Molecular Analysis of 250 Patients with Autosomal Recessive Congenital Ichthyosis: Evidence for Mutation Hotspots in *ALOXE3* and Allelic Heterogeneity in *ALOX12B*

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In recent years several new genes for autosomal recessive congenital ichthyosis (ARCI) have been identified. However, little is known about the molecular epidemiology and pathophysiology of this genetically and clinically heterogeneous group of severe disorders of keratinization. ARCI is characterized by intense scaling of the whole integument often associated with erythema. We and others have shown that mutations in *ALOX12B* and *ALOXE3*, coding for the lipoxygenases 12R-LOX and eLOX-3 predominantly synthesized in the epidermis, can underlie this rare condition. Here we have surveyed a large group of 250 patients with ARCI for mutations in these two genes. We have identified 11 different previously unreported mutations in *ALOX12B* and *ALOXE3* in 21 ARCI patients from 19 unrelated families and demonstrated that mutations in the two genes are the second most common cause for ARCI in this cohort of patients. Examination of the molecular data revealed allelic heterogeneity for *ALOX12B* and two mutational hotspots in *ALOXE3*. Functional analysis of all missense mutations and a splice site mutation demonstrated that complete loss of function of the enzymes underlies the phenotype. Our findings further establish the pivotal role of the 12-lipoxygenase pathway during epidermal differentiation.

Journal of Investigative Dermatology (2009) **129,** 1421–1428; doi:10.1038/jid.2008.409; published online 8 January 2009

INTRODUCTION

Lipoxygenases (LOX) represent a widely distributed family of non-heme, iron-containing dioxygenases that catalyze the regioselective and stereoselective dioxygenation of fatty acid substrates containing one or more (Z,Z)-1,4-pentadiene moieties (reviewed by Brash, 1999; Kühn and Thiele, 1999). Within the mammalian LOX family, a distinct subclass of epidermis-type LOX has been found to be preferentially synthesized in the skin and few other epithelial tissues (Brash *et al.*, 1997, 2007; Boeglin *et al.*, 1998; Kinzig *et al.*, 1999; Heidt *et al.*, 2000; Krieg *et al.*, 2002). The genes for the human epidermal LOX, 15-LOX-2, 12R-LOX, and eLOX-3, map closely together on human chromosome 17p13.1 (Krieg *et al.*, 2001). Their differentiation-dependent expression pattern in epithelial tissues suggests a common physiological role in the regulation of proliferation and differentiation of epithelial cells, especially keratinocytes. The epidermal 12R-LOX and eLOX-3 differ from all other mammalian LOX in their unique structural and enzymatic features (Boeglin *et al.*, 1998; Kinzig *et al.*, 1999; Krieg *et al.*, 1999) as both proteins contain an extra domain located at the surface of the catalytic subunit. 12R-LOX represents the only mammalian LOX that forms products with R chirality, and, unlike all other LOX, eLOX-3 does not exhibit dioxygenase activity but acts as a hydroperoxide isomerase (Yu *et al.*, 2003). Both enzymes participate in the same pathway and convert arachidonic acid via 12R-hydroperoxy-5Z,8Z,10E,14Z-eicosatetraenoic acid (12R-HPETE) to the corresponding hepoxilin-like

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Abbreviations: 12R-HPETE, 12R-hydroperoxy-5Z,8Z,10E,14Z-eicosatetraenoic acid; ARCI, autosomal recessive congenital ichthyosis; LOX, lipoxygenase

Received 8 July 2008; revised 3 November 2008; accepted 6 November 2008; published online 8 January 2009

epoxyalcohol, 8R-hydroxy-11R,12R-epoxyeicosatrienoic acid. Mutations in *ALOX12B* and *ALOXE3*, the genes for 12R-LOX and eLOX-3, were found in patients with autosomal recessive congenital ichthyosis (ARCI; Jobard *et al.*, 2002; Eckl *et al.*, 2005). We and others have shown that those mutations completely eliminate the catalytic activity of the LOX enzymes, suggesting that mutational inactivation of either 12R-LOX or eLOX-3 is causally linked to the ARCI phenotype (Eckl *et al.*, 2005; Yu *et al.*, 2005). Furthermore, 12R-LOX deficiency in the mouse clearly recapitulates the human phenotype (Epp *et al.*, 2007; Moran *et al.*, 2007).

ARCI, including lamellar ichthyosis, non-bullous congenital ichthyosiform erythroderma, and congenital fine scaling, forms a clinically and genetically heterogeneous group of severe keratinization disorders with a prevalence of approximately 1 in 200,000 persons in the European and northern American populations (Traupe, 1989; Williams *et al.*, 2005; Oji and Traupe, 2006; Schmuth *et al.*, 2007). Affected newborns often present with a so-called collodion membrane. After loss of this encasement in the first weeks of life, patients exhibit a generalized scaling, which varies from patient to patient in extent, color, and degree of adherence. An underlying erythema is often seen, but mostly mild. Further features may include alopecia, hypohydrosis, as well as marked hyperlinearity and hyperkeratosis of the palms and soles. Although mutations in six genes, *TGM1*, *ALOX12B*, *ALOXE3*, *ABCA12*, *Ichthyin*, and *CYP4F22*, have been found, more loci must exist, as 30–40% of all ARCI patients do not have mutations in any of these genes. Only transglutaminase 1, 12R-LOX, and eLOX-3, the products of *TGM1*, *ALOX12*B, and *ALOXE3*, respectively, have been analyzed functionally so far, and only the pathophysiology of ARCI caused by deficiency of transglutaminase 1 has been analyzed in more detail.

Here we present 11 previously unreported inactivating mutations in either *ALOX12B* or *ALOXE3*, seen in 19 independent ARCI cases out of 250 patients analyzed. In contrast to *ALOX12B*, showing extended allelic heterogeneity, we have identified two mutational hotspots in *ALOXE3*. Using an *in vitro* assay, we have further demonstrated the ablation of enzyme activity of mutant 12R-LOX and eLOX-3.

RESULTS

Mutation analysis in ALOX12B and ALOXE3

Consanguineous families were prescreened for regions of homozygosity as described elsewhere (Mizrachi-Koren *et al.*, 2005; Lugassy *et al.*, 2008). After analyzing *TGM1* by direct sequencing, mutation analysis was extended by sequencing all 30 exons and exon/intron boundaries of *ALOX12B* and *ALOXE3*. In a series of 250 patients, we identified mutations in *ALOX12B* or *ALOXE3* in a total of 17 patients each. We found eight different mutations in *ALOX12B* previously unreported to our knowledge, of which two were insertions, two deletions, two missense mutations, one splice-site mutation (which we found in two independent patients), and one mutation of the initial methionine codon (Table 1). In *ALOXE3*, two new deletions and one exonic splice site mutation were detected. In three patients, the second mutation remained undetected as we expect compound heterozygosity for those patients, and may reside in regulatory regions or farther in the introns or represent larger deletions. All mutations were excluded from a panel of 100 matched control persons.

Table 1. Summary of previously unreported mutations in *ALOX12B* and *ALOXE3* including two hotspot mutations identified in patients with ARCI in this study

Mutation	Protein	Exon	Frequency[1]
ALOX12B			
c.1A>G	p.Met1?	1	1
c.583T>C	p.Phe195Leu	5	1
c.942_943insTTTA	p.Ala316ProfsX59	8	1
c.1144A>G	p.Lys382Glu	9	1
c.1153delG	p.Val385TyrfsX30	9	1
c.1272_1273insC	p.Lys425GlnfsX24	9	1
c.1625_1626delAA	p.Lys542ArgfsX13	12	2
c.1654+3A>G	splice defect	12	2
ALOXE3			
c.434G>A	p.Arg119GlyfsX12	3	2
c.700C>T[2]	p.Arg234X	6	11
c.719delA	p.Lys240ArgfsX40	6	1
c.1031_1039del9	p.Gln344_Ala347delinsPro	8	2
c.1889C>T[3]	p.Pro630Leu	14	15

[1]Number of independent chromosomes with the mutation identified in 250 ARCI patients described here and by Eckl *et al.*, 2005.
[2]Mutation described previously by Jobard *et al.*, 2002.
[3]Mutation described previously by Eckl *et al.*, 2005.

Patient ISA carried two homozygous missense mutations in *ALOXE3*, predicted to result in p.Arg145His and p.Leu237Met, respectively. Both mutant enzymes, however, were shown to be enzymatically active (Eckl *et al.*, 2005). We then tested a double mutant, which also showed normal LOX activity. As the first mutation, c.434G>A, altered the last base of exon 3 and prediction of splice sites (Reese *et al.*, 1997; Nalla and Rogan, 2005) suggested an effect on RNA splicing, we used a mini-gene assay to analyze the RNA. We cloned genomic DNA of the patient spanning intron 1 to exon 5 of *ALOXE3* into a eukaryotic expression vector and transfected the construct into HEK 293 cells. Analysis of total RNA revealed that the mutation c.434G>A leads to complete skipping of exon 3 (Figure 1). A second, heterozygous splice site mutation, c.1654+3A>G in *ALOX12B*, was seen in two unrelated patients. Again, tissue samples from these patients were not available, however, the mutation alters one of the highly conserved intronic residues of the donor splice site. The probability for correct splicing is clearly reduced as predicted by neural network analysis (Reese *et al.*, 1997). The mutation c.1A>G destroys the translation start codon. Still an alternative start site may be used, however, there are only two further potential start sites, as confirmed upon prediction with NetStart (Pedersen and

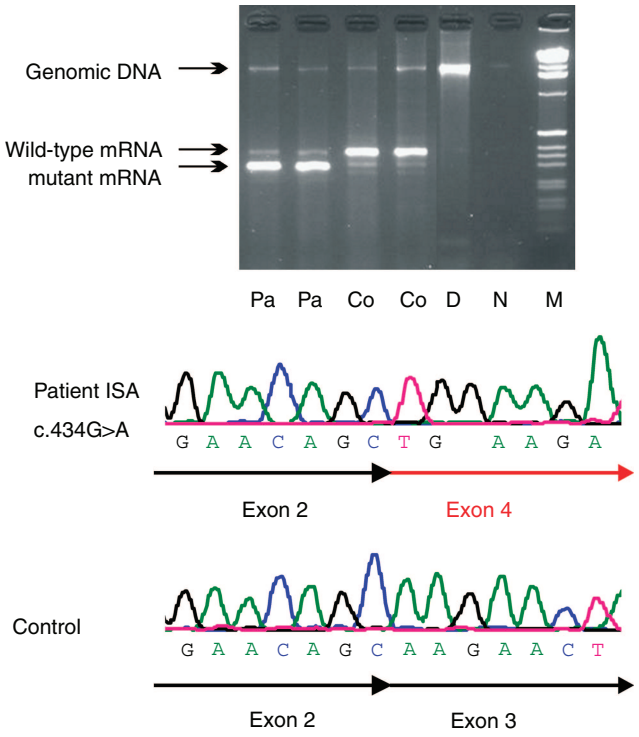

Figure 1. Analysis of the transcription of mutation c.434G>A in *ALOXE3* using a mini-gene assay. Genomic DNA of patient ISA spanning intron 1 to exon 5 was cloned and expressed. RNA analysis revealed a shortened fragment from exon 2 to exon 4 (upper panel). Sequence analysis showed complete skipping of exon 3 (lower panel). Pa, patient; Co, control; D, DNA template; N, negative control; M, molecular weight standard.

Nielsen, 1997), at positions 257 and 313 bases downstream of nucleotide c.1, resulting in a frameshift and in an almost complete loss of the N-terminal LH2 domain, respectively. In both cases, the mutation was expected to result in the synthesis of a nonfunctional peptide.

Analysis of recurrent mutations

Additionally, we found the previously reported mutations p.Arg234X and p.Pro630Leu of *ALOXE3* (Jobard *et al.*, 2002; Eckl *et al.*, 2005) in 10 unrelated families from Germany: 4 patients were compound heterozygous for both mutations, 2 and 3, respectively, were homozygous for either p.Arg234X or p.Pro630Leu, and 1 patient was compound heterozygous for mutations p.Pro630Leu and p.Lys240ArgfsX4. Thus, we found the mutation p.Arg234X on 8 and the mutation p.Pro630Leu on 11 independent chromosomes, in addition to the patients described earlier.

To search for a common origin of these two mutations, we performed microsatellite marker analysis around the *LOX* gene cluster (Krebsová *et al.*, 2001; Lugassy *et al.*, 2008) with all families carrying at least one of the two mutations but did not detect a shared haplotype. The analysis showed homozygosity for patients FE7 and FE10 but no common haplotype.

Phenotype

A total of 21 patients from 19 unrelated families, originating from Germany, Austria, Spain, Belgium, Israel, the United Arab Emirates, and Sri Lanka, were clinically assessed in detail. These patients carried previously unreported mutations in *ALOX12B* or *ALOXE3*; moreover, we have included our patients with recurrent mutations p.Arg234X and p.Pro630Leu in *ALOXE3*. Consanguinity was only reported for families FE1, FB2, and ISA. Neonates born with collodion membrane often showed a mild to moderate manifestation compared with other types of congenital ichthyosis. Ectropion or eclabion, mostly mild, was only present in one-third of the cases. Children and adults showed a generalized scaling with mild to moderate erythema. Scales were mostly whitish to light brown, discrete to moderate in adherence, and small in size (Figure 2a). Moreover, more than half of the patients (13 out of 19 patients) showed a striking palmoplantar hyperlinearity with or without mild keratoderma (Figure 2b). In contrast to ichthyosis vulgaris, patients showed a mild keratotic lichenification that also included the elbow or popliteal fossa and the dorsa of the extremities (Figure 2c and d). Heat intolerance because of a reduced sweating ability (hypohidrosis) was an accompanying clinical problem in almost all patients. One patient showed a peculiar kink of the external ear helix (Figure 2e). The clinical features are summarized in Table S1.

Enzymatic activity of 12R-LOX and eLOX-3

All three new missense mutations found in *ALOX12B* and the in-frame deletion c.1031_1039del9 in *ALOXE3* were cloned by site-directed mutagenesis and expressed in HEK 293 cells. Sonic homogenates were adjusted after Western blot analysis and incubated with their genuine substrates, that is, arachidonic acid for 12R-LOX and 12R-HPETE in case of eLOX-3. 12R-LOX converts arachidonic acid to the primary product 12R-HPETE, which is rapidly transformed to its reduced derivative, 12R-HETE; eLOX-3 converts 12R-HPETE to 8R-hydroxy-11R,12R-epoxyeicosa-5Z,9E,14Z-trienoic acid. RP-HPLC analyses of incubations with 12R-LOX mutants revealed no reaction products, similar to extracts from cells expressing β-galactosidase, indicating complete loss of enzymatic activity for all 12R-LOX mutants (Figure 3a). Correspondingly, the in-frame deletion in eLOX-3 resulted in complete loss of enzyme activity (Figure 3b). The insertions and deletions that result in a frameshift are supposed to be deleterious and have therefore not been tested for enzyme activity.

DISCUSSION

Since the first publication of mutations in *ALOX12B* and *ALOXE3* describing a total of 6 mutations in patients with non-bullous congenital ichthyosiform erythroderma from the Mediterranean area (Jobard *et al.*, 2002), 21 further mutations in *ALOX12B* and 3 mutations in *ALOXE3* have been published so far (Figure 4; Table S2; Eckl *et al.*, 2005; Ashoor *et al.*, 2006; Lesueur *et al.*, 2007; Harting *et al.*, 2008). Here we have completed the first analysis of *ALOX12B* and *ALOXE3* in a large group of patients representing the whole phenotypic spectrum of ARCI. In a total of 250 independent patients, mutations in *TGM1* accounted for 38% of the ARCI cases (data not shown), and mutations in *ALOX12B* and

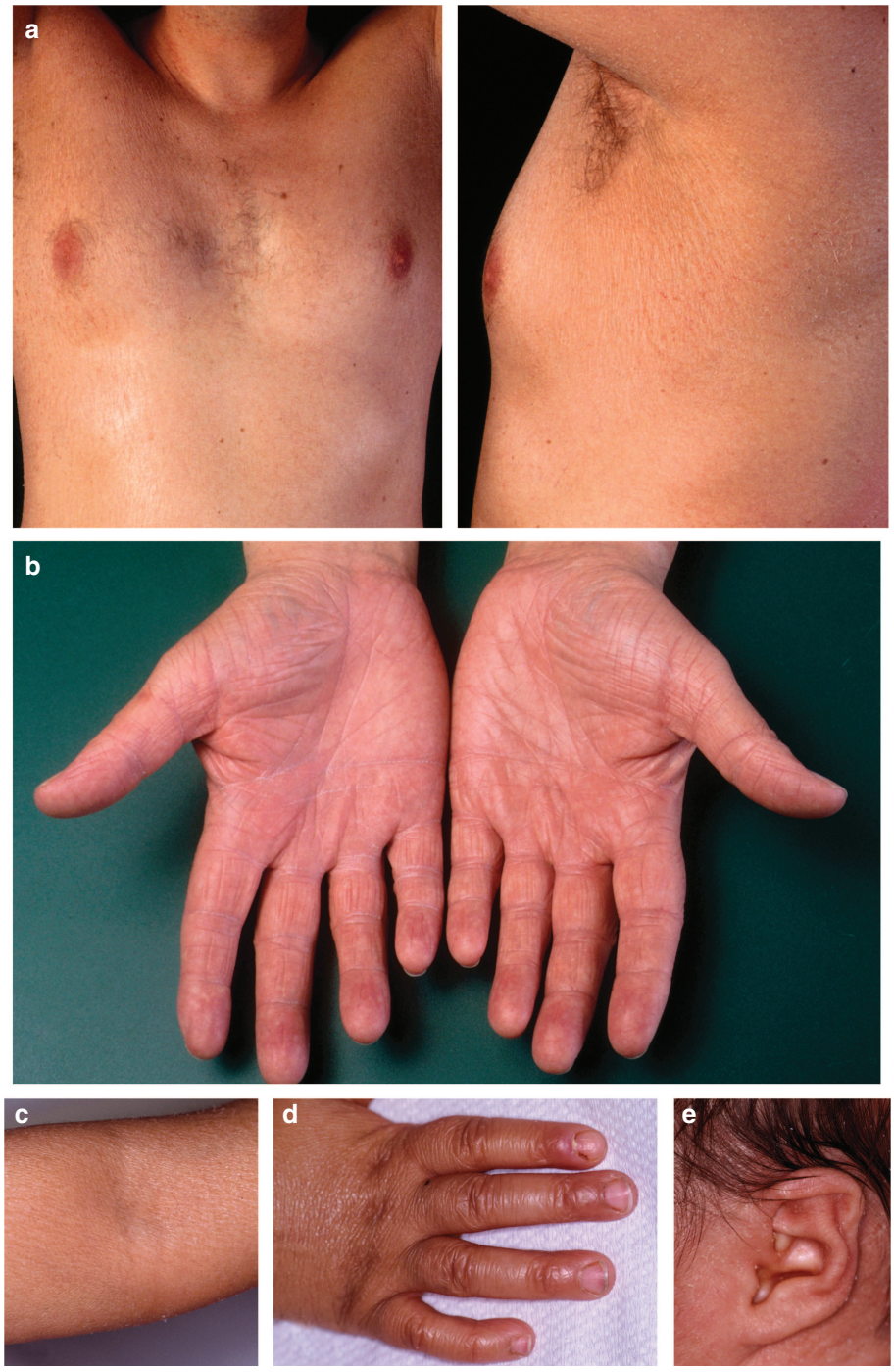

Figure 2. Clinical presentation of patients with epidermal lipoxygenase deficiency. (**a** and **b**) Clinical presentation of adults: the light scaling (**a**) and hyperlinearity of the hands (**b**) in a patient with mutations in *ALOXE3* were reminiscent of ichthyosis vulgaris but the ichthyosis was present at birth, and the patient showed a mild keratotic lichenification of the overall integument (patient FE3). (**c**–**e**) Clinical presentation of children: the keratotic lichenification also included the elbow fossa and dorsa of the extremities (**c**, patient FE1; **d**, FE4). Additionally, there was a peculiar kink of the external ear helix in patient FE4 (**e**).

ALOXE3 were found in 17 patients each, thus each representing 6.8% of the cases. We have identified three new mutations in *ALOXE3* and eight new mutations in *ALOX12B* in ARCI patients from Germany, Spain, Belgium, Israel, the United Arab Emirates, and Sri Lanka. Thus our findings also demonstrate extended allelic heterogeneity of ARCI.

Surprisingly, we found 6 of 8 mutations in *ALOX12B* to be deletions, insertions, or splice site mutations leading to frameshift, in contrast to the mutations known so far, which consisted in 21 missense mutations and only 4 mutations resulting in premature termination codons. The same was true for *ALOXE3*, where we found two deletions and a splice site mutation. Truncating mutations are likely to result in loss

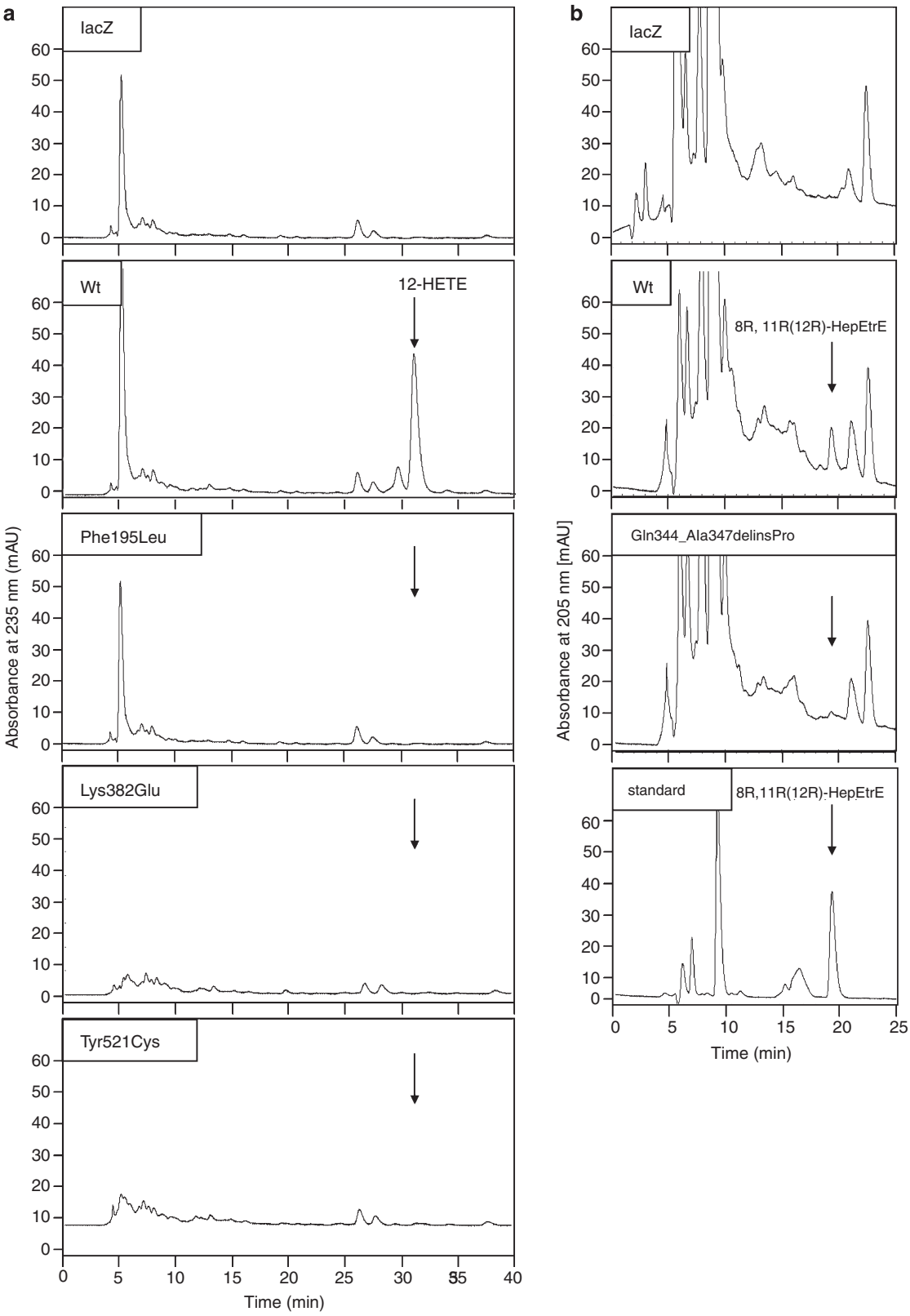

Figure 3. RP-HPLC analysis of the products formed by wild-type or mutant LOX. Homogenates from HEK-293 cells transiently transfected with pcDNA3 constructs containing wild-type or mutant 12R-LOX and eLOX-3 or the β-galactosidase (lacZ) coding region were incubated in TE buffer with (**a**) 100 μM arachidonic acid or (**b**) 50 μM 12R-HPETE for 15 minutes at 37 °C. Products were extracted with methanol/dichloromethane, dried under vacuum, redissolved in methanol/water/acetic acid (82:18:0.01 by volume), injected on a 4 μm YMC-Pack ODS-H80 column, and eluted at 0.5 ml min^{-1}. The eluates were monitored at 235 and 205 nm, respectively. Authentic 12-HETE and 8R-hydroxy-11R,12R-epoxyeicosatrienoc acid (8R,11R(12R)-HepEtrE) were used as standards. The retention times of 12-HETE and 8R,11R(12R)-HepEtrE were 32.2 and 19.3 minutes, respectively.

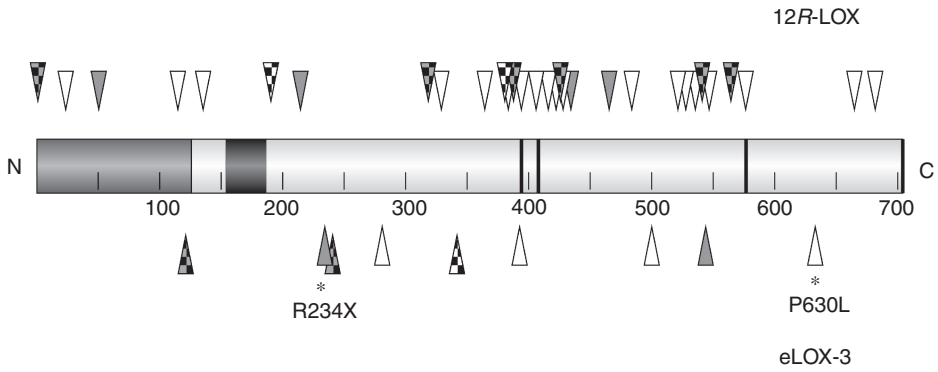

Figure 4. Schematic representation of the domain organization of 12R-LOX and eLOX-3 and survey of known mutations associated with ARCI. The amino-acid sequences of 12R-LOX and eLOX-3 are very similar to each other. 12R-LOX and eLOX-3 contain an N-terminal β-barrel LH2 domain (dark gray), a C-terminal catalytic lipoxygenase domain (white) from position 126, and an inserted specific extra domain (black). Putative iron ligands of the active site are shown in black (Gillmor *et al.*, 1997; Boeglin *et al.*, 1998; Krieg *et al.*, 2001). Mutation sites are represented by arrowheads (upper part: 12R-LOX; lower part: eLOX-3); missense (white) and truncating (gray) mutations are differentiated. Previously unreported mutations described here are checkered. The two mutational hotspots in eLOX-3 are marked by asterisks.

of protein activity. All missense and in-frame mutations found in either *ALOX12B* or *ALOXE3* were analyzed functionally. The mutants were properly expressed in HEK 293 cells but completely lacked enzymatic activity. These results indicate that ARCI is caused by inactivating mutations in *ALOX12B* and *ALOXE3* regardless of whether or not the mutant protein is synthesized.

In contrast to most other studies, the families reported here were mostly not consanguineous. This is of interest with regard to the distribution of the mutations in different genes for ARCI and within a gene. The mutations described here were distributed throughout the entire genes (Figure 4). There was a minor focus on exons 9 and 12 in *ALOX12B* in the regions encoding the substrate-binding pocket of the enzyme. However, most mutations were private ones, either heterozygous or homozygous by descent, and no mutation was found on more than four different chromosomes. In contrast, only a total of nine different mutations are known in *ALOXE3*, three of these described here, although the overall frequency of mutations is identical in both *LOX* genes. Our results reveal that this is due to the high frequency of mutations p.Arg234X and p.Pro630Leu in eLOX-3. These two mutations were seen in 10 families described here and 5 previously reported kindreds (Jobard *et al.*, 2002; Eckl *et al.*, 2005). In total, p.Arg234X was found on 13 alleles and p.Pro630Leu on 15 alleles in 12 families from Germany, 2 from the Czech Republic, and 1 from France so far. Microsatellite analysis did not support a founder effect except in case of the Czech families with mutation c.700C>T (data not shown). Thus, p.Arg234X and p.Pro630Leu appear to represent true mutational hotspots. In case of c.700C>T the CG to TG transition may be due to the spontaneous deamination of 5-methylcytosine in the dinucleotide CpG. This type of mutation often associates with mutational hotspots and accounts for approximately 37% of all transitions (Krawczak *et al.*, 1998; Antonarakis *et al.*, 2000; Cooper, 2002). Moreover, these data imply that *ALOXE3* is at least partly methylated in the germ lines, which is in accordance with previous observations (Monk *et al.*, 1987, 1991; Koeberl *et al.*, 1990; Bestor, 1996; Bestor and Tycko, 1996). The high frequency of the mutation c.1889C>T cannot be explained easily. Again this is a transition, which is the more common mutation type as compared to transversions, however, it does not affect a CpG dinucleotide. Both mutational hotspots in *ALOXE3* are probably due to structural features of the gene beyond its primary structure.

In concordance to our previous findings (Eckl *et al.*, 2005) we saw only mild to moderate, light to light-brown scaling with no or moderate erythema in all patients. In contrast, patients with severe transglutaminase deficiency caused by inactivating mutations in *TGM1* mostly show a severe lamellar ichthyosis, often with brown to dark-brown scaling (Hennies *et al.*, 1998; Oji and Traupe, 2006). The clinical findings were independent of mutation position and type. There were some differences between patients with mutations in *ALOX12B* and those with *ALOXE3* mutations. Patients with mutations in *ALOX12B* showed a lighter and more discrete scaling in comparison to an often brownish and moderately adherent scaling seen in patients with *ALOXE3* mutations (Table S1). This finding, scored and averaged over all body sites, was not significant though using Fisher's exact test on a 2×2 table ($P = 0.067$). However, erythema was significantly more pronounced in patients with *ALOX12B* mutations ($P = 0.0017$). Remarkably, all patients with *ALOX12B* mutations showed mild hyperkeratosis of palms and soles with accentuated palmoplantar creases. Keratoderma was absent in 9/12 patients with mutations in *ALOXE3*, and the other three only showed a very discrete palmar or plantar keratosis ($P = 0.00071$). In total, 76% (13/17) of the patients were born as collodium babies or showed a partial collodium membrane. Interestingly, 6/8 patients complained about itching, and 15/17 reported reduced or completely absent sweating ability.

In summary, we have identified 11 previously unreported mutations in *ALOX12B* and *ALOXE3* in patients with ARCI. All the mutations were loss-of-function mutations, either impairing enzyme activity or ablating protein synthesis, thus confirming the crucial role played by 12R-LOX and eLOX-3

during epidermal barrier formation. In the first analysis of a large group of patients with ARCI, we have demonstrated that mutations in *ALOX12B* or *ALOXE3* account for almost 15% of ARCI cases. Although *ALOX12B* mutations are distributed over the gene, there are two significant hotspots for mutations in *ALOXE3*. These findings are important for the design of diagnostic strategies for ARCI and may form the basis of further studies into the molecular characterization of the epidermal LOX pathway during epidermal differentiation.

MATERIALS AND METHODS

DNA samples

All patients were diagnosed as having ARCI based on common clinical criteria. Family history and clinical data were recorded by specialized dermatologists experienced in keratinization diseases using a standardized questionnaire that collected data, among others, on findings at birth, color and form of scales and extent of erythema on various body sites, and presence or absence of palmoplantar keratoderma and hyperlinearity on palms and soles at the time of examination. The study was conducted according to Declaration of Helsinki principles and approved by the ethical committee of the Medical Faculty of the University of Cologne. Blood samples were collected after written informed consent. DNA was prepared according to standard methods.

Mutation detection

A total of 250 ARCI cases including 15 families described before (Eckl *et al.*, 2005) were screened for mutations. *ALOX12B* (GenBank NM_001139.2) and *ALOXE3* (NM_021628.1) consist of 15 exons each. As described earlier (Eckl *et al.*, 2005), all exons and exon/intron boundaries were analyzed for mutations by amplifying each exon and directly sequencing the PCR product using the BigDye Terminator v.1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) on a 3730 DNA Analyzer (Applied Biosystems). Primer sequences are available upon request.

Haplotype analysis

Microsatellite marker analysis was performed in all families that showed common mutations in *ALOXE3* (c.700C>T or c.1889C>T). Eight markers covering 14 cM were chosen in and around the epidermal *LOX* gene cluster (Krebsová *et al.*, 2001; Lugassy *et al.*, 2008) and amplified in singleplex reactions. Products were pooled, run on a DNA Analyzer (3730; Applied Biosystems) and analyzed with GeneMapper software version 3.0 (Applied Biosystems).

Synthesis of mutant recombinant 12R-LOX and eLOX-3

cDNA of human 12R-LOX and eLOX-3 has been cloned in the eukaryotic expression vector pcDNA3 (Eckl *et al.*, 2005). Site-directed mutagenesis was performed in the construct for each missense mutation found using the Site Directed Mutagenesis Kit (Stratagene, Amsterdam, the Netherlands). Primers were designed manually following the manufactures' recommendations. For the mutation c.1031_1039del9 in *ALOXE3* a deletion primer pair was designed to generate an in-frame deletion of three residues in the recombinant protein. Transformation was done in SURE II super-competent cells (Strategene). Clones were analyzed by complete sequencing using primers T7 and Sp6 and gene-specific primers. Confirmed mutant clones, wild-type controls and negative control pcDNA/lacZ were transfected into HEK-293 cells seeded in 10 cm dishes using the CalPhos method (Clontech, Mountain View, CA). Cells were harvested 48 hours after transfection, washed and homogenized by sonication. Cell lysates were used for western blot analysis and enzymatic assays. All transfection experiments were done in triplicates.

Western blot analysis

Antibodies against human 12R-LOX and eLOX3 were used as described (Eckl *et al.*, 2005). Western blotting was performed using standard techniques after running a 7.5% Laemmli gel. Detection was done using either rabbit anti-12R-LOX or rabbit anti-eLOX-3 antibody. Anti-β-actin antibody was used as an expression control for all samples.

Enzyme assay and product analysis

For the 12R-LOX activity assay, homogenates were incubated with arachidonic acid. For assaying eLOX-3 activity, the homogenates were incubated with 12R-HPETE. The incubations were terminated by the addition of sodium formate buffer. The products were extracted with a modification of the Bligh and Dyer procedure as described (Chang *et al.*, 1996). The final mixture was centrifuged and the organic phase was removed and evaporated. The products were dissolved in ethanol and stored at −70 °C until further analysis. The products were analyzed by RP-HPLC on a 4 μm YMC-Pack ODS-H80 column (25cm × 0.46 cm; YMC Europe, Schermbeck, Germany) with a 1 cm guard column using the solvent system of methanol/water/acetic acid (82:18:0.01 by volume) and a flow rate of 0.5 ml minute^{-1}. Elution was monitored at 205, 235, and 300 nm on a Bio-Tek Kontron 540 diode array detector.

Splice assay

Genomic DNA constructs spanning exons 2–5 of *ALOXE3* from patient ISA and unaffected control persons were cloned into the eukaryotic expression vector pcDNA3.1/V5-His TOPO (Invitrogen, Carlsbad, CA) and transformed into TOP10 competent cells (Invitrogen). After complete sequencing correct clones were transfected into HEK-293 cells and total RNA was isolated 48 hours after transfection. After cDNA synthesis using standard procedures with oligo(dT) priming, specific PCR was performed with primers located in exons 2 and 4. Products were extracted from agarose gels and sequenced.

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

We thank all patients and their families for providing samples. This work was supported by the German Federal Ministry for Education and Research with a grant to the Network for Ichthyoses and Related Keratinization Disorders (NIRK) to HCH, HT, and MLP, and with grants from the Deutsche Forschungsgemeinschaft to HCH, ES, and PK, the European Commission in the 6th Framework Programme for Research to the network GeneSkin (HCH and HT), and the German Support Group Ichthyosis (Selbsthilfe Ichthyose e.V.) to HCH.

SUPPLEMENTARY MATERIAL

Table S1. Summary of clinical features of 20 ARCI patients from 19 families. (a) Patients with mutations in *ALOX12B*. (b) Patients with mutations in *ALOXE3*.

Table S2. Summary of mutations in *ALOX12B* and *ALOXE3* identified in patients with ARCI.

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