clubbing | N | Y | Acitretine |
| 7 | Moderate LI | Y | white* | Y | N | Y | Y | N | N | Y | N | N | N/A | Cream |
| 8 | LI | Y | Brown* | Y | Y | N | N | N | N | Y | N | N | N/A | Cream |
| 9 | CI | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A |
| 10 | LI | Y | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A |
| 11 | LI without retinoids | N | N* | N | Y | N/A | N/A | N | N | Y | N | N | Y | Acitretine |
| 12 | CI | N | N/A | N/A | N/A | N/A | N | N | N/A | N/A | N/A | N/A | Y | Cream |
| 13 | CI | N | N | N | N/A | N | N/A | N/A | N | N | N | N | N/A | Cream |
| 14 | Moderate LI | Y | N/A | Y | Y | N | N | N | N | N | N | N | N/A | Cream |
| 15 | CI | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A |
| 16 | CIE | N | N | Y | N | Y | Y | N | N | Y | N | N | Y | Cream |
| 17 | LI | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A |
| 18 | CIE | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A |
| 19 | CI | N/A | N/A | N/A | Y | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A |
| 20 | Mixed LI | Y | N/A* | N | Y | N | Y | Y | N | N | N | N | Y | Cream |
| 21 | Severe LI | Y | N/A | N | Y | N/A | N | N | N | N | Y | N | N/A | N/A |
| 22 | CI | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A |
| 23 | CI | N | N | N | N/A | N/A | N/A | N/A | N | N | N | N/A | Y | Cream |
| 24 | Severe LI | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | Y | N/A | N/A | N/A |
| 25 | CIE | Y | N/A | N/A | N/A | N/A | N | N/A | N | N | N | N/A | Y | Acitretine |
| 26 | Severe LI | N/A | Fine white | N/A | Y | N/A | Y | N/A | N/A | Sparse | N | N | N/A | Etretinate |
| 27 | LI | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A |

(Continues)

TABLE 2 (Continued)

| No. | Diagnosis | CB | Scale color | Reticulate pattern | PPK | Yellowish PPK | Erythema | Ectropion | Eclabium | Hair | Ears | Nails | Moucosae | Heat intolerance | Treatment |
|-----|---|-----|-------------|--------------------|-----|---------------|----------|-----------|----------|------|------|-----------|----------|------------------|---------------|
| 28 | CI | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A |
| 29 | CIE | Y | white | N/A | Y | N/A | Y | Y | Y | N | N | Clubbing | N | N/A | Cream |
| 30 | CI | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A |
| 31 | LI | N/A | Brown | N/A | Y | N/A | Y | Y | N | N | N | N | N | N/A | N/A |
| 32 | Very severe LI | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A |
| 33 | CI | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A |
| 34 | CI | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A |
| 35 | CI | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A |
| 36 | CI | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A |
| 37 | CI | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A |
| 38 | CI | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A |
| 39 | CI | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A |
| 40 | CI | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A |
| 41 | CIE | N/A | N/A | N/A | Y | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A |
| 42 | CIE | N/A | N/A | N/A | Y | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A |
| 43 | CI | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A |
| 44 | CI | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A |
| 45 | CIE | N/A | N/A | N/A | Y | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A |
| 46 | CI | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A |
| 47 | Moderate LI | Y | white | Y | Y | N | Y | N | N | N | N | N | N/A | N/A | Cream |
| 48 | CI | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A |
| 49 | CI | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A |
| 50 | CIE | Y | white | Y | N/A | N | Y | Y | N | N | N | Clubbing | N/A | N/A | Cream |
| 51 | - Retinoids LI, + retinoids normal skin | N/A | N | Y | Y | N | N | N | N | N | N | N | N/A | Y | Alltretinoine |
| 52 | CIE | N/A | white | N | Y | Y | Y | N/A | N | N | N | N/A | N/A | N | Actretine |
| 53 | CIE | N | N | Y | Y | Y | N | N/A | N | N | N | Y | N/A | N | Cream |
| 54 | LI | N/A | N | Y | Y | Y | Y | Y | Y | N | N | Dystrophy | N/A | N/A | Actretine |
| 55 | CIE | N | N/A | N/A | Y | N | Y | N | N | N | N | N | N/A | N/A | Cream |
| 56 | CIE | N/A | White | N | Y | Y | Y | N | N | N | N | N | N/A | N/A | Actretine |
| 57 | CI | N | N/A | N/A | Y | N/A | Y | N | N | N | N | N | N | N/A | Actretine |

(Continues)

TABLE 2 (Continued)

| No. | Diagnosis | CB | Scale color | Reticulate pattern | PPK | Yellowish PPK | Erythema | Ectropion | Eclabium | Hair | Ears | Nails | Mousoae | Heat intolerance | Treatment |
|-----|--------------------------------|-----|-------------|--------------------|------------|---------------|----------|-----------|----------|------|--------------|----------------------------|---------|------------------|-------------|
| 58 | CI | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A |
| 59 | NCIE | N | Brown/white | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A |
| 60 | CI | N | Fine white | N/A | N/A | Face | Yes | N | N | N | Yes | N | N | N/A | N/A |
| 61 | CI | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A |
| 62 | CI | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A |
| 63 | CI | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A |
| 64 | CI | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A |
| 65 | LI/CI | N/A | N/A | N/A | N | N/A | Y | Y | N | N | N | N | N | Y | Moisturizer |
| 66 | LI/CI | N | N/A | N/A | N | N/A | Y | N | N | N | N | N | N | Y | Moisturizer |
| 67 | LI/CI | N | N/A | N/A | Y | N/A | Y | N | N | N | N | N | N | Y | Moisturizer |
| 68 | LI/CI | N | N/A | N/A | N | N/A | Y | N | N | N | Sligh- ht | N | N | Y | Moisturizer |
| 69 | LI | N | N/A | N/A | N | N/A | Y | N | N | N | N | N | N | Y | Moisturizer |
| 70 | LI/CI | N/A | N/A | N/A | Y | N/A | Y | N | N | N | N | N | N | Y | Moisturizer |
| 71 | CI | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A |
| 72 | CI | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A |
| 73 | CI | N | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A |
| 74 | CI | N | Fine white | N/A | Y | N/A | N/A | N | N | N | N | N | N | N/A | N/A |
| 75 | Severe LI | Y | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A |
| 76 | Severe inflammatory ichthyosis | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | Mild hourglass nails | N/A | N/A | N/A |
| 77 | CI | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A |
| 78 | CI | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A |
| 79 | LI | N | Brown* | N | Y | N | Y | N | N | N | N | N | N | N/A | Acitretine |
| 80 | LI | N | N/A | N/A | Y | N/A | N | N | N | N | N | N | N | N/A | Cream |
| 81 | CI | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A |
| 82 | CI | N/A | N/A | N/A | Calluses | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | Cream |
| 83 | LI | N | N/A | N/A | N | N/A | N | N | N | N | N | N | N | N | Cream |
| 84 | LI | N/A | N/A | N/A | Y | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A |
| 85 | CI | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A |
| 86 | | Y | N/A | N/A | Mild, feet | N/A | Scarcely | N | N | N | N | N | N | Y | |

(Continues)

TABLE 2 (Continued)

| No. | Diagnosis | CB | Scale color | Reticulate pattern | PPK | Yellowish PPK | Erythema | Ectropion | Eclabium | Hair | Ears | Nails | Moucosae | Heat intolerance | Treatment | |
|-----|----------------------|-----|---------------------------|--------------------|----------------|---------------|----------|-----------|----------|------|------|-------|----------|------------------|-----------|----------------------|
| | LI (moderate-severe) | | | | | | | | | | | | | | | |
| 87 | LI | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | Cream + keratolysis |
| 88 | LI | N/A | Mainly trunk | N/A | Y | Y | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | Cream |
| 89 | CIE | N/A | N/A | Y | N | N | Y | N/A | N | N | N | N | N/A | N/A | N/A | Allitretinoine |
| 90 | moderate LI | N | hite | N | N | N | Y | N/A | N | N | N | N | N/A | N/A | N/A | Cream |
| 91 | CI | N/A | N/A | N/A | Y | Y | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A |
| 92 | CI | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | Cream |
| 93 | CI | N/A | N/A | N/A | Focal/ striata | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A |
| 94 | LI | N/A | White | Y | Mutilating | N | N/A | N/A | N/A | N/A | Y | Y | N/A | N/A | N/A | Acitretine |
| 95 | CIE | N | Fine white/moderate brown | N/A | Y | N | Slight | N | N | N | N | N | N | Slight | N | Acitretine |
| 96 | CI | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A |
| 97 | CI | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A |
| 98 | CI | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A |
| 99 | CI | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A |
| 100 | CI | N/A | N/A | Y | N | N/A | Y | N/A | N/A | N/A | N | N/A | N/A | Y | N/A | Acitretine, Daivonex |
| 101 | CI | N | Brown | N/A | N | N/A | N/A | N | N | N | N | N | N | Slight | N | Cream |

Abbreviations: CB, collodion baby; CI, congenital ichthyosis; CIE, congenital ichthyosiform erythroderma; LI, lamellar ichthyosis; PPK, palmo-plantar keratoderma; N, no; N/A, no information on this feature; Y, yes.



FIGURE 3 Phenotypic spectrum of patients with ARCI having mutations in *NIPAL4*. Variability of the ichthyotic phenotypes is demonstrated in four patients with different mutations in *NIPAL4*: (a–c) P32 (p.(Ala145*), homozygous) shows a more severe lamellar ichthyosis (LI) than patients carrying missense mutations in *NIPAL4*. (d–f) P22 (p.(Ala176Asp), homozygous), large scales at body front; hyperlinear palms. (g–i) P30 (p.(Gly142Val), homozygous) with small scales at neck, body front only slightly affected, and keratoderma at palms. (j–l) P74 (p.(Ala176Asp)/p.(Gly230Arg)) with fine white scales at neck and over the front of the body. (m–o) Plantar keratoderma with a frequent predilection for pressure point areas (m = sister of P51, n = P2, o = P56). (p, q) Treatment with oral retinoids improved the LI phenotype switching to a more pronounced erythroderma, here shown by the example of two siblings (p), body front, P2; (q), lower back under acitretin, brother of P2

acid position 155 of *NIPAL4* (NP_001092757.1) followed by the generation of a premature stop codon (p.(Val155Alafs*23); data not shown). This truncated version of *NIPAL4* is highly likely to be dysfunctional as it contains only 155 amino acids of the wild type protein and thereby it may contribute to the observed ichthyosis phenotype.

In case of splice site variant c.223+5G>C, in vitro examination of *NIPAL4* mRNA from P76 showed a complete loss of *NIPAL4* mRNA,

which confirmed its pathogenicity (Figure 2c). In addition to this, it becomes evident that *NIPAL4* is expressed in peripheral blood lymphocytes, which makes it possible to analyze *NIPAL4* mRNA without the need for a skin biopsy. Consequently, this offers an easily accessible and simple method to understand the effect of potential splice site mutations on a molecular level.

In our study we were able to provide substantial evidence for the existence of a mutational hot spot at position c.527 in *NIPAL4*, which had

been detected before in smaller cohorts of ARCI patients with mutations in *NIPAL4* (Alavi et al., 2012; Dahlqvist et al., 2007; Lefèvre et al., 2004). First, c.527C>A, p.(Ala176Asp) was detected in pedigrees from a variety of different origins such as Scandinavia, central Europe, and North Africa. Consequently, any founder effects being responsible for the high frequency of this mutation can be ruled out. Second, the appearance of

c.527C>A, p.(Ala176Asp) was not associated with a consanguineous family background since it was only detected in 46% of consanguineous families with a homozygous mutation in *NIPAL4* and whereas it was even found in 73% of nonconsanguineous families with a homozygous mutation profile. This tendency of c.527C>A, p.(Ala176Asp) to appear spontaneously further supports the existence of a mutational hot spot.

Due to the large number of families of ARCI patients with mutations in *NIPAL4* included in this cohort, the frequency of missense mutation c.527C>A, p.(Ala176Asp) in patients with *NIPAL4* mutations could be estimated more precisely. Dahlqvist et al. (2007) detected this mutation on 37 out of 54 alleles (69%), and it is present in our cohort in 124 out of 202 alleles (61%). In this context, it has to be noted that c.527C>A, p.(Ala176Asp) was present in 18 out of 19 patients who were compound heterozygotes.

The mechanism, which causes this highly frequent mutation, remains elusive. Position 176 is located in the middle of a predicted transmembrane domain with an alpha-helical secondary structure (UniProt) and this alanine residue is conserved in mammals (cross-species analysis, data not shown). Thus, a change from alanine, which is a strong alpha former, to aspartate, which is a weak alpha former (HGMD professional), might affect correct secondary structure formation and thereby might impair the proper functioning of *NIPAL4* as Mg^{2+} -transporter.

Retrospective investigation of potential clinical genotype-phenotype correlations supports the general view that there are no clinical characteristics of ARCI disease, which can be solely attributed to mutations in *NIPAL4*. It has been observed by Alavi et al. (2012) that there is a tendency towards a yellowish PPK. However, it neither appears consistently in ARCI patients with mutations in *NIPAL4* nor is it associated with a specific type of mutation. In our cohort, we have data about presence or absence and clinical features of PPK in 51 patients. Thirty-eight of them have PPK, some of them show yellowish PPK (Table 2). To confirm a correlation between nonsense and splice site mutations with a more severe phenotype, more patients with clinical data are necessary. However, a correlation of such variants with more severe phenotypes has already been reported for other ARCI-causing genes. The most impressive example in this context affects mutations in *ABCA12*: While missense

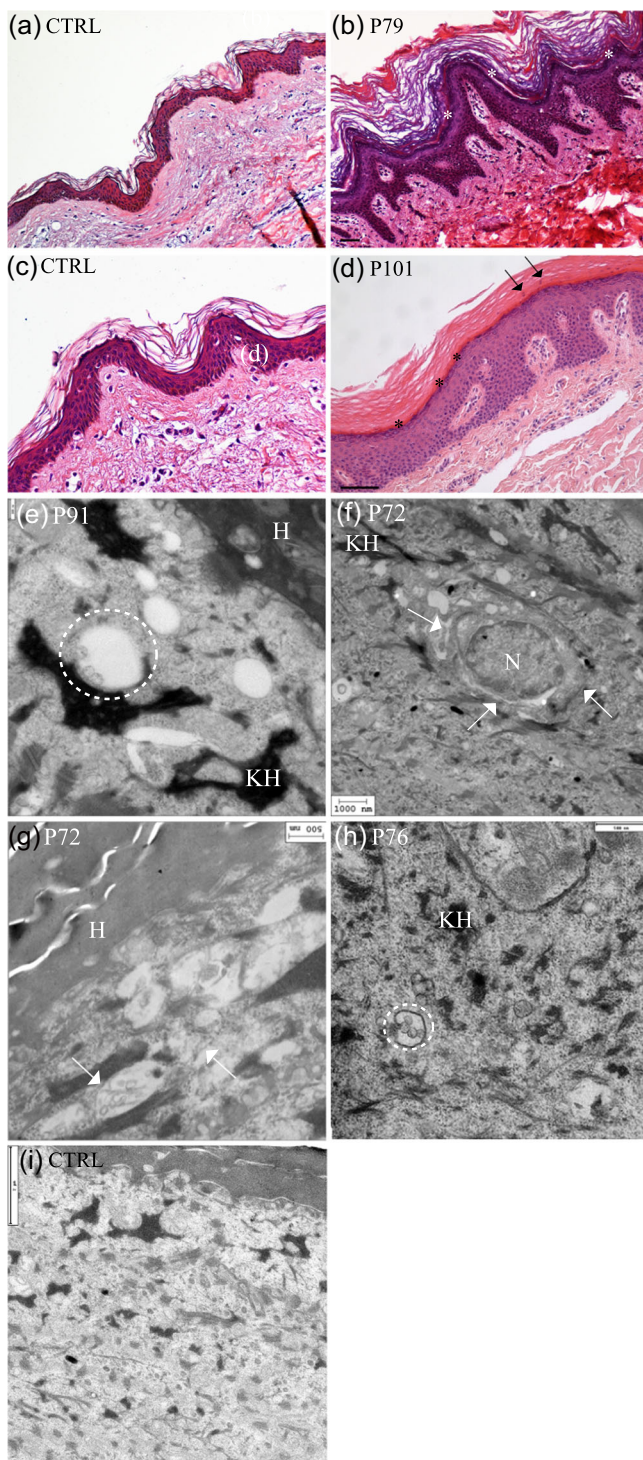


FIGURE 4 Morphological and ultrastructural examination of skin specimens shows a variety of epidermal alterations caused by mutations in *NIPAL4*.

(a–d) In hematoxylin and eosin (H&E) staining P79 (b) and P101 (d) show a slight epidermal hyperplasia with a hyperkeratosis and granular layer cells with large nuclei (asterisks). In P79 the stratum corneum is extremely expanded while P101 has a milder hyperkeratosis with an additional parakeratosis (arrows). Scale bars 50 μ m. Ultrastructural aberrations in patients comprise vesicular complexes within granular layer cells, empty, or containing some small marginal vesicles ((e), P91; (h), P76, encircled). P72 with a novel missense mutation (c.695C>G, (p.Tyr232Arg) homozygous) displayed granular layer cells with accumulation of irregular vesicles ((f) arrows) or extended areas with irregular vesicular complexes ((g) arrows). (i) Morphological aspects of a normal granular layer without vesicular structures characteristic for *NIPAL4* mutations. KH, keratohyalin granule; N, nucleus; H, horny layer. Bars indicate 200 nm (e), 1 μ m (f), 500 nm (g), 500 nm (h), 2 μ m (i)

mutations in this gene cause LI (Lefèvre et al., 2003), truncating mutations result in the life-threatening condition of harlequin ichthyosis (Akiyama et al., 2005; David kelsell et al., 2005). Consequently, it is likely that truncating mutations in *NIPAL4* also result in a more severe form of ARCI disease. Nevertheless, two patients in our study with severe ARCI carried the highly recurrent missense mutation c.527C>A, p.(Ala176Asp). This reflects findings in two consanguineous Pakistani families (Wajid et al., 2010) with the same homozygous mutation in *NIPAL4*, who showed severe ichthyosis phenotypes with fine white scales over the whole body and face. In one of these Pakistani families, some members had a brownish reticulated lamellar ichthyosis similar to patients in our cohort, who carried c.527C>A, p.(Ala176Asp) in a homozygous state. Hence, in case of c.527C>A, p.(Ala176Asp) different clinical outcomes are possible, which might also be due to other genetic modifiers.

Ultrastructural analysis showed characteristic morphological alterations like vesicular complexes, which can also be found in 12R lipoygenase knockout mice (Epp et al., 2007) and, very rarely, in single patients with mutations in *ALOXE3* or *CYP4F22*. These ultrastructural markers were elaborately described by Arnold et al. (1988) and Dahlqvist et al. (2007); in the latter communication, involving 27 cases of 18 families compared with 18 patients with ARCI but without consistent EM findings, the clear association with mutations in *ICHTHYIN*, now *NIPAL4*, was proven. One ARCI patient did not fulfill the EM criteria but carried a *NIPAL4* mutation indicating that *NIPAL4* deficiency does not necessarily result in a distinct clinical and also morphological phenotype. Concerning the origin of these vacuolar and membrane material, both groups assumed that these structures present defective lamellar bodies. However, there are intact lamellar bodies (in contrast to e.g., harlequin ichthyosis with mutations in *ABCA12*), and in view of deposition of excess membrane material in ichthyosis with mutations in *PNPLA1* and *FATP4*, a more general disturbance of membrane trafficking and respective fatty acid components is more likely to play a role. Interestingly, the novel missense mutation c.695C>G in P72 caused a morphological phenotype resembling to some degree to *PNPLA1* morphology (Grall et al., 2012). These observations hint to a strong association of ARCI pathogenesis and the reaction chain leading to intact ω -esterified ultralong chain fatty acid.

In summary, we present a large cohort of 101 families affected with ARCI carrying mutations in *NIPAL4*. We expand the mutational spectrum by presenting 16 novel mutations in *NIPAL4*. We provide insights to the distribution of mutations within *NIPAL4*, the frequency of their occurrence and potential genotype-phenotype correlations.

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CONFLICTS OF INTEREST

The authors declare that there are no conflict of interests.

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