ULTRASTRUCTURAL AND GENETIC STUDIES IN ICHTHYOSES

Krisztina Becker M.D.
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Leader of the program: Prof. Dr. András Falus Ph.D., D.Sc.
Tutor: Prof. Dr. Sarolta Kárpáti Ph.D., D.Sc.
'Ichthyosis' comprises a group of clinically different disorders underlying different genetic causes, sharing the common features of hereditary nature and dry, scaly skin due to disorders of cornification.

Frequently occurring mild vulgar (autosomal dominant and X recessive inheritance), rare severe congenital forms (lamellar ichthyosis and congenital ichthyosiform erythroderma) and ichthyotic skin symptoms associated with disturbances in other organ systems (X-dominant ichthyosis, Chanarin-Dorfman disease, Sjögren-Larsson syndrome) are clinically distinguished within this group of disorders.

Further, genetic heterogeneity is present in the sub-groups sharing similar clinical symptoms (lamellaris ichthyosis (LI) or congenital ichthyosiform erythroderma (CIE)), thus the distinction between different subgroups is not possible based on the clinical symptoms alone. Previous studies failed to establish any phenotype-genotype correlation.

Electron microscopic examination of subcellular abnormalities may help to distinguish between macro-morphologically similar diseases and in certain cases it may show the localisation of the suggested genetic error.

Causative genes have been identified and mutations have been described in several hereditary skin diseases, as in certain sub-groups of ichthyoses using new genetic methods introduced in the past few years.

1994 LI has been mapped to chromosome 14 and subsequently mutations of the TGM1 gene, encoding keratinocyte transglutaminase (TGase 1) have been identified in vast majority of the families with LI and some families with CIE.

Transglutaminase (TGase) 1, localised in the cells of the upper granular and horny layer is a membrane-associated enzyme consisting of 815 amino acids. It is the largest and major enzyme of the five known TGases expressed in the epidermis, which plays a key role in the formation of the cornified cell envelope (CCE). It catalyses by formation of \( \gamma \)-glutamyl-\( \varepsilon \)-lysine isodipeptide bonds not only cross-linking of CCE precursor proteins on the inner side of the plasma membrane but the ester-linkage of ceramides to cell envelope proteins, too. TGase 1 takes thus part in formation of the corneocyte lipid envelope (CLE).

1999 X-dominant ichthyosis (Conradi-Hünermann-Happle syndrome) has been identified as caused by one of a genetic defects in the postsqualene cholesterol biosynthesis and mutations of the 3\( \beta \)-hydroxysteroid-\( \Delta 8 \)-\( \Delta 7 \)-isomerase (also called emopamil-binding protein, EBP) gene have been described.

Molecular-biological - biochemical correlation of the genotype and phenotype are still unknown in most of the disorders. Ultrastructural characteristics may help to define the phenotype more precise. Disclosure of mutations connected to the ultrastructural phenotype may help to classify these inherited diseases and may widen our knowledge about genotype-phenotype correlation.
AIM OF THE STUDY

- Collection, clinical description, classification and documentation of patients with severe forms of ichthyosis (autosomal recessive lamellar ichthyosis and congenital ichthyosiform erythroderma).
- Diagnostic, clinical characterisation and documentation of patients with associated ichthyosis (X-dominant ichthyosis/ Conradi-Hünermann-Happle syndrome, Chanarin-Dorfman disease, Comèl-Netherton syndrome).
- Electron microscopic examination of specimen obtained from the lesional skin in order to assess ultrastructural characteristics.
- Disclosure of mutations underlying the clinical symptoms and comparison with the ultrastructural features found in order to define phenotype-genotype correlation more precise.

MATERIAL AND METHODS

Patients and clinical description
Among the 9 families studied, history and clinical symptoms proposed in 7 cases the existence of autosomal recessive congenital ichthyosis (ARCI). Symptoms of six patients fulfilled the criteria of ARCI, two of them showed formation of thick scales without erythema corresponding LI, four of them had erythroderma of various severity corresponding CIE. In one case the mild clinical symptoms resembled vulgar ichthyosis, but anamnestic data suggested the possibility of ARCI. Electron microscopic examination of skin samples were carried out in all cases studied, mutation analysis of the TGM1 gene has been carried out in the 7 cases corresponding the clinical criteria of ARCI. One patient with mild osseal and ocular symptoms (X-dominant ichthyosis, Conradi-Hünermann-Happle syndrome), and one patient with skin symptoms (severe ichthyotic hair loss) associated with jaundice, lipoid vacuolisation of the liver, lipid vacuoles in the granulocytes of the peripheral blood smear and minor anomalies (Chanarin-Dorfman syndrome) referred to an associated ichthyosis.

Blood and skin samples
Skin biopsy was obtained under local anaesthesia. DNA was isolated from peripheral blood lymphocytes of the proband and family members according to standard techniques. All participating patients and family members gave written informed consent to the histology and genetic studies.

PCR amplification
All fifteen TGM1-exons were amplified by PCR using intronic primers in order to include the exon/intron boundaries in the mutation analysis. Primer pairs for PCR were as described previously, except of exon 1 (sense and antisense): 5’-GCA CGG CCT CTG ATA GTG TGG-3’ and 5’-TAG GAA TCA GCC TGG TGC CAG-3’, exon 2,3 (sense and antisense): 5’-ACT GGC TGG ACT ACC TGG TTA-3’ and 5’-GCC TCT CCC CAC CAA ACA TAG-3’; exon 7 (antisense): 5’-CCT TAG GCC TCT CTC TGT
TGT TAA CAC-3’; exon 10 (sense and antisense): 5’-TGA TGA CCT TGT TCC AGA GGC CAC-3’ and 5’-TGT ATA ATG AGT GAC TTG CCC CG-3’; exon 12 (sense) 5’-CAC AAC AAG TGT TCC TGC CAA GTG-3’; exon 13 (antisense): 5’-GTC CTT ATC CCC TGG CCT TCA CTC-3’; exon 14 (antisense) 5’-AGA CAG AGA GGG AGC AAA GCT GG-3’.

All five EBP-exons were amplified using intronic primers. Primer pairs for PCR were (sense and antisense): exon 1, 5’-GGG ATG TGA CAG AGC GCG AG-3’ and 5’-CGC GTA GCC GGG GAG AGC-3’; exon 2, 5’-ATT CGG TCC ATT TAC ATT TCTC-3’ and 5’-CAA ATC CCA TCC CAC AGC-3’; exon 3, 5’-CAC AAC AAG TGT TCC TGC CAA GTG-3’ and 5’-CAA ATC CCA TCC CAC AGC-3’; exon 4, 5’-ATT CGG TCC ATT TAC ATT TCTC-3’ and 5’-CAA ATC CCA TCC CAC AGC-3’; exon 5, 5’-ATT CGG TCC ATT TAC ATT TCTC-3’ and 5’-CAA ATC CCA TCC CAC AGC-3’ as described previously. Amplification conditions were 95 °C for 5 min, 95 °C for 45 s, 60-63 °C for 30 s and 72 °C for 30 s for 40 cycles in a TouchGene thermal cycler (Techne Cambridge Ltd UK).

Conformation sensitive gel electrophoresis
Heteroduplex analysis was performed in a vertical gel containing 8% polyacrylamid, 10% ethylene glycol, 15% formamide and 20×GT buffer. Sample preparation consisted of denaturation at 98 °C for 5 min followed by a 55 min incubation on 68 °C before loading. Heteroduplexes were visualised by staining with SYBR Green I. (Sigma)

Automated sequence analysis
Bands of altered mobility detected by heteroduplex analysis were directly sequenced using ABI Prism 310 automated sequencing system (Applied Biosystem). TGM1 nucleotides were numbered according to the published sequence of Kim et al. (1992). In the case of EBP the numbering of nucleotids started with the beginning of the coding sequence (A of ATG=1), according to GenBank H.sapiens mRNA for phenylalkylamine binding protein (accession number: Z37986)

Allele-specific PCR analysis
Allele-specific PCR was performed with the following primers: for the A3366G mutation (sense): 5’ATC CCT CCT TCC GGT TTG ACCA 3’ and for the G4073C transversion (antisense): 5’ACG CCA GCA AAG AGC CAG CACT3’. The primer pairs generated a fragment of 252 bp and 275 bp respectively, proving the presence of the relevant mutation.

Restriction endonuclease digestion
with Alw26I, RsaI (PROMEGA, Madison, WI, USA) and Aci I (NEW ENGLAND BioLabs Inc.) was carried out according to the manufacturers recommendations. Bands were detected in 8% polyacrylamide gel and were visualised with ethidium bromide.

Ultrastructural analysis was performed on samples fixed in 4% paraformaldehyde and 1% glutaraldehyde, embedded in Poly/Bed 812 Embedding Media (Polysciences, Inc.). Morphological analysis was performed using Philips C10 transmission electron microscope.
Enzyme and immuno-histochemical procedures

Transglutaminase activity assay was performed on unfixed cryostat sections of the skin by monitoring the covalent incorporation of monodansyl cadaverine (Sigma Chemicals Co, USA) using 12 µM monodansyl cadaverine in Tris-HCL buffer at pH 7.4, containing 5 mM CaCl₂. Control sections were incubated by adding 20 mM EDTA. After blocking the reaction with 10 mM EDTA in Tris-HCL, sections were incubated with anti-dansyl rabbit IgG antibodies (Molecular Probes Inc, Europe) diluted 1:100 in PBS, followed by incubation with anti-rabbit sheep IgG coupled with Cy3 (Amersham Life Science Inc.) 1:800 in PBS.

The presence of Tgase 1 protein was detected with mouse monoclonal anti-human keratinocyte transglutaminase antibodies (B.C1) diluted 1:20 in 0.1% BSA followed by incubation with sheep anti-mouse IgG coupled with Cy3 (Amersham Life Science Inc) 1:200 in PBS. The sections were examined and photographed, using fixed times on a Leitz Laborlux S, Wild MPS epifluorescens equipment.

RESULTS

Among the 9 families studied, clinical symptoms referred in six, the history of the patient in one case to the existence of ARCI.

In two of the patients symptoms of other organ systems associated to skin symptoms proposed the existence of associated ichthyosis (X-dominant ichthyosis/Conradi-Hünermann-Happle syndrome and Chanarin-Dorfman syndrome). Electron microscopy confirmed the clinical diagnoses in the two cases corresponding associated ichthyosis. In the case of X-dominant ichthyosis ultrastructural examination proved vacuole-formation in the epidermal cells, needle-like inclusions described in the literature could not be found. Mutation in the EBP gene, recently disclosed in the background of the disease was identified in our patient; this R147G transversion has not been published so far. The diagnosis of our patient with Chanarin-Dorfman syndrome has been confirmed by biochemical studies as well, the ultrastructure proved lipid vacuoles in the epidermal cells as described in the literature.

Among the six patients fulfilling the clinical criteria of ARCI two patients showed symptoms of lamellar ichthyosis. Ultrastructure of the skin samples showed in both cases rectangular cholesterol clefts (IC type II) in the cells of the horny layer, an ultrastructural feature most frequently found among cases with TGM1 mutations, based on previous experiences. Analysis of the TGM1 gene identified in both case mutations in the TGM1 gene. In the first case we found two previously described TGM1 mutations (A3366G/ V378L) in a compound heterozygous pattern, in the second case only one mutation (R553P) could be found, not reported in the literature so far.

Four patients had skin symptoms fulfilling the clinical criteria of congenital ichthyosiform erythroderma. Ultrastructure of the skin sample in one of the patients proved typical rectangular clefts in the str. corneum (IC type II) and mutation analysis of the TGM1 gene identified the presence of the previously reported mutation V378L. Based on ultrastructural findings we classify our patient as an erythrodermic phenotype of LI similar to CIE. Based on literary data retinoid treatment may play a role in the formation of erythroderma. Clinical symptoms of the real phenotype could be observed during the follow up period after stopping the administration of retinoids.
Electron microscopy of the skin samples proved in two of our patients lipid droplets in the horny cells and disorganised lamellar structures in the intercellular space (IC type I). In one of the patients no mutation of the TGM1 gene could be found. In the second patient two novel nonsense mutations (Y503X/S669X) of the TGM1 gene could be identified in a compound heterozygous pattern, resulting in truncation of the C-terminal end domain of the TGase 1 enzyme. Dramatic reduction in the presence of the protein and impairment of TGase activity by these mutations was confirmed by enzyme- and immuno-histochemical studies.

Ultrastructure of the epidermis in our fourth patient showing the clinical symptoms of congenital ichthyosiform erythroderma showed incomplete cornification of the horny layer, lack of keratohyalin granules, and granules in the cells of the granular and horny layer containing granular electrondense material. These ultrastructural features did not fit the ultrastructure of ARCI and presumed Comèl-Netherton syndrome. Increased total IgE levels of the patient corroborate this presupposition; no mutation in the TGM1 gene was found. Lack of hairshaft-anomaly (trichorrhexis nodosa) formally contradicts our presumption, though this feature may manifest even in older age. We follow up the patient, observing her for Comèl-Netherton syndrome in future.

In the patient, presenting first as an adult, the mild skin symptoms observed resembled vulgar type ichthyosis and only history of erythroderma and severe skin condition as a newborn, and recurring inflammation in the skin folds arose the possibility of ARCI. Electron microscopic examination of the skin proved infrequently seen features of abnormal perinuclear membrane structures in the granular layer and elongated multi-layer membranes in the horny cells. Based on ultrastuctural findings we diagnosed our patient to have ARCI. Mutation analysis of the TGM1 gene proved no abnormalities.

**CONCLUSIONS AND UTILITY OF THE RESULTS**

Our results proved the importance of ultrastructure, a morphological examination of the highest resolution in the more exact diagnosis of ichthyoses. Using this investigation the possibility of more accurate diagnosis and classification is given within the heterogenous group of ichthyosis. Cases not classified or misdiagnosed based on clinical features could be identified by this investigation (CIE→Comèl-Netherton syndrome, or vulgar type ichthyosis→ARCI).

To forecast a prognosis is easier by classification even in absence of knowledge concerning accurate genetic background of the disease (eg. dominant/recessive inheritance, role of anticipation etc. in the genetic guidance). Exact diagnosis and classification is of pivotal interest in planning genetic studies, because search for mutations in different genes is requested in different diseases and they have increasing availability with the development of genetics. (eg. Comèl-Netherton syndrome and mutations in the SPINK5 gene)

Ultrastructural features may help in planning genetic studies not only by clearing up diagnostic/classification questions. In small, clinically homogenous but genetically heterogenous groups (eg. ARCI) it may act as a screen, because some ultrastructural characteristics may increase the probability of the presence of a mutation. (eg. IC type II ultrastructure→TGM1 mutation). Electron microscopic examination may act as a guide
by selecting patients for mutation analysis of a certain gene. This will not only increase the efficiency, but the cost-effectiveness of mutation analyses, too. Premise of these is of course, that further systematic (in rare diseases international) studies, comparing different mutations with different ultrastructural morphologies, delineate specificity of ultrastructure. Genetic studies and mutation analyses in well-diagnosed and accurately classified patients opens the possibility of prenatal diagnosis, as it got practically available for our patients with TGM1 and EBP mutations following successful mutation analysis. Definition of the genotype based on exact diagnosis/classification of the phenotype will open the perspective for these patients for somatic gene-therapy in future.

This paper comprises the results of ultrastructural and genetic studies made in the group of congenital and associated ichthyoses. First mutation analyses on ichthyoses in Hungary are reported. In this study four novel, unpublished mutations, and two previously reported mutations have been identified. The study did not reveal any clear-cut phenotype-genotype correlation between the clinical symptoms and the underlying mutations, but some relations between ultrastructural phenotype and genotype could be found.

Our study proved that ultrastructural analysis plays a pivotal role in exact differentiation of clinically similar diseases and may suit for planning genetic studies.

**PUBLICATIONS REPORTED IN THE TOPIC OF THE STUDY**

1. Szalai S, Szalai Cs, Becker K, Török É. Keratin 9 mutations in the coil 1A region in epidermolytic palmoplantar keratoderma *Pediatric Dermatology* 1999;16 (6):430-435  **IF: 0.874**


ABSTRACTS REPORTED IN THE TOPIC OF THE STUDY


