MUTATION ANALYSIS AND GENE EXPRESSION STUDIES IN DARIER’S DISEASE AND HAILEY-HAILEY DISEASE

Emőke Rácz M.D.
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Leader of the program: Dr. András Falus Ph.D., D.Sc.
Tutor: Dr. Sarolta Kárpáti Ph.D., D.Sc.
INTRODUCTION

Darier’s disease (dyskeratosis follicularis, MIM 124200) is an autosomal dominantly inherited skin disorder with a prevalence of 1 in 30000-50000. It is characterized by skin-coloured or yellow-brown keratotic papules on the seborrheic areas of the skin; the papules often form large, crusted, confluent plaques. Mutations in the \textit{ATP2A2} gene encoding the sarco/endoplasmic reticulum calcium ATPase 2 (SERCA2) were identified as the cause of the disease. Hailey-Hailey disease (benign chronic familial pemphigus, MIM 169600) is characterized by recurrent vesicles and erosions, particularly involving flexural areas. Mutations in the \textit{ATP2C1} gene encoding the human secretory pathway calcium ATPase 1 (hSPCA1) were identified in the background of Hailey-Hailey disease, another autosomal dominantly inherited skin disease. A common histopathologic feature of the two diseases is the loss of cell-to-cell adhesion (acantholysis) and abnormal keratinization.

SERCA2 pumps are found in the membrane of the endoplasmic reticulum (ER). They catalyze the hydrolysis of ATP coupled with the translocation of Ca\textsuperscript{2+} ions from the cytosol to the lumen of the ER. The hSPCA1 protein is a transmembrane protein of the Golgi membrane. It has been shown to transport both Ca\textsuperscript{2+} and Mn\textsuperscript{2+} from the cytoplasm into the Golgi lumen. Both pumps play a pivotal role in intracellular Ca\textsuperscript{2+} homeostasis and signalling.

SERCA2 and hSPCA1 are expressed in all tissues examined, however, the symptoms of Darier’s disease and Hailey-Hailey disease are confined to the skin.
AIMS OF THE STUDY

To analyse the mutations of the \textit{ATP2A2} gene in Darier’s disease and mutations of the \textit{ATP2C1} gene in Hailey-Hailey disease in Hungarian patients using DNA extracted from peripheral blood lymphocytes.

To reveal genotype-phenotype correlations by coupling the results of the mutation analysis to the clinical data of the patients.

To study the process of calcium-induced differentiation in keratinocytes from patients with Hailey-Hailey disease \textit{in vitro}.

METHODS

\textit{Mutation analysis}

DNA was obtained from peripheral EDTA blood samples using standard protocols. All patients gave written informed consent to the genetic studies. All exons of the studied genes, that is 21 exons of the \textit{ATP2A2} gene in patients with Darier’s disease and 28 exons of the \textit{ATP2C1} gene in patients with Hailey-Hailey disease, were amplified by polymerase chain reaction (PCR). The amplicons were screened for sequence variations by heteroduplex analysis using conformation-sensitive gel electrophoresis (CSGE). Amplicons with divergent migration patterns on CSGE were reamplified from genomic DNA by PCR and products were sequenced in forward and reverse orientation using ABI Prism 310 automated sequencing system. In addition, CSGE and sequencing if necessary were used to exclude the possibility of
polymorphisms in a cohort of 50 control individuals. Accession numbers for the nucleotide and protein sequences were M23115 (GenBank) and P16615 (SwissProt), respectively, for the ATP2A2 gene and NT005612 (GenBank) and P98194 (SwissProt) for the ATP2C1 gene.

Keratinocyte culture
Normal adult keratinocytes and keratinocytes from Hailey-Hailey disease patients were obtained from surgical skin margins and punch biopsies, respectively. Keratinocytes were grown in 0.06 mM calcium until 60-70% confluent and then either switched to 1.2 mM calcium to stimulate involucrin expression or maintained at 0.06 mM calcium. After 24 hours cells were harvested and total cell lysates were used for Western blotting with anti-involucrin and anti-filaggrin antibodies, or for RNA extraction and quantitative RT-PCR.

Protein Extraction and Western Immunoblotting:
Total protein content of the cells was extracted using standard protocols. Subsequently Western immunoblotting was performed using 10% acrylamide gel SDS-PAGE, as described previously. The primary antibody used was mouse anti-involucrin antibody. Protein expression was normalized using an antibody for β-actin. To quantify the decrease in involucrin protein in HHD keratinocytes, densitometry was performed on the chemiluminescence photo images. Density values were normalized to β-actin expression within same samples.

RNA extraction and real-time quantitative PCR
Total RNA extraction was performed with commercially available kit. On-column DNase digestion step was included to avoid detection of DNA remnants by the nucleic acid stain SYBR Green in the PCR step. Real-time quantitative PCR was performed on a ABI 7900 machine using SYBR green detection as previously described. The 18S ribosomal protein gene was used as an internal reference control to normalize relative levels of gene expression. Primer sequences were: INVfw: TCCTCCTCCAGTCAATACCC, rev: GCTGATCCCTTTGTGTT; 18Sfw: GTAACCCGTGTTAACCCATT, rev: CCATCCACCGGTAGTAGCCG. All reactions were performed in triplicate. Data were analysed using the \(2^{-\Delta\Delta CT}\) method.

**Luciferase assay**

The regulatory activity of calcium on the involucrin promoter in normal vs. Hailey-Hailey cells was analysed by the Dual-Luciferase Reporter Assay System. The involucrin promoter construct (a gift from Yuko Oda) consisted of the 3.7 kb fragment of the human involucrin promoter (-2461 to +1228 bp of the involucrin gene, numbered with transcription start site as +1) subcloned into a pGL-3 basic vector to drive firefly luciferase. An internal control plasmid with the SV40 promoter alone to drive the Renilla reniformis luciferase was used to normalize for transfection efficiency.

Healthy adult and Hailey-Hailey keratinocytes at 40% confluency were cotransfected with the two constructs using Superfect Transfection Reagent according to the instruction manual.
The regulatory activity of calcium on the involucrin promoter was monitored by the relative luciferase activities.

**Message RNA degradation assay**

Keratinocytes were grown in 0.06 mM Ca\(^{2+}\) to 70-80% confluence. Ca\(^{2+}\) concentration of the medium was switched to 1.2 mM for 4 hours to stimulate the transcription of the involucrin mRNA, then 10 µg/ml actinomycin D, a compound known to inhibit transcription by intercalation into DNA, was added to inhibit further mRNA synthesis and cells were harvested at 3-hour intervals up until 12 hours. Results were expressed as a percentage of the initial mRNA levels present at the onset of actinomycin D treatment.

**RESULTS**

In Hungarian patients with Darier’s disease we found 11 heterozygous mutations in the *ATP2A2* gene, 9 of which were novel. The mutations are summarized in the table below.

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Consequence</th>
<th>Amino acid change</th>
<th>Protein domain</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. 116A→C</td>
<td>missense</td>
<td>N39T</td>
<td>S1</td>
</tr>
<tr>
<td>2. 482C→A</td>
<td>missense</td>
<td>A161D</td>
<td>β-strand</td>
</tr>
<tr>
<td>3. 492G→C</td>
<td>missense</td>
<td>R164S</td>
<td>β-strand</td>
</tr>
<tr>
<td>4. 542C→G</td>
<td>missense</td>
<td>T181R</td>
<td>β-strand</td>
</tr>
<tr>
<td>5. 558insT</td>
<td>Frame shift</td>
<td>PTC +5aa</td>
<td>β-strand</td>
</tr>
<tr>
<td>6. 1288-6A→G</td>
<td>New acceptor splice site, frame shift</td>
<td>PTC +21aa</td>
<td>P-domain</td>
</tr>
</tbody>
</table>


<table>
<thead>
<tr>
<th></th>
<th>Mutation</th>
<th>Type</th>
<th>Effect</th>
<th>Domain/Region</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.</td>
<td>1320delT</td>
<td>Frame shift</td>
<td>PTC+8as</td>
<td>P-domain, phosphorylation site</td>
</tr>
<tr>
<td>8.</td>
<td>1821delC</td>
<td>Frame shift</td>
<td>PTC+3as</td>
<td>Nucleotide-binding (N)-domain</td>
</tr>
<tr>
<td>9.</td>
<td>2104G→A</td>
<td>Missense</td>
<td>D702N</td>
<td>Hinge domain</td>
</tr>
<tr>
<td>10.</td>
<td>2369A→C</td>
<td>Missense</td>
<td>Q790P</td>
<td>M6</td>
</tr>
<tr>
<td>11.</td>
<td>2727T→A</td>
<td>Non-sense</td>
<td>C909X</td>
<td>M8</td>
</tr>
</tbody>
</table>

In patients with Hailey-Hailey disease we identified 3 novel, heterozygous mutations in the *ATP2C1* gene. In a patient whose symptoms were induced by environmental contact allergens, we found a nonsense mutation, Q506X in exon 17. In a 65-year-old male patient with a 41-year-long history of severe recurrent symptoms a single-nucleotide-insertion, 1085insA was detected. In a 55-year-old woman we identified a heterozygous gross deletion, nt+4-+29del, through which a part of the 5’ non-coding region of the *ATP2C1* gene is deleted from one allele of the gene. This region contains the binding site of the Sp-1 transcription factor, without which transcription from the *ATP2C1* gene is minimal.

At high calcium concentrations Hailey-Hailey keratinocytes had lower levels of involucrin compared to normal keratinocytes, while filaggrin levels remained unchanged. HHD keratinocytes both expressed lower absolute levels of involucrin, and also proved to be less responsive to raised extracellular Ca²⁺.

To determine whether the decrease in protein expression was caused by decreased mRNA levels, we next assessed involucrin mRNA levels using real-time quantitative PCR. As a result of calcium stimulation, involucrin mRNA levels in normal cells increased 3-
fold, while in Hailey-Hailey keratinocytes only a 1.3-fold increase was detected. Next we examined whether the decrease in the involucrin mRNA levels are caused by a defect in the calcium responsiveness of the involucrin promoter. The regulatory activity of calcium on the involucrin promoter in normal vs. Hailey-Hailey cells was analysed by a dual luciferase assay system. In this experiment calcium increased the expression of the involucrin promoter 4-fold in healthy adult keratinocytes and 6-fold in HHD keratinocytes, which indicates that the calcium-responsive AP-1 site of the involucrin promoter responds well to calcium in Hailey-Hailey keratinocytes. The defect in involucrin synthesis therefore is not due to a defect in involucrin promoter activation.

To address whether the decrease in the involucrin mRNA levels was associated with increased mRNA degradation, we treated keratinocytes with 1 μg/ml Actinomycin D to inhibit mRNA synthesis at high calcium conditions, extracted RNA 6 hours after treatment and performed real-time quantitative PCR. We found that the degradation of involucrin mRNA was increased in HHD keratinocytes.

**DISCUSSION AND CONCLUSIONS**

Studying the ATP2A2 gene in Hungarian patients with Darier’s disease, we found 11 distinct, heterozygous mutations, 9 of which were novel. Next to two deletions, one insertion and a new splice-site generating intronic nucleotide change we detected 6 missense and one non-sense mutations. In the ATP2C1 gene of Hungarian patients with Hailey-
Hailey disease we identified three novel mutations: one nucleotide change leading to a STOP codon, one insertion and one gross deletion affecting the 5’ noncoding region of the gene. No clear correlation between genotype and phenotype could be observed in this cohort of patients.

Keratinocytes from patients with Hailey-Hailey disease have elevated cytosolic Ca**2+** levels, and are less responsive to increasing levels of extracellular calcium than their normal counterparts. We found decreased protein and mRNA levels of involucrin, a marker of the early keratinocyte differentiation was decreased in HHD keratinocytes. The activation of the involucrin promoter upon calcium stimulation was normal or increased. The degradation of the involucrin mRNA transcripts was increased in HHD keratinocytes. To understand the significance of these in vitro data, further in vivo experiments will be indispensable. Our results raise the question whether the degradation of mRNA transcripts of other structural proteins are also increased.

The thesis summarizes the first mutation analysis data in patients with Darier and Hailey-Hailey diseases from Hungary.
PUBLICATIONS IN THE FIELD OF THE THESIS


