Cooper, G.M., Shendure, J., 2011. Needles in stacks of needles: finding disease-causal variants in a wealth of genomic data. Nat. Rev. Genet. 12, 628–640.
De Paepe, A., Malfait, F., 2012. The Ehlers–Danlos syndrome, a disorder with many faces. Clin. Genet. 82, 1–11.
De Paepe, A., Nuytinck, L., Hausser, I., Anton-Lamprecht, I., Naeyaert, J.M., 1997. Mutations in the COL5A1 gene are causal in the Ehlers–Danlos syndromes I and II. Am. J. Hum. Genet. 60, 547–554.
Fine, J.D., Johnson, L.B., Weiner, M., Suchindran, C., 2008. Cause-specific risks of childhood death in inherited epidermolysis bullosa. J. Pediatr. 152, 276–280.
Genomes Project, C., Abecasis, G.R., Altshuler, D., Auton, A., Brooks, L.D., Durbin, R.M., Gibbs, R.A., Hurles, M.E., McVean, G.A., 2010. A map of human genome variation from population-scale sequencing. Nature 467, 1061–1073.
Hindorff, L.A., Sethupathy, P., Junkins, H.A., Ramos, E.M., Mehta, J.P., Collins, F.S., Manolio, T.A., 2009. Potential etiologic and functional implications of genome-wide association loci for human diseases and traits. Proc. Natl. Acad. Sci. U. S. A. 106, 9362–9367.
Hoischen, A., Gilissen, C., Arts, P., Wieskamp, N., van der Vliet, W., Vermeer, S., Steehouwer, M., de Vries, P., Meijer, R., Seiqueros, J., Knoers, N.V., Buckley, M.F., Scheffer, H., Veltman, J.A., 2010. Massively parallel sequencing of ataxia genes after array-based enrichment. Hum. Mutat. 31, 494–499.
International Human Genome Sequencing, C., 2004. Finishing the euchromatic sequence of the human genome. Nature 431, 931–945.
Kimura, M., 1983. The Neutral Theory Of Molecular Evolution. Cambridge Univ. Press, New York.
Ku, C.S., Cooper, D.N., Polychronakos, C., Naidoo, N., Wu, M., Soong, R., 2012. Exome sequencing: dual role as a discovery and diagnostic tool. Ann. Neurol. 71, 5–14.
MacArthur, D.G., Tyler-Smith, C., 2010. Loss-of-function variants in the genomes of healthy humans. Hum. Mol. Genet. 19, R125–R130.
McGrath, J.A., Stone, K.L., Begum, R., Simpson, M.A., Dopping-Hepenstal, P.J., Liu, L., McMillan, J.R., South, A.P., Pourreyron, C., McLean, W.H., Martinez, A.E., Mellerio, J.E., Parsons, M., 2012. Germline mutation in EXPH5 implicates the Rab27B effector protein Slac2-b in inherited skin fragility. Am. J. Hum. Genet. 91, 1115–1121.
Metzker, M.L., 2010. Sequencing technologies — the next generation. Nat. Rev. Genet. 11, 31–46.
Moore, B., Hu, H., Singleton, M., De La Vega, F.M., Reese, M.G., Yandell, M., 2011. Global analysis of disease-related DNA sequence variation in 10 healthy individuals: implications for whole genome-based clinical diagnostics. Genet. Med. 13, 210–217.
Ng, P.C., Henikoff, S., 2001. Predicting deleterious amino acid substitutions. Genome Res. 11, 863–874.
Pettersson, E., Lundeberg, J., Ahmadian, A., 2009. Generations of sequencing technologies. Genomics 93, 105–111.
Ritelli, M., Dordoni, C., Venturini, M., Chiarelli, N., Quinzani, S., Traversa, M., Zoppi, N., Vascellaro, A., Wischmeijer, A., Manfredini, E., Garavelli, L., Calzavara-Pinton, P., Colombi, M., 2013. Clinical and molecular characterization of 40 patients with classic Ehlers–Danlos syndrome: identification of 18 COL5A1 and 2 COL5A2 novel mutations. Orphanet J. Rare Dis. 8, 58.
Schaffer, J.V., 2012. Molecular diagnostics in genodermatoses. Semin. Cutan. Med. Surg. 31, 211–220.
Singleton, A.B., 2011. Exome sequencing: a transformative technology. Lancet Neurol. 10, 942–946.
Smith, S.M., Birk, D.E., 2012. Focus on molecules: collagens V and XI. Exp. Eye Res. 98, 105–106.
Stenmark, H., 2009. Rab GTPases as coordinators of vesicle traffic. Nat. Rev. Mol. Cell Biol. 10, 513–525.
Stone, E.A., Sidow, A., 2005. Physicochemical constraint violation by missense substitutions mediates impairment of protein function and disease severity. Genome Res. 15, 978–986.
Watson, J.D., Crick, F.H., 1953. Molecular structure of nucleic acids; a structure for deoxyribose nucleic acid. Nature 171, 737–738.
Wenstrup, R.J., Langland, G.T., Willing, M.C., D'Souza, V.N., Cole, W.G., 1996. A splice-junction mutation in the region of COL5A1 that codes for the carboxyl propeptide of pro alpha 1(V) chains results in the gravis form of the Ehlers–Danlos syndrome (type I). Hum. Mol. Genet. 5, 1733–1736.

REPORT

Mutations in *GRHL2* Result in an Autosomal-Recessive Ectodermal Dysplasia Syndrome

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Grainyhead-like 2, encoded by *GRHL2*, is a member of a highly conserved family of transcription factors that play essential roles during epithelial development. Haploinsufficiency for *GRHL2* has been implicated in autosomal-dominant deafness, but mutations have not yet been associated with any skin pathology. We investigated two unrelated Kuwaiti families in which a total of six individuals have had lifelong ectodermal defects. The clinical features comprised nail dystrophy or nail loss, marginal palmoplantar keratoderma, hypodontia, enamel hypoplasia, oral hyperpigmentation, and dysphagia. In addition, three individuals had sensorineural deafness, and three had bronchial asthma. Taken together, the features were consistent with an unusual autosomal-recessive ectodermal dysplasia syndrome. Because of consanguinity in both families, we used whole-exome sequencing to search for novel homozygous DNA variants and found *GRHL2* mutations common to both families: affected subjects in one family were homozygous for c.1192T>C (p.Tyr398His) in exon 9, and subjects in the other family were homozygous for c.1445T>A (p.Ile482Lys) in exon 11. Immortalized keratinocytes (p.Ile482Lys) showed altered cell morphology, impaired tight junctions, adhesion defects, and cytoplasmic translocation of GRHL2. Whole-skin transcriptomic analysis (p.Ile482Lys) disclosed changes in genes implicated in networks of cell-cell and cell-matrix adhesion. Our clinical findings of an autosomal-recessive ectodermal dysplasia syndrome provide insight into the role of GRHL2 in skin development, homeostasis, and human disease.

Grainyhead-like 2 (GRHL2) is a mammalian homolog of *Drosophila* protein grainy head (GRH), which, along with GRHL1 and GRHL3, has a role in epithelial morphogenesis.[1,2] This family of transcription factors controls the development and differentiation of multicellular epithelia by regulating genes germane to cell junction formation and proliferation.[3,4] Biologically, GRHL2 contributes to formation of the epithelial barrier and wound healing, as well as neural-tube closure, maintenance of the mucociliary airway epithelium, and tumor suppression.[5–11] *GRHL2* (MIM 608576) has been shown to regulate *TERT* (MIM 187270) expression and to enhance proliferation of epidermal keratinocytes; it also impairs keratinocyte differentiation through transcription inhibition of genes clustered at the epidermal differentiation complex[12] and regulates epithelial morphogenesis by establishing functional tight junctions.[13]

GRHL2 is also present in the cochlear duct,[14] and mutations in human *GRHL2* have been found in progressive autosomal-dominant hearing loss (DFNA28 [MIM 608641]),[15,16] and other polymorphic sequence variants in *GRHL2* have been implicated in age-related hearing impairment and noise-induced hearing loss.[17–19] To date, however, the role of GRHL2 in skin biology has not been well established. Causing severe facial and neural-tube defects, *Grhl2* knockout is embryonically lethal in mice,[17,20] and mutant zebrafish display inner-ear defects and abnormal swimming positions.[18] In contrast, *Grhl1*$^{-/-}$ mice show hair loss and palmoplantar keratoderma, as well as abnormal desmosome cell junctions and dysregulated terminal differentiation in keratinocytes.[21] Moreover, *Grhl3*$^{-/-}$ embryos fail to establish a normal epidermal barrier and display defective embryonic wound repair.[22] Thus, unlike for GRHL1 and GRHL3, there is currently a lack of data associating GRHL2 with skin pathology. In this report, however, we have identified two families in which affected subjects have developmental defects affecting skin, oral mucosa, and teeth (as well as hearing and lungs), thus implicating *GRHL2* in an autosomal-recessive ectodermal dysplasia syndrome.

We investigated two unrelated Kuwaiti families, both consanguineous, in which clinically similar features were present in a total of six affected individuals (Figures 1A and 1B). The clinical features were noted in early infancy and comprised short stature ($\leq 25^{th}$ percentile), nail dystrophy and/or loss, oral mucosa and/or tongue pigmentation, abnormal dentition (delay, hypodontia, enamel hypoplasia), keratoderma affecting the margins of the palms

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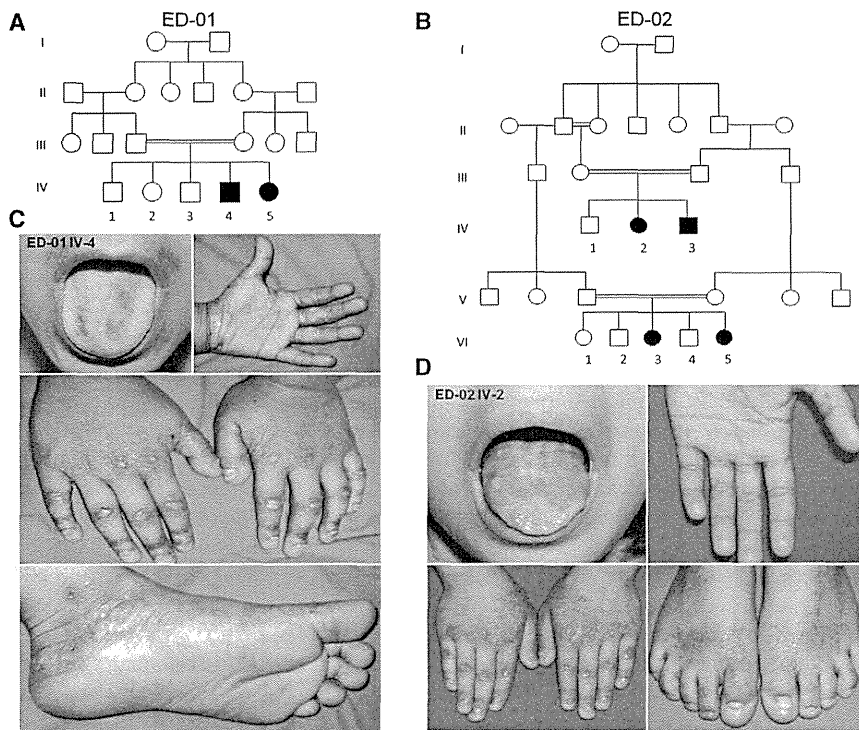

Figure 1. Pedigrees and Clinical Features of This Autosomal-Recessive Ectodermal Dysplasia Syndrome
(A and B) Two unrelated consanguineous pedigrees with a total of six affected individuals.
(C and D) An affected 8-year-old male (from pedigree ED-01) and an affected 12-year-old female (from pedigree ED-02) both show features of tongue hyperpigmentation, skin thickening around the margins of the palms and soles, hypoplastic finger and toe nails, knuckle pads on the fingers, and atrophic wrinkling on the dorsal aspects of the hands and feet. Additional clinical images from other subjects are shown in Figure S1.

and soles, and focal hyperkeratosis of the dorsal aspects of the hands and feet (Figures 1C and 1D; Figure S1, available online). No individual showed any wound-healing defect, blistering tendency, hair or sweating abnormalities, or other developmental anomalies. Two affected sisters (ED-02 VI-3 and VI-5) had dysphagia with evident esophageal strictures. Three individuals (ED-01 IV-4 and IV-5 and ED-02 VI-3) developed sensorineural deafness in early infancy, and three others (ED-01 IV-4 and IV-5 and ED-02 IV-2) had bronchial asthma. One individual (ED-01 IV-4) had severe iron-deficiency anemia requiring blood transfusion. Laboratory tests (full blood count, serum biochemistry, immunoglobulin levels, and thyroid-function tests) were otherwise within the normal range for all affected individuals. None of the parents had any skin, hair, teeth, nail, or hearing abnormalities.

To investigate the etiology of the condition, we first assessed lesional skin biopsies taken from three affected individuals (ED-01 IV-4 and ED-02 VI-3 and VI-5) by using immunohistochemistry and transmission electron microscopy. The subjects' legal guardians provided written informed consent according to a protocol approved by the St. Thomas' Hospital Ethics Committee (Molecular basis of inherited skin disease: 07/H0802/104). Blood and skin samples (ellipse of skin taken under local anesthesia by 1% lignocaine) were obtained in adherence to the Declaration of Helsinki guidelines. Light microscopy showed mild acanthosis and hyperkeratosis (Figure S2), but transmission electron microscopy of the skin was unremarkable—it showed no clear abnormalities in keratinocytes, hemidesmosomes, or desmosome cell-cell junctions. Likewise, skin immunolabeling using a panel of antibodies that target basement membrane extracellular matrix proteins (collagen IV and collagen VII), epidermal-adhesion-associated proteins (desmoglein 1, keratin 1, keratin 14, and desmoplakin), and markers of terminal differentiation (filaggrin) showed normal intensity and labeling patterns when affected skin from two affected individuals was compared to skin from a normal control subject (Figure S3; see Supplemental Data for methods and antibody details). However, we noted increased staining for the proliferation marker Ki-67 in the epidermis (Figure S4). Collectively, the skin-biopsy findings were not diagnostic for any known inherited skin disease.

We then used whole-exome sequencing to identify a candidate gene or genes. We extracted genomic DNA from peripheral blood from two affected individuals (ED-01 IV-4 and ED-02 VI-3). We performed whole-exome capture by using in-solution hybridization (Agilent All Exon Kit V4) and generated sequencing on the Illumina HiSeq 2000. Resulting reads were aligned to the reference human genome (UCSC Genome Browser hg19, GRCh37) with the Novoalign software package (Novocraft Technologies). Duplicate reads, resulting from PCR clonality or optical duplicates, and reads mapping to multiple locations were excluded from downstream analysis. A summary of exome-coverage data is presented in Table S1. Sixteen previously unreported homozygous mutations were identified (ten in family ED-01 and six in family ED-02). The only gene containing homozygous variants common to both subjects was *GRHL2* (Table S2). The respective mutations were c.1192T>C (p.Tyr398His) in exon 9 and c.1445T>A (p.Ile482Lys) (RefSeq NM_024915.3) in exon 11. These mutations were confirmed by Sanger sequencing (Figures 2A and 2B) and were also shown to segregate with the disease phenotype in other affected pedigree members. Both mutations are located in the DNA binding site of *GRHL2* (Figure 2C) and are predicted to be "probably damaging" by PolyPhen-2 analysis (scores 0.984 and 0.994 for

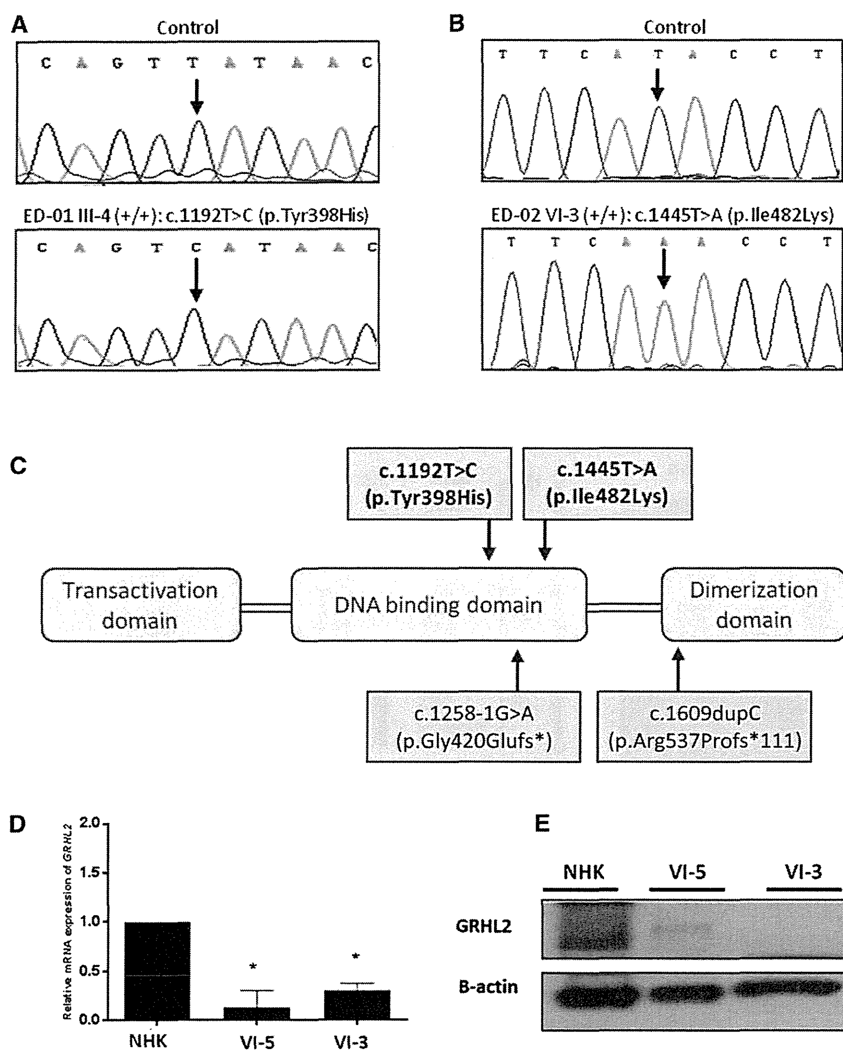

Figure 2. Autosomal-Recessive Mutations in *GRHL2* Lead to Reduced Gene Expression and Protein Levels

(A and B) Sanger sequencing confirmed the presence of different homozygous missense mutations in *GRHL2* in affected subjects from both pedigrees.

(C) Schematic representation of the functional domains of GRHL2. The recessive missense mutations we identified (top) are located within the DNA binding domain; the previously reported heterozygous splice-site or deletion mutations that cause autosomal-dominant deafness are also illustrated (bottom).

(D) qPCR for *GRHL2* expression in cultured keratinocytes showed reduced expression in two affected subjects from pedigree ED-02 (*$p < 0.05$ in comparison to control cells). Error bars represent the SD from three independent experiments.

(E) Immunoblotting using cultured keratinocyte whole-cell lysates revealed markedly reduced or undetectable amounts of GRHL2 in these same individuals.

c.1192T>C and c.1445T>A, respectively). Neither variant has been observed by the 1000 Genomes Project or detected in ~1,200 control in-house exomes or in 260 ethnically matched control chromosomes.

GRHL2 can be detected in the nuclear fraction of normal human keratinocytes in culture (but is subsequently lost in cell senescence).[23] We cultured keratinocytes from two of the skin biopsies (ED-02 VI-3 and ED-02 VI-5) by standard methods and used these cells to examine *GRHL2* expression and GRHL2 localization (see Supplemental Data for methods); both were found to be reduced (Figures 2D and 2E). We then isolated primary keratinocytes from one of the affected individuals (ED-02 VI-5) and immortalized these cells at passage 1 (see Supplemental Data for methods). The phenotype of these cells was assessed by confocal microscopy. *GRHL2* mutant cells showed a less cuboidal, elongated phenotype and failed to form intact cell junctions, as seen in control immortalized keratinocytes. Notably, there was a reduction in cell membrane labeling for E-cadherin (adherens junctions) and zona-occludens-2 (tight junctions) (Figure S5). GRHL2 staining in control keratinocytes was seen both at cell-cell contact areas and within the nucleus (Figure 3A), whereas in mutant cells, the signal was not at the periphery and instead showed a fragmented punctate nuclear localization (Figure 3B). To assess the effects of the mutations on keratinocyte cell function, we also performed assays of cell adhesion and de-adhesion (see Supplemental Data for methods). No differences were noted for cell adhesion between mutant and control cells (Figure 3C), but mutant cells detached from fibronectin much faster than normal human keratinocyte controls after exposure to trypsin (Figure 3D).

Next, we assessed the transcriptome profile by using RNA extracted from whole skin from two individuals in pedigree ED-02. RNA from healthy control skin was obtained from discarded abdominoplasty tissue from plastic surgeons and used as four pooled samples. RNA extraction was performed with the Ambion mirVana miRNA Isolation kit (Invitrogen) according to the manufacturer's instructions. RNA was amplified with the Illumina TotalPrep RNA Amplification Kit, and subsequent gene-expression profiling was performed with the Illumina array HumanHT-12 v4 Expression BeadChip Kit according to the manufacturer's instructions. Gene-expression data were then analyzed with GenomeStudio software (Illumina). A prefiltering set was determined for significantly modulated expression (detection p value < 0.01; signal intensity fold change ≥ 2.0) between affected and control skin. A comprehensive functional-enrichment analysis was then performed with (1) the Database for Annotation, Visualization, and Integrated Discovery (v.6.7), based on the Gene

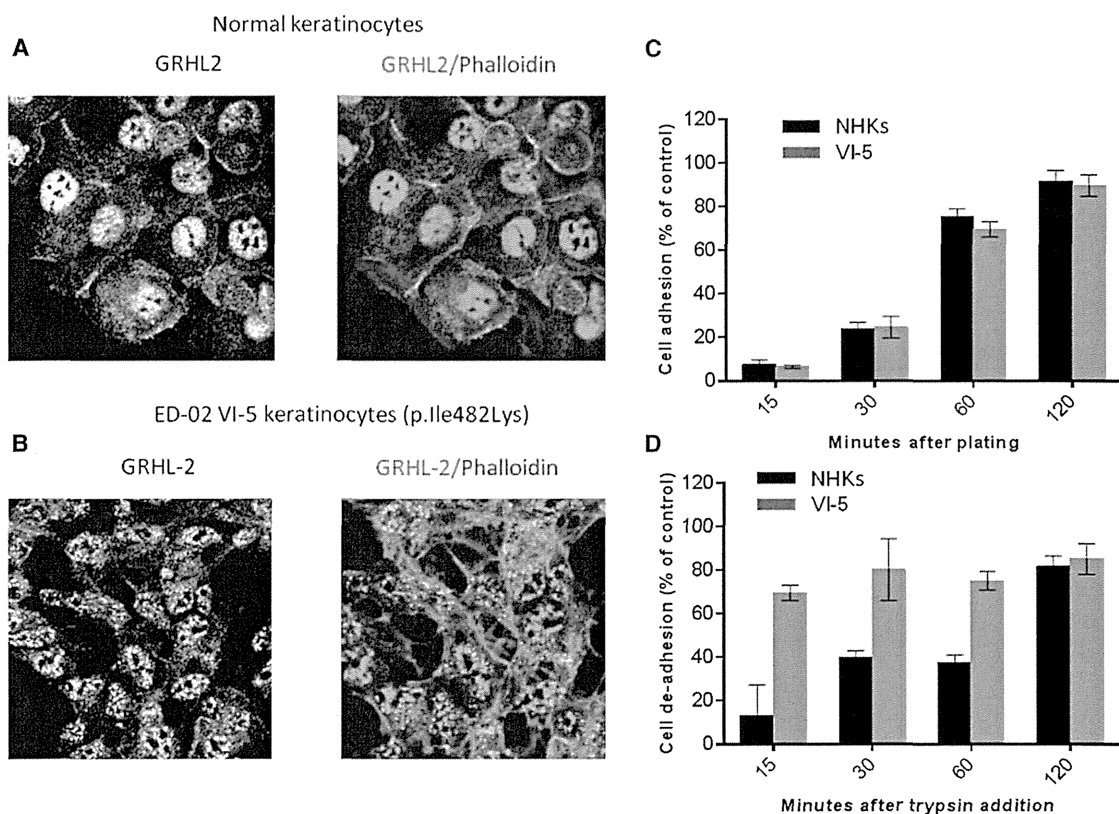

Figure 3. Impact of *GRHL2* Mutations on Keratinocyte Cell Biology
(A) Confocal microscopy in normal keratinocytes revealed nuclear, cytoplasmic, and membranous labeling for an antibody raised against GRHL2.
(B) In contrast, keratinocytes from an affected subject showed an altered pattern of antibody localization within the nucleus and a lack of any cell membrane labeling.
(C) Cell-adhesion assays showed no difference between wild-type and mutant keratinocytes. Error bars represent the SD from three independent experiments.
(D) In contrast, mutant cells showed more rapid detachment in trypsin de-adhesion assays. Error bars represent the SD from three independent experiments. NHK stands for normal human keratinocyte.

Ontology (GO) database (see Web Resources), and (2) the GeneGo Metacore software (Thomson Reuters), a systems-biology analysis tool based on a curated database of human protein-protein and protein-DNA interactions, transcription factors, signaling, and metabolic pathways. Comparison of the affected individuals' skin with the skin of healthy age- and site-matched control individuals identified 1,457 gene transcripts that were significantly altered: 668 upregulated (≥2-fold change) and 789 downregulated (≤0.5-fold change) transcripts for ED-02 VI-5. For ED-02 VI-3, 1,141 gene transcripts were altered: 466 upregulated and 675 downregulated. Of these changes in gene expression, 359 upregulated and 344 downregulated gene transcripts were common to both affected subjects. Evaluation of the changes in gene expression by functional-enrichment analysis identified several enriched GO pathways, processes, networks, and disease-associated transcripts, some of which are germane to the known functions of GRHL2. The top three upregulated GO pathways were linked to protein-folding maturation, cytoskeleton remodeling, and transcriptional control of lipid biosynthesis (involving genes encoding proopiomelanocortins and mitochondrial enzymes involved in metabolic pathways) (Tables S3–S10). Among the most significantly upregulated GO networks were the signal-transduction pathways and intermediate-filament remodeling (Table S7). Conversely, immune-response signaling; migration-inhibitory-factor-induced cell adhesion, migration, and angiogenesis; and networks of cell-cell and cell-matrix adhesion were downregulated (Table S8).

With regard to skin differentiation and barrier formation, selected alterations in gene expression are presented in Table S11. We also verified potential changes by performing quantitative PCR (qPCR) with RNA from whole skin of three individuals from the two pedigrees, as well as immortalized keratinocytes and primary fibroblasts from one affected person (see Supplemental Data for methods and controls). We observed reduced expression of *GRHL2* for all templates and a contrasting increase in *GRHL1* (MIM 609786) and *GRHL3* (MIM 608317) expression: unique and cooperative roles for this transcription factor family have been previously documented.[24] The most marked skin-barrier-associated gene changes were upregulation of aquaporin-encoding genes *AQP5* (MIM

600442) and *AQP7* (MIM 602974), the latter of which was expressed 50×–100× more in affected skin than in control skin. Gain-of-function mutations in *AQP5* have previously been associated with a form of autosomal-dominant nonepidermolytic palmoplantar keratoderma (MIM 600962).[25] Two-fold or greater reduction in gene expression was noted for *S100A8* (MIM 123885) and *S100A9* (MIM 123886), known targets for *GRHL1*. Previously, it has also been shown that GRHL2 enhances skin-barrier function by upregulating the tight-junction components claudins 3 and 4 and also Rab25, which localizes claudin 4 to tight junctions.[26] In affected people, we noted increases in *CLDN3* (MIM 602910), *CLDN4* (MIM 602909), and *RAB25* (MIM 612942) expression in whole skin (transcriptome and qPCR) and cultured keratinocytes (qPCR). Increased claudin 4 immunolabeling was also noted in the skin of two affected individuals (Figure S5).

Labeling for the proliferation marker Ki-67 was increased in the affected subjects' skin (Figure S4). This indicates that suprabasal keratinocytes are subject to an abnormal terminal-differentiation program, which provides a possible explanation for thickening of the epidermis and impairment of the epidermal barrier in our affected individuals with mutant *GRHL2*, although it is unclear why the most prominent skin scaling was found around the margins of the soles. We also noted reduced expression of *TERT* in the affected individuals' skin and keratinocytes. Overexpression of *GRHL2* in normal keratinocytes increases telomerase activity and increases replicative life span (*TERT* and *PCNA* [MIM 176740]). In contrast, knockdown of *GRHL2* represses the expression of these genes.[12]

The impact of *GRHL2* mutations on cell morphology has been previously described.[4] Lung epithelial cells transduced with *Grhl2* small hairpin RNA flatten in culture and lose their cuboidal morphology into an expanded cell phenotype. Knocking down *Grhl2* in lung epithelial cell lines leads to downregulation of *Cldn4* and *Cdh1*.[9] In our subjects' keratinocytes, immunostaining with E-cadherin showed reduced expression and qPCR showed downregulation of this transcript (Figure S6).

In addition to being expressed in skin, *Grhl2* and *GRHL2* are highly expressed in the inner ear, the lung epithelium, the ureteric bud of the kidney, the olfactory epithelium, the urogenital tract, the gastric mucosa, and human breast cancer cells.[4,8–11,18,27,28] With regard to the clinical phenotype in our affected individuals, aside from the changes affecting the skin and oral mucosa, the other main features comprised deafness and asthma, although this was variably present. Three subjects (ED-01 IV-4 and IV-5 and ED-02 VI-3) had deafness that developed in early infancy (c.f. the later-onset deafness in other families with GRHL2 haploinsufficiency).[15,16] Of note, none of the heterozygous carriers of either missense mutation in *GRHL2* had any deafness. The significance of GRHL2 in vertebrate inner-ear development is well established,[16] but the lack of deafness in the heterozygotes (and some homozygotes) in our pedigrees indicates a different functional effect of the missense mutations. Deafness is not a common feature of ectodermal dysplasia syndromes, although hearing loss can result from abnormalities in p63 and Notch signaling (morphological defects in organ of Corti)[29] and mutations in connexins 26 and 30 (altered endolymph ion homeostasis).[30] In contrast, mutagenesis studies in *Grhl2* have indicated a probable different pathophysiology for deafness with enlarged otocysts, absent otoliths, and malformed semicircular canals.[16]

The observation that three of the affected individuals (ED-01 IV-4 and IV-5 and ED-02 IV-2) had clinical symptoms of asthma is also noteworthy because the top enriched GO disease among the downregulated transcripts in our microarray data was asthma (Table S10). Previous in situ hybridization analyses have indicated that *Grhl2* is the only family member that is highly expressed in distal lung epithelium throughout development, although the particular cells expressing *Grhl2* have not been identified, nor has its functional role in the lung epithelium been fully established.[4] *Grhl1* and *Grhl3*, in contrast, are expressed in the embryonic lung epithelium, but later their expression is reduced in bronchi and bronchioles and is undetectable in the alveolar lung epithelium.[4,27] The potential relevance of other sequence variants in *GRHL2* to sporadic or familial cases of human asthma and other obstructive-airway diseases remains to be determined.

In summary, GRHL2, a member of a family of highly conserved transcription factors, is implicated in epithelial morphogenesis across a number of species. We have used whole-exome sequencing to identify *GRHL2* mutations underlying an ectodermal dysplasia syndrome in two families, and our data expand the current knowledge about the role of *GRHL2* in human disease and epithelial cell biology.

Supplemental Data

Supplemental Data include 6 figures and 13 tables and can be found with this article online at http://dx.doi.org/10.1016/j.ajhg.2014.08.001.

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Web Resources

The URLs for data presented herein are as follows:

Ensembl Genome Browser, http://www.ensembl.org/index.html
Gene Expression Omnibus, http://www.ncbi.nlm.nih.gov/geo/
Gene Ontology Consortium, http://www.geneontology.org/
Online Mendelian Inheritance in Man (OMIM), http://www.omim.org/
Primer3, http://frodo.wi.mit.edu/primer3/
PubMed, http://www.ncbi.nlm.nih.gov/PubMed/
RefSeq, http://www.ncbi.nlm.nih.gov/RefSeq
UCSC Genome Browser, http://genome.ucsc.edu/

Accession Numbers

The Gene Expression Omnibus accession number for the microarray data reported in this paper is number GSE56486.

References

1. Venkatesan, K., McManus, H.R., Mello, C.C., Smith, T.F., and Hansen, U. (2003). Functional conservation between members of an ancient duplicated transcription factor family, LSF/Grainyhead. Nucleic Acids Res. *31*, 4304–4316.
2. Stramer, B., and Martin, P. (2005). Cell biology: master regulators of sealing and healing. Curr. Biol. *15*, R425–R427.
3. Werth, M., Walentin, K., Aue, A., Schönheit, J., Wuebken, A., Pode-Shakked, N., Vilianovitch, L., Erdmann, B., Dekel, B., Bader, M., et al. (2010). The transcription factor grainyhead-like 2 regulates the molecular composition of the epithelial apical junctional complex. Development *137*, 3835–3845.
4. Varma, S., Cao, Y., Tagne, J.B., Lakshminarayanan, M., Li, J., Friedman, T.B., Morell, R.J., Warburton, D., Kotton, D.N., and Ramirez, M.I. (2012). The transcription factors Grainy-head-like 2 and NK2-homeobox 1 form a regulatory loop that coordinates lung epithelial cell morphogenesis and differentiation. J. Biol. Chem. *287*, 37282–37295.
5. Bray, S.J., and Kafatos, F.C. (1991). Developmental function of Elf-1: an essential transcription factor during embryogenesis in Drosophila. Genes Dev. *5*, 1672–1683.
6. Narasimha, M., Uv, A., Krejci, A., Brown, N.H., and Bray, S.J. (2008). Grainy head promotes expression of septate junction proteins and influences epithelial morphogenesis. J. Cell Sci. *121*, 747–752.
7. Cieply, B., Farris, J., Denvir, J., Ford, H.L., and Frisch, S.M. (2013). Epithelial-mesenchymal transition and tumor suppression are controlled by a reciprocal feedback loop between ZEB1 and Grainyhead-like-2. Cancer Res. *73*, 6299–6309.
8. Werner, S., Frey, S., Riethdorf, S., Schulze, C., Alawi, M., Kling, L., Vafaizadeh, V., Sauter, G., Terracciano, L., Schumacher, U., et al. (2013). Dual roles of the transcription factor grainyhead-like 2 (GRHL2) in breast cancer. J. Biol. Chem. *288*, 22993–23008.
9. Varma, S., Mahavadi, P., Sasikumar, S., Cushing, L., Hyland, T., Rosser, A.E., Riccardi, D., Lu, J., Kalin, T.V., Kalinichenko, V.V., et al. (2014). Grainyhead-like 2 (GRHL2) distribution reveals novel pathophysiological differences between human idiopathic pulmonary fibrosis and mouse models of pulmonary fibrosis. Am. J. Physiol. Lung Cell. Mol. Physiol. *306*, L405–L419.
10. Mlacki, M., Darido, C., Jane, S.M., and Wilanowski, T. (2014). Loss of Grainy head-like 1 is associated with disruption of the epidermal barrier and squamous cell carcinoma of the skin. PLoS ONE *9*, e89247.
11. Xiang, J., Fu, X., Ran, W., Chen, X., Hang, Z., Mao, H., and Wang, Z. (2013). Expression and role of grainyhead-like 2 in gastric cancer. Med. Oncol. *30*, 714.
12. Chen, W., Xiao Liu, Z., Oh, J.E., Shin, K.H., Kim, R.H., Jiang, M., Park, N.H., and Kang, M.K. (2012). Grainyhead-like 2 (GRHL2) inhibits keratinocyte differentiation through epigenetic mechanism. Cell Death Dis. *3*, e450.
13. Senga, K., Mostov, K.E., Mitaka, T., Miyajima, A., and Tanimizu, N. (2012). Grainyhead-like 2 regulates epithelial morphogenesis by establishing functional tight junctions through the organization of a molecular network among claudin3, claudin4, and Rab25. Mol. Biol. Cell *23*, 2845–2855.
14. Wilanowski, T., Tuckfield, A., Cerruti, L., O'Connell, S., Saint, R., Parekh, V., Tao, J., Cunningham, J.M., and Jane, S.M. (2002). A highly conserved novel family of mammalian developmental transcription factors related to Drosophila grainyhead. Mech. Dev. *114*, 37–50.
15. Peters, L.M., Anderson, D.W., Griffith, A.J., Grundfast, K.M., San Agustin, T.B., Madeo, A.C., Friedman, T.B., and Morell, R.J. (2002). Mutation of a transcription factor, TFCP2L3, causes progressive autosomal dominant hearing loss, DFNA28. Hum. Mol. Genet. *11*, 2877–2885.
16. Vona, B., Nanda, I., Neuner, C., Müller, T., and Haaf, T. (2013). Confirmation of GRHL2 as the gene for the DFNA28 locus. Am. J. Med. Genet. A. *161A*, 2060–2065.
17. Van Laer, L., Van Eyken, E., Fransen, E., Huyghe, J.R., Topsakal, V., Hendrickx, J.J., Hannula, S., Mäki-Torkko, E., Jensen, M., Demeester, K., et al. (2008). The grainyhead like 2 gene (GRHL2), alias TFCP2L3, is associated with age-related hearing impairment. Hum. Mol. Genet. *17*, 159–169.
18. Han, Y., Mu, Y., Li, X., Xu, P., Tong, J., Liu, Z., Ma, T., Zeng, G., Yang, S., Du, J., and Meng, A. (2011). Grhl2 deficiency impairs otic development and hearing ability in a zebrafish model of the progressive dominant hearing loss DFNA28. Hum. Mol. Genet. *20*, 3213–3226.
19. Li, X., Huo, X., Liu, K., Li, X., Wang, M., Chu, H., Hu, F., Sheng, H., Zhang, Z., and Zhu, B. (2013). Association between genetic variations in GRHL2 and noise-induced hearing loss in Chinese high intensity noise exposed workers: a case-control analysis. Ind. Health *51*, 612–621.
20. Rifat, Y., Parekh, V., Wilanowski, T., Hislop, N.R., Auden, A., Ting, S.B., Cunningham, J.M., and Jane, S.M. (2010). Regional neural tube closure defined by the Grainy head-like transcription factors. Dev. Biol. *345*, 237–245.
21. Wilanowski, T., Caddy, J., Ting, S.B., Hislop, N.R., Cerruti, L., Auden, A., Zhao, L.L., Asquith, S., Ellis, S., Sinclair, R., et al. (2008). Perturbed desmosomal cadherin expression in grainy head-like 1-null mice. EMBO J. *27*, 886–897.
22. Ting, S.B., Caddy, J., Hislop, N., Wilanowski, T., Auden, A., Zhao, L.L., Ellis, S., Kaur, P., Uchida, Y., Holleran, W.M., et al. (2005). A homolog of Drosophila grainy head is essential for epidermal integrity in mice. Science *308*, 411–413.
23. Chen, W., Dong, Q., Shin, K.H., Kim, R.H., Oh, J.E., Park, N.H., and Kang, M.K. (2010). Grainyhead-like 2 enhances the human telomerase reverse transcriptase gene expression by inhibiting DNA methylation at the 5′-CpG island in normal human keratinocytes. J. Biol. Chem. *285*, 40852–40863.
24. Boglev, Y., Wilanowski, T., Caddy, J., Parekh, V., Auden, A., Darido, C., Hislop, N.R., Cangkrama, M., Ting, S.B., and Jane, S.M. (2011). The unique and cooperative roles of the Grainy

head-like transcription factors in epidermal development reflect unexpected target gene specificity. Dev. Biol. *349*, 512–522.

25. Blaydon, D.C., Lind, L.K., Plagnol, V., Linton, K.J., Smith, F.J., Wilson, N.J., McLean, W.H., Munro, C.S., South, A.P., Leigh, I.M., et al. (2013). Mutations in AQP5, encoding a water-channel protein, cause autosomal-dominant diffuse nonepidermolytic palmoplantar keratoderma. Am. J. Hum. Genet. *93*, 330–335.
26. Tanimizu, N., and Mitaka, T. (2013). Role of grainyhead-like 2 in the formation of functional tight junctions. Tissue Barriers *1*, e23495.
27. Kang, X., Chen, W., Kim, R.H., Kang, M.K., and Park, N.H. (2009). Regulation of the hTERT promoter activity by MSH2, the hnRNPs K and D, and GRHL2 in human oral squamous cell carcinoma cells. Oncogene *28*, 565–574.
28. Auden, A., Caddy, J., Wilanowski, T., Ting, S.B., Cunningham, J.M., and Jane, S.M. (2006). Spatial and temporal expression of the Grainyhead-like transcription factor family during murine development. Gene Expr. Patterns *6*, 964–970.
29. Terrinoni, A., Serra, V., Bruno, E., Strasser, A., Valente, E., Flores, E.R., van Bokhoven, H., Lu, X., Knight, R.A., and Melino, G. (2013). Role of p63 and the Notch pathway in cochlea development and sensorineural deafness. Proc. Natl. Acad. Sci. USA *110*, 7300–7305.
30. Terrinoni, A., Codispoti, A., Serra, V., Bruno, E., Didona, B., Paradisi, M., Nisticò, S., Campione, E., Napolitano, B., Diluvio, L., and Melino, G. (2010). Connexin 26 (GJB2) mutations as a cause of the KID syndrome with hearing loss. Biochem. Biophys. Res. Commun. *395*, 25–30.

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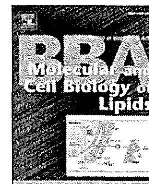

Review

The roles of ABCA12 in epidermal lipid barrier formation and keratinocyte differentiation☆

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ABSTRACT

ATP-binding cassette (ABC) transporters form a large superfamily of transporters that bind and hydrolyze ATP to transport various molecules across limiting membranes or into vesicles. The ABCA subfamily members are thought to transport lipid materials. ABCA12 is a keratinocyte transmembrane lipid transporter protein associated with the transport of lipids via lamellar granules. ABCA12 is considered to transport lipids including ceramides to form extracellular lipid layers in the stratum corneum of the epidermis, which is essential for skin barrier function. *ABCA12* mutations are known to underlie the three major types of autosomal recessive congenital ichthyoses: harlequin ichthyosis, lamellar ichthyosis and congenital ichthyosiform erythroderma. *ABCA12* mutations result in defective lipid transport via lamellar granules in the keratinocytes, leading to ichthyosis phenotypes from malformation of the stratum corneum lipid barrier. Studies on ABCA12-deficient bioengineered models have revealed that lipid transport by ABCA12 is required for keratinocyte differentiation and epidermal morphogenesis. Defective lipid transport due to loss of ABCA12 function leads to the accumulation of intracellular lipids, including glucosylceramides and gangliosides, in the epidermal keratinocytes. The accumulation of gangliosides seems to result in the apoptosis of $Abca12^{-/-}$ keratinocytes. It was reported that AKT activation occurs in $Abca12^{-/-}$ granular-layer keratinocytes, which suggests that AKT activation serves to prevent the cell death of $Abca12^{-/-}$ keratinocytes. This article is part of a Special Issue entitled The Important Role of Lipids in the Epidermis and their Role in the Formation and Maintenance of the Cutaneous Barrier. Guest Editors: Kenneth R. Feingold and Peter Elias.

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1. Introduction

ATP-binding cassette (ABC) transporters form a large superfamily [1]. They bind and hydrolyze ATP to transport various molecules across limiting membranes or into vesicles [2]. The ABCA subfamily members are thought to transport lipid materials [3]. The ABC transporter A12 (ABCA12) is known to be a key molecule in keratinocyte lipid transport (Fig. 1) [1,4–6]. This article comprehensively reviews the physiologic roles and pathogenic importance of *ABCA12* as a keratinocyte lipid transporter in the context of keratinocyte differentiation, and epidermal morphology and lipid barrier formation.

2. ABCA subfamily transporters and human diseases

Mutations in ABCA subfamily genes have been shown to cause several genetic diseases. The ABCA subfamily, of which the *ABCA12* gene is a member, comprises 17 members (ABCA1–ABCA17), several of which are essential for lipid transport and secretion [7]. ABCA11 is a pseudogene, and no orthologous counterparts for ABCA14–ABCA17 have been documented in the human genome [8].

mRNA levels of ABCA2, ABCA3 and ABCA7 in the ABCA subfamily were reported to be upregulated after sustained cholesterol influx [9,10], suggesting that ABCA transporters are involved in the transmembrane transport of endogenous lipids [11]. Transporters in the ABCA subfamily are thought to be involved in the transmembrane transport of cholesterol [12–14]. Interestingly, ABCA3 functions in pulmonary surfactant lipid secretion through the production of lamellar-type granules within lung alveolar type II cells [15,16]. This lamellar granule is similar but not identical to the lamellar granules seen in epidermal keratinocytes.

Three genes in the ABCA subfamily, including ABCA12, have been implicated in the development of genetic diseases affecting cellular lipid transport. In the phylogenetic tree of ABCA subfamily proteins, ABCA3 is very close to ABC12 [1]. ABCA3 is known to aid lipid secretion from alveolar type II cells via lamellar granules [15], and an ABCA3

Abbreviations: ABC, ATP-binding cassette; ABCA12, ATP-binding cassette transporter sub-family A member 12; CIE, congenital ichthyosiform erythroderma; HDL, high-density lipoprotein; HI, harlequin ichthyosis; LG, lamellar granule; LI, lamellar ichthyosis; PPAR, peroxisome proliferator-activated receptor; TINCR, terminal differentiation-induced non-coding RNA

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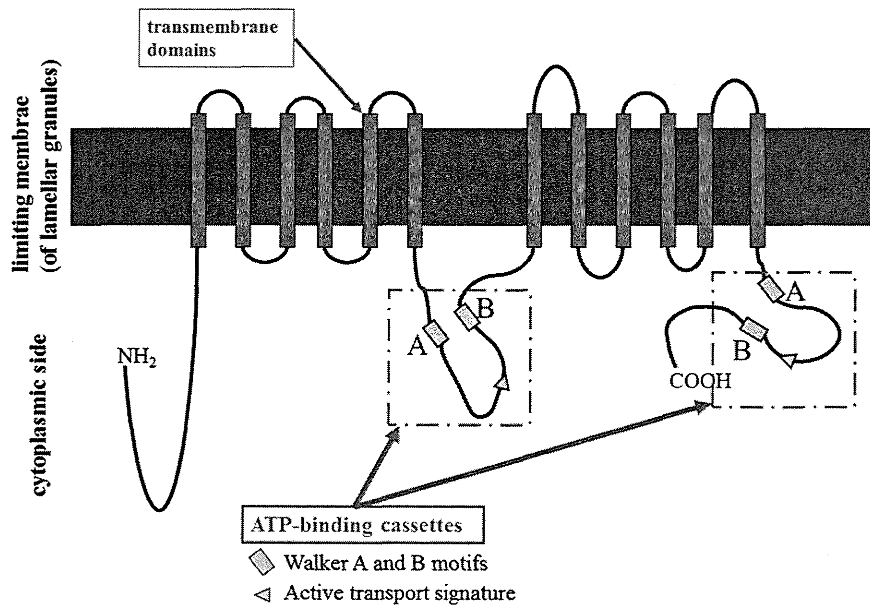

Fig. 1. ABCA12 protein and domain structure. ABCA12 is a full ABC transporter containing two ATP-binding cassettes (adapted from Reference No. [4]).

deficiency was reported to underlie a fatal lung surfactant deficiency in newborns (surfactant metabolism dysfunction-3, MIM #610921) [16], a condition that often leads to death shortly after birth.

ABCA1 is the key member of the ABCA subfamily. Mutations in the human ABCA1 gene cause familial alpha-lipoprotein deficiency syndrome (high density lipoprotein deficiency type I, Tangier disease, MIM #205400), which suggests that ABCA1 is a significant regulator of high-density lipoprotein metabolism [17–19].

3. *ABCA12* mutations and ichthyoses: genetic diagnosis and genotype/phenotype correlations

Mutations in *ABCA12* are known to cause the three major phenotypes of autosomal recessive congenital ichthyoses: harlequin ichthyosis (HI) [4,20], lamellar ichthyosis and congenital ichthyosiform erythroderma [21,22]. HI has the most severe phenotype of any ichthyosis subtype. Affected patients have plate-like scales over the whole body, severe ectropion and eclabium (Fig. 2) [4].

My review of the literature on *ABCA12* mutations in patients with ARCI summarized 56 *ABCA12* mutations in 66 unrelated families, including 48 HI, 10 lamellar ichthyosis and 8 congenital ichthyosiform erythroderma families in 2010 [23]. *ABCA12* mutations have been reported among autosomal recessive congenital ichthyosis patients with African, European, Pakistani/Indian and Japanese backgrounds in most parts of the world. Of the 56 mutations, 36% (20) are nonsense, 25% (14) are missense, 20% (11) comprise small deletions, 11% (6) are splice-site, 5% (3) are large-deletion and 4% (2) are insertion mutations [23]. At least 62.5% (35) of all reported mutations are predicted to result

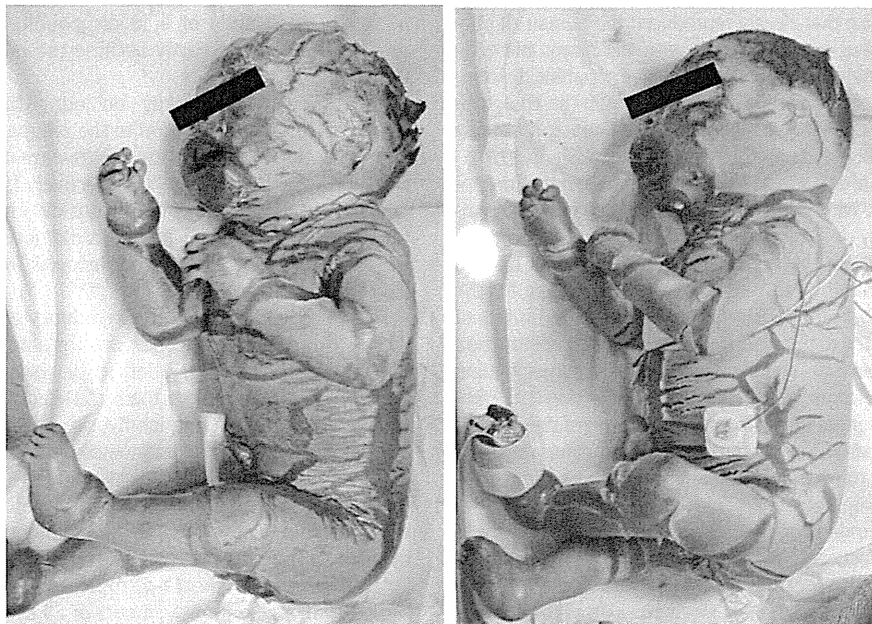

Fig. 2. Typical clinical features of harlequin ichthyosis (HI) seen in two affected babies (adapted from Reference No. [4]).

in truncated proteins. There is no obvious mutation hot spot in *ABCA12* mutations, although mutations underlying the lamellar ichthyosis phenotype are clustered in the domain of the first ATP-binding cassette [24].

It was reported from a study of 45 HI cases that HI is genetically homogeneous, with a 100% mutation rate in one or both alleles of ABCA12 [25]. In the two other major phenotypes of autosomal recessive ichthyosis, LI and congenital ichthyosiform erythroderma, *ABCA12* mutations were frequently found in CIE families (five of eight families), but not in LI families from our study in the Japanese population [26]. *ABCA12* are the major causative genes also in congenital ichthyosiform erythroderma, at least in the Japanese population, although this situation clearly differs from that of Europe [26].

As for genotype/phenotype correlations resulting from *ABCA12* mutations, typically homozygotes or compound heterozygotes with truncation *ABCA12* mutations lead to an HI phenotype [23]. Only a few exceptional cases have been reported [27]. Some missense *ABCA12* mutations within highly conserved transmembrane regions are able to cause drastic alterations in the protein structure and function, resulting in severe phenotypes that are similar to those of truncation mutation patients [27].

Recently, we confirmed that the *ABCA12* mRNA expression by cutaneous epithelial cells can be analyzed using total RNA extracted from pulled hairs without the need for invasive skin biopsies [28]. tRNA was extracted from the hair roots, and cDNAs were obtained by reverse transcription-PCR using the tRNA. In an HI patient, the expression of *ABCA12* mRNA can be evaluated by quantitative PCR using the cDNAs to determine whether there is RNA decay. Sequencing of cDNA can also demonstrate the aberrant splicing patterns that are due to splice-site mutations.

4. ABCA12-deficient bioengineered models

Several bioengineered disease models of ABCA12 deficiency have been established to study the pathomechanisms of ichthyosis phenotypes resulting from defective ABCA12 function and to obtain clues toward developing novel treatments for ichthyosis with ABCA12 deficiency.

We transplanted cultured keratinocytes from patients with HI and succeeded in reproducing HI skin lesions in immunodeficient mice [29]. The reconstituted HI lesions demonstrated similar abnormalities to those seen in HI patients' skin. Furthermore, we generated *Abca12*-disrupted (*Abca12*$^{-/-}$) mice that closely reproduced the human HI phenotype, including intensive hyperkeratosis with eclabium and skin fissures [30]. Lamellar granule malformation, defective ceramide distribution and severe skin barrier dysfunction were apparent in the epidermis [30]. The *Abca12*$^{-/-}$ mice also showed lung alveolar collapse immediately after birth [30]. Lamellar bodies in alveolar type II cells from *Abca12*$^{-/-}$ mice showed malformation, and surfactant protein B, an essential component of alveolar surfactant, was deficient in the *Abca12*$^{-/-}$ mice [30]. Another group independently developed *Abca12*$^{-/-}$ mice that also had the clinical features of HI [31]. In the *Abca12*-disrupted HI model mice, a lack of desquamation of keratinized cells was seen, accounting for the five-fold thickening of the *Abca12*$^{-/-}$ stratum corneum [31]. This lack of desquamation was suggested to be associated with a significant reduction in linoleic esters of long-chain ϖ-hydroxyceramides and a corresponding increase in their glucosylceramide precursors in the skin [31]. ϖ-Hydroxyceramides are essential for intact barrier function of the stratum corneum. A mouse strain harboring a homozygous spontaneous missense mutation was reported to demonstrate skin symptoms similar to ichthyosis [32]. Analysis of lipids in *Abca12* mutant epidermis showed defects in lipid components, indicating that *Abca12* plays an important role in maintaining lipid homeostasis in the skin [32].

A zebrafish model of ichthyosis was also established by morpholino-mediated knockdown of the *abca12* gene [33]. In the skin of the *abca12*-deficient zebrafish, morphological changes that were akin to those seen in the epidermis of human HI patients were observed [33]. This fish model might serve as a useful tool for studying abnormalities in epidermal morphogenesis in keratinization disorders caused by ABCA12 deficiency.

5. Pathomechanisms of ichthyosis due to *ABCA12* mutations

Several striking morphologic abnormalities have been reported in the epidermis of HI patients, including aberrant lamellar granules in the keratinocyte granular layer and malformation of the extracellular lipid lamellae in the stratum corneum [34–37]. ABCA12 deficiency subsequently results in disturbed lipid transport by lamellar granules in the upper epidermal keratinocytes, leading to malformation of the intercellular lipid layers in the stratum corneum of HI patients [4].

Cultured epidermal keratinocytes from an HI patient carrying *ABCA12* loss-of-function mutations demonstrated defective glucosylceramide transport, and this abnormality was reversible by in vitro *ABCA12* corrective gene transfer [4]. Glucosylceramide transport in the cytoplasm has been studied on cultured keratinocytes from a total of three patients harboring *ABCA12* mutations. One patient was homozygous for the splice-site mutation c.3295-2A>G [4], and another was compound heterozygous for p.Ser387Asn and p.Thr1387del [38]. Only one heterozygous mutation, p.Ile1494Thr, was identified in the other patient [39]. Keratinocytes cultured from each of the three patients clearly showed disturbed glucosylceramide transport in the cytoplasm.

Furthermore, in the epidermal keratinocytes from two congenital ichthyosiform erythroderma patients with *ABCA12* mutations, malformation of lamellar granules was seen [39]. Electron microscopy revealed abnormal, defective lamellar granules in the cytoplasm of granular layer keratinocytes, along with some normal-appearing lamellar granules [39].

The intercellular lipid layers of the stratum corneum are indispensable for epidermal barrier function. In ichthyotic skin with ABCA12 deficiency, malformation of the lipid layers is thought to lead to a serious loss of epidermal barrier function and probably to result in extensive compensatory hyperkeratosis [40].

In this context, there may be possible candidate therapies for ichthyosis lesions based upon the identified pathogenic mechanisms, i.e., malformation of the intercellular lipid layers in the stratum corneum. Topical application of lipid contents similar to lamellar granule-derived lipids might be effective for the ichthyosis lesions that result from ABCA12 deficiency. However, no clear evidence supporting the efficacy of topical application of lipid compounds has been obtained in the model mice, to date, partly because the model mice die shortly after birth [30–32].

We tried fetal therapy on our *Abca12*$^{-/-}$ HI model mice, with systemic administration to the pregnant mother mice of retinoid or dexamethasone, which are effective treatments for neonatal HI and neonatal respiratory distress, respectively [30]. Neither treatment was effective on the skin phenotype, nor did either extend the survival of the *Abca12*$^{-/-}$ mice [30]. Retinoids did not improve the phenotypes in in vivo studies on cultured keratinocytes from the model mice, either [41].

Disturbed differentiation of keratinocytes is seen in the lesional epidermis, and the pathomechanisms for autosomal recessive congenital ichthyosis due to ABCA12 deficiency are hypothetically explained by the "differentiation defect theory." Defective keratinocyte differentiation in ABCA12-deficient ichthyosis is described in detail later (see Section 7: ABCA12 and keratinocyte differentiation and apoptosis).

HI patients often die in the perinatal period. However, in most patients who survive beyond the neonatal period, the phenotype gradually improves from a few weeks to several months after birth. To reveal the mechanisms of phenotypic recovery, we investigated grafted skin and keratinocytes from *Abca12*-disrupted (*Abca12*$^{-/-}$) mice [41]. We observed remarkable improvements of all the abnormalities seen in the model mice in *Abca12*$^{-/-}$ skin grafts kept in a dry environment. The increased transepidermal water loss due to barrier defect was dramatically

mitigated in the grafted *Abca12*$^{-/-}$ skin kept in a dry environment. Abnormal ceramide distribution, defective differentiation-specific protein expression and profilaggrin/filaggrin conversion were seen in the primary-culture of *Abca12*$^{-/-}$ keratinocytes, but these abnormalities were resolved in ten-passage sub-cultured *Abca12*$^{-/-}$ keratinocytes [41]. These findings suggest that *Abca12*$^{-/-}$ epidermal keratinocytes restore normal differentiation during maturation [41]. The exact mechanisms of this restoration of keratinocyte differentiation remain to be clarified, but the mitigation of keratinocyte differentiation defects may give us a clue to novel therapies for the ichthyosis phenotype.

6. Lipid transport by ABCA12 in epidermal keratinocytes

For skin barrier function, extracellular lipids, including ceramides and cholesterols, are considered to be essential [42]. ABCA12 is expressed in almost the entire epidermis, but in normal human epidermis most of the expression is in the upper spinous and granular layers [5]. We clarified that ABCA12 is localized in lamellar granules (LGs) in the granular layer keratinocytes and suggested that ABCA12 works in lipid transport via LGs, to form the intercellular lipid layers in the stratum corneum [4]. LGs are part of the continuous tubular network from the Golgi apparatus to the cell membrane. Thus, we investigated the localization of ABCA12 in the epidermal keratinocytes in comparison with the localization of Golgi apparatus markers, LG-associated proteins and transglutaminase 1 [5]. We used antibodies against well-established marker molecules of each part of the Golgi apparatus–LG-cell membrane network, i.e. the GM130, anti-TGN-46 and anti-transglutaminase 1 antibodies (B.C1), as markers for cis-Golgi, trans-Golgi and cell membrane, respectively. The results clearly demonstrated that ABCA12 localizes from the Golgi apparatus to LGs at the cell periphery in keratinocytes of the upper epidermis. Our findings suggested that ABCA12 transports lipids from the Golgi apparatus to LGs predominantly in the granular layer cells [5]. Furthermore, double-labeling immunofluorescence staining apparently showed that ABCA12 is localized from the Golgi apparatus (overlapped staining with cis-Golgi marker GM130 and trans-Golgi marker TGN-46) to the cell periphery (close to the plasma membrane stained with cell membrane-bound transglutaminase 1) in cultured keratinocytes. ABCA12 fails to colocalize with transglutaminase 1 both in vivo and in cultured keratinocytes. Thus, ABCA12 is considered to be present only very sparsely on the cell membrane [5].

In addition, double-labeling immunofluorescence staining demonstrated that the majority of ABCA12 colocalizes with glucosylceramide in the cytoplasm in the upper spinous and granular layer keratinocytes [5]. Immunofluorescence staining of ultrathin cryosections apparently showed that ABCA12 and glucosylceramide staining almost completely overlap in the cytoplasm of granular layer keratinocytes [5]. Postembedding immunoelectron microscopy clarified that ABCA12 and glucosylceramide localize in the limiting membrane and the lamellar structure of LGs, respectively, in the uppermost granular layer keratinocytes (Fig. 3) [5]. Under immunoelectron microscopy using ultrathin cryosections, glucosylceramide immunogold labeling was observed in the lamellar structures of the LGs. ABCA12 labeling was seen on or close to the limiting membrane of LGs in the uppermost granular layer keratinocytes [5].

From these findings, we hypothesize that ABCA12 is probably a membrane lipid transporter within the limiting membrane of the trans-Golgi network and LGs. ABCA12 may play a role in the transport of lipids from the cytoplasm to the trans-Golgi network and LGs, whereby the lipid is finally transported to the keratinocyte periphery via the trans-Golgi network and LGs [4,5]. It was confirmed biochemically that ABCA12 deficiency impairs glucosylceramide accumulation in lamellar granules and that ABCA12 transports glucosylceramide to the inner side of lamellar granules [43]. Furthermore, it was reported that ceramides up-regulate ABCA12 expression via PPAR delta-mediated signaling pathway, providing a substrate-driven, feed-forward mechanism for regulation of ABCA12 [44,45]. Ceramides increase the

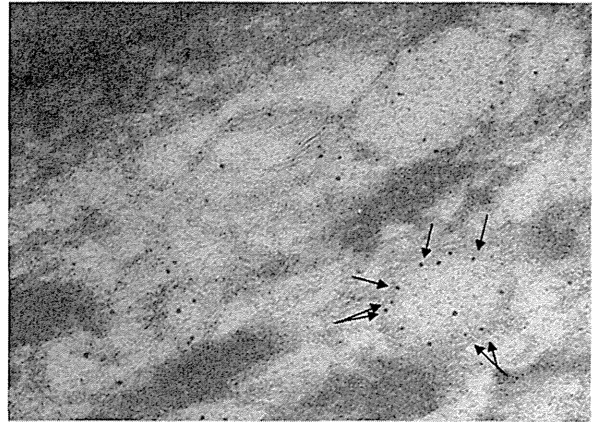

Fig. 3. Postembedding immunoelectron microscopy with cryosubstitution, and cryofixation on anti-ABCA12 antibodies. 5-nm gold labeling for ABCA12 (arrows) is seen on the limiting membrane of lamellar granules in the cytoplasm of the granular layer keratinocytes (adapted from Reference No. [5]).

expression of PPARδ, but not of other PPARs or LXRs [44], although PPARβ/δ, PPARγ and LXR activators all stimulate ABCA12 expression in human keratinocytes dose- and time-dependently [46].

7. ABCA12 and keratinocyte differentiation and apoptosis

Recently, ABCA12 has been widely accepted as a keratinocyte differentiation marker. It was reported that the PPARα agonists clofibrate, docasohexaenoic acid and WY-14,643 produce mild to moderate keratinocyte hyperplasia, increased stratification (particularly of the granular and cornified layers) and elevated levels of differentiation markers including ABCA12 [47]. Furthermore, a 3.7-kilobase non-coding RNA called terminal differentiation-induced non-coding RNA (TINCR) was reported to control human epidermal differentiation in a post-transcriptional manner [48]. TINCR is required for high messenger RNA abundance of key differentiation genes, including ABCA12 [48]. Non-coding double-strand RNA was reported to induce TLR3-dependent increased expression of ABCA12 in human keratinocytes, resulting in increased epidermal lipid and lamellar bodies [49]. Non-coding double-strand RNA can stimulate some events in keratinocyte differentiation that are important for skin barrier repair and maintenance via ABCA12 upregulation [49].

Conversely, ABCA12 deficiency is suggested to lead to differentiation defects in keratinocytes. Fetuses affected with HI start developing their ichthyotic phenotypes while in the amniotic fluid, where the barrier function of the stratum corneum is not required. In light of this, barrier defects might not be involved directly in the pathogenesis of the HI phenotype, at least during the fetal period. Thus, in the "differentiation defect theory" of HI pathogenesis, disturbed keratinocyte differentiation is speculated to play a key role in the formation of the HI skin lesions. Indeed, three-dimensional culture studies of the epidermal keratinocytes have revealed that HI keratinocytes differentiate poorly, judging from morphologic criteria, and show reduced expression of keratin 1 and defective conversion from profilaggrin to filaggrin [50].

The expression of keratinocyte late-differentiation-specific molecules is dysregulated in an *ABCA12*-ablated organotypic co-culture system, which is an in vitro model of HI skin lesions [51]. In addition, mutant mice carrying a homozygous spontaneous missense mutation showed that loss of Abca12 function leads to the premature differentiation of basal keratinocytes [32]. In contrast, in our *Abca12*$^{-/-}$ HI model mice, immunofluorescence and immunoblotting studies demonstrated defective profilaggrin/filaggrin conversion and reduced expression of the differentiation-specific molecules loricrin, kallikrein 5 and transglutaminase 1 in *Abca12*$^{-/-}$ neonatal epidermis, although their mRNA expression was up-regulated [41]. The

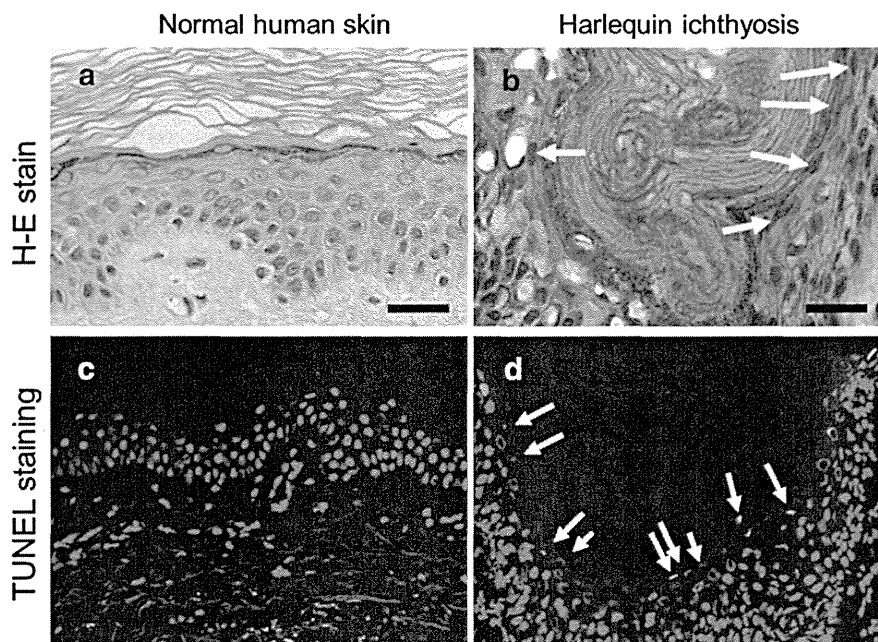

Fig. 4. Epidermal keratinocytes in HI patient's skin frequently show apoptosis. The nuclei of the granular-layer keratinocytes are condensed (b, white arrows) in the HI patient's epidermis. The nuclei are positive TUNEL labeling (d, white arrows). Condensed, apoptotic nuclei are rare in the control normal epidermis (a, c) (adapted from Reference No. [53]).

mechanism by which these abnormalities occur in HI keratinocytes is still unknown. They might be the direct consequence of a lack of lipids required for intact LG formation, leading to the disturbed secretion of LG-associated enzymes, or they might be caused by abnormal lipid accumulation that activates certain cell signaling pathways, resulting in the aberrant keratinocyte differentiation [52].

Furthermore, we have suggested that keratinocyte apoptosis is involved in the pathomechanisms of HI [53] (Fig. 4). Defective lipid transport due to loss of ABCA12 function leads to the accumulation of intracellular lipids, including glucosylceramides and gangliosides [4,43]. Studies by Wang et al. [54] and Sun et al. [55] showed that the elevation of ganglioside levels in the keratinocytes leads to their apoptosis. In this context, we speculate that the accumulation of gangliosides results in the apoptosis of *Abca12*$^{-/-}$ keratinocytes. In addition, we have demonstrated that the AKT signaling pathway helps *Abca12*$^{-/-}$ keratinocytes to survive during keratinization [53]. We also revealed that PPARδ and RXRα are candidate anti-apoptotic molecules in *Abca12*$^{-/-}$ keratinocytes [53].

In light of the various data, it is probable that the differentiation defects and apoptosis of ABCA12 deficient keratinocytes are secondary to the primary lipid abnormalities due to disturbed lipid transport caused by ABCA12 deficiency.

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References

[1] T. Annilo, S. Shulenin, Z.Q. Chen, I. Arnould, C. Prades, C. Lemoine, C. Maintoux-Larois, C. Devaud, M. Dean, P. Denèfle, M. Rosier, Identification and characterization of a novel ABCA subfamily member, ABCA12, located in the lamellar ichthyosis region on 2q34, Cytogenet. Genome Res. 98 (2002) 169–176.

[2] P. Borst, R.O. Elferink, Mammalian ABC transporters in health and disease, Annu. Rev. Biochem. 71 (2002) 537–592.

[3] F. Peelman, C. Labeur, B. Vanloo, S. Roosbeek, C. Devaud, N. Duverger, P. Denèfle, M. Rosier, J. Vandekerckhove, M. Rosseneu, Characterization of the ABCA transporter subfamily: identification of prokaryotic and eukaryotic members, phylogeny and topology, J. Mol. Biol. 325 (2003) 259–274.

[4] M. Akiyama, Y. Sugiyama-Nakagiri, K. Sakai, J.R. McMillan, M. Goto, K. Arita, Y. Tsuji-Abe, N. Tabata, K. Matsuoka, R. Sasaki, D. Sawamura, H. Shimizu, Mutations in ABCA12 in harlequin ichthyosis and functional rescue by corrective gene transfer, J. Clin. Invest. 115 (2005) 1777–1784.

[5] K. Sakai, M. Akiyama, Y. Sugiyama-Nakagiri, J.R. McMillan, D. Sawamura, H. Shimizu, Localization of ABCA12 from Golgi apparatus to lamellar granules in human upper epidermal keratinocytes, Exp. Dermatol. 16 (2007) 920–926.

[6] M. Akiyama, The roles of ABCA12 in keratinocyte differentiation and lipid barrier formation in the epidermis, Dermatoendocrinol 3 (2011) 107–112.

[7] M. Akiyama, Pathomechanisms of harlequin ichthyosis and ABCA transporters in human diseases, Arch. Dermatol. 142 (2006) 914–918.

[8] A.P. Piehler, M. Hellum, J.J. Wenzel, E. Kaminski, K.B.F. Haug, P. Kierulf, W.E. Kaminski, The human ABC transporter pseudogene family: evidence for transcription and gene-pseudogene interference, BMC Genomics 9 (2008) 165.

[9] J. Klucken, C. Buchler, E. Orsó, W.E. Kaminski, M. Porsch-Ozcürümez, G. Liebisch, M. Kapinsky, W. Diederich, W. Drobnik, M. Dean, R. Allikmets, G. Schmitz, ABCG1 (ABC8), the human homolog of the *Drosophila* white gene, is a regulator of macrophage cholesterol and phospholipid transport, Proc. Natl. Acad. Sci. U. S. A. 97 (2000) 817–822.

[10] W.E. Kaminski, A. Piehler, K. Püllmann, M. Porsch-Ozcürümez, C. Duong, G.M. Bared, C. Büchler, G. Schmitz, Complete coding sequence, promoter region, and genomic structure of the human ABCA2 gene and evidence for sterol-dependent regulation in macrophages, Biochem. Biophys. Res. Commun. 281 (2001) 249–258.

[11] G. Schmitz, T. Langmann, Structure, function and regulation of the ABC1 gene product, Curr. Opin. Lipidol. 12 (2001) 129–140.

[12] J. Weng, N.L. Mata, S.M. Azarian, R.T. Tzekov, D.G. Birch, G.H. Travis, Insights into the function of Rim protein in photoreceptors and etiology of Stargardt's disease from the phenotype in abcr knockout mice, Cell 98 (1999) 13–23.

[13] M.R. Hayden, S.M. Clee, A. Brooks-Wilson, J. Genest Jr., A. Attie, J.J. Kastelein, Cholesterol efflux regulatory protein, Tangier disease and familial high-density lipoprotein deficiency, Curr. Opin. Lipidol. 11 (2000) 117–122.

[14] E. Orsó, C. Broccardo, W.E. Kaminski, A. Böttcher, G. Liebisch, W. Drobnik, A. Götz, O. Chambenoit, W. Diederich, T. Langmann, T. Spruss, M.F. Luciani, G. Rothe, K.J. Lackner, G. Chimini, G. Schmitz, Transport of lipids from Golgi to plasma membrane is defective in Tangier disease patients and Abc1-deficient mice, Nat. Genet. 24 (2000) 192–196.

[15] G. Yamano, H. Funahashi, O. Kawanami, L.X. Zhao, N. Ban, Y. Uchida, T. Morohoshi, J. Ogawa, S. Shioda, N. Inagaki, ABCA3 is a lamellar body membrane protein in human lung alveolar type II cells, FEBS Lett. 508 (2001) 221–225.

[16] S. Shulenin, L.M. Nogee, T. Annilo, S.E. Wert, J.A. Whitsett, M. Dean, ABCA3 gene mutations in newborns with fatal surfactant deficiency, N. Engl. J. Med. 350 (2004) 1296–1303.

[17] M. Bodzioch, E. Orsó, J. Klucken, T. Langmann, A. Böttcher, W. Diederich, W. Drobnik, S. Barlage, C. Büchler, M. Porsch-Ozcürümez, W.E. Kaminski, H.W. Hahmann, K. Oette, G. Rothe, C. Aslanidis, K.J. Lackner, G. Schmitz, The gene encoding ATP-binding cassette transporter 1 is mutated in Tangier disease, Nat. Genet. 22 (1999) 316–318.

[18] A. Brooks-Wilson, M. Marcil, S.M. Clee, L.H. Zhang, K. Roomp, M. van Dam, L. Yu, C. Brewer, J.A. Collins, H.O. Molhuizen, O. Loubser, B.F. Ouelette, K. Fichter, K.J. Ashbourne-Excoffon, C.W. Sensen, S. Scherer, S. Mott, M. Denis, D. Martindale, J. Frohlich, K. Morgan, B. Koop, S. Pimstone, J.J. Kastelein, J. Genest Jr., M.R. Hayden, Mutations in ABC1 in Tangier disease and familial high-density lipoprotein deficiency, Nat. Genet. 22 (1999) 336–345.
[19] S. Rust, M. Rosier, H. Funke, J. Real, Z. Amoura, J.C. Piette, J.F. Deleuze, H.B. Brewer, N. Duverger, P. Denèfle, G. Assmann, Tangier disease is caused by mutations in the gene encoding ATP-binding cassette transporter 1, Nat. Genet. 22 (1999) 352–355.
[20] D.P. Kelsell, E.E. Norgett, H. Unsworth, M.T. Teh, T. Cullup, C.A. Mein, P.J. Dopping-Hepenstal, B.A. Dale, G. Tadini, P. Fleckman, K.G. Stephens, V.P. Sybert, S.B. Mallory, B.V. North, D.R. Witt, E. Sprecher, A.E. Taylor, A. Ilchyshyn, C.T. Kennedy, H. Goodyear, C. Moss, D. Paige, J.I. Harper, B.D. Young, I.M. Leigh, R.A. Eady, E.A. O'Toole, Mutations in ABCA12 underlie the severe congenital skin disease harlequin ichthyosis, Am. J. Hum. Genet. 76 (2005) 794–803.
[21] V. Oji, G. Tadini, M. Akiyama, C. Blanchet Bardon, C. Bodemer, E. Bourrat, P. Coudiere, J.J. DiGiovanna, P. Elias, J. Fischer, P. Fleckman, M. Gina, J. Harper, T. Hashimoto, I. Hausser, H.C. Hennies, D. Hohl, A. Hovnanian, A. Ishida-Yamamoto, W.K. Jacyk, S. Leachman, I. Leigh, J. Mazereeuw-Hautier, L. Milstone, F. Morice-Picard, A.S. Paller, G. Richard, M. Schmuth, H. Shimizu, E. Sprecher, M. Van Steensel, A. Taïeb, J.R. Toro, P. Vabres, A. Vahlquist, M. Williams, H. Traupe, Revised nomenclature and classification of inherited ichthyoses: results of the First Ichthyosis Consensus Conference in Sorèze 2009, J. Am. Acad. Dermatol. 63 (2010) 607–641.
[22] M. Akiyama, Updated molecular genetics and pathogenesis of ichthiyoses, Nagoya J. Med. Sci. 73 (2011) 79–90.
[23] M. Akiyama, *ABCA12* mutations and autosomal recessive congenital ichthyosis: a review of genotype/phenotype correlations and of pathogenic concepts, Hum. Mutat. 31 (2010) 1090–1096.
[24] C. Lefèvre, S. Audebert, F. Jobard, B. Bouadjar, H. Lakhdar, O. Boughdene-Stambouli, C. Blanchet-Bardon, R. Heilig, M. Foglio, J. Weissenbach, M. Lathrop, J.F. Prud'homme, J. Fischer, Mutations in the transporter ABCA12 are associated with lamellar ichthyosis type 2, Hum. Mol. Genet. 12 (2003) 2369–2378.
[25] S. Rajpopat, C. Moss, J. Mellerio, A. Vahlquist, A. Ganemo, M. Hellstrom-Pigg, A. Ilchyshyn, N. Burrows, G. Lestringant, A. Taylor, C. Kennedy, D. Paige, J. Harper, M. Glover, P. Fleckman, D. Everman, M. Fouani, H. Kayserili, D. Purvis, E. Hobson, C. Chu, C. Mein, D. Kelsell, E. O'Toole, Harlequin ichthyosis: a review of clinical and molecular findings in 45 cases, Arch. Dermatol. 147 (2011) 681–686.
[26] K. Sakai, M. Akiyama, T. Yanagi, J.R. McMillan, T. Suzuki, K. Tsukamoto, H. Sugiyama, Y. Hatano, M. Hayashitani, K. Takamori, K. Nakashima, H. Shimizu, ABCA12 is a major causative gene for non-bullous congenital ichthyosiform erythroderma, J. Invest. Dermatol. 129 (2009) 2306–2309.
[27] H. Umemoto, M. Akiyama, T. Yanagi, K. Sakai, Y. Aoyama, A. Oizumi, Y. Suga, Y. Kitagawa, H. Shimizu, New insight into genotype/phenotype correlations in ABCA12 mutations in harlequin ichthyosis, J. Dermatol. Sci. 61 (2011) 136–139.
[28] T. Takeichi, K. Sugiura, K. Matsuda, M. Kono, M. Akiyama, Novel ABCA12 splice site deletion mutation and ABCA12 mRNA analysis of pulled hair samples in harlequin ichthyosis, J. Dermatol. Sci. 69 (2013) 259–261.
[29] Y. Yamanaka, M. Akiyama, Y. Sugiyama-Nakagiri, K. Sakai, M. Goto, J.R. McMillan, M. Ota, D. Sawamura, H. Shimizu, Expression of the keratinocyte lipid transporter ABCA12 in developing and reconstituted human epidermis, Am. J. Pathol. 171 (2007) 43–52.
[30] T. Yanagi, M. Akiyama, H. Nishihara, K. Sakai, W. Nishie, S. Tanaka, H. Shimizu, Harlequin ichthyosis model mouse reveals alveolar collapse and severe fetal skin barrier defects, Hum. Mol. Genet. 17 (2008) 3075–3083.
[31] Y. Zuo, D.Z. Zhuang, R. Han, G. Isaac, J.J. Tobin, M. McKee, R. Welti, J.L. Brissette, M.L. Fitzgerald, M.W. Freeman, ABCA12 maintains the epidermal lipid permeability barrier by facilitating formation of ceramide linoleic esters, J. Biol. Chem. 283 (2008) 36624–36635.
[32] I. Smyth, D.F. Hacking, A.A. Hilton, N. Mukhamedova, P.J. Meikle, S. Ellis, K. Slattery, J.E. Collinge, C.A. de Graaf, M. Bahlo, D. Sviridov, B.T. Kile, D.J. Hilton, A mouse model of harlequin ichthyosis delineates a key role for Abca12 in lipid homeostasis, PLoS Genet. 4 (2008) e1000192.
[33] Q. Li, M. Frank, M. Akiyama, H. Shimizu, S.Y. Ho, C. Thisse, B. Thisse, E. Sprecher, J. Uitto, Abca12-mediated lipid transport and Snap29-dependent trafficking of lamellar granules are crucial for epidermal morphogenesis in a zebrafish model of ichthyosis, Dis. Model. Mech. 4 (2011) 777–785.
[34] B.A. Dale, K.A. Holbrook, P. Fleckman, J.R. Kimball, S. Brumbaugh, V.P. Sybert, Heterogeneity in harlequin ichthyosis, an inborn error of epidermal keratinization: variable morphology and structural protein expression and a defect in lamellar granules, J. Invest. Dermatol. 94 (1990) 6–18.
[35] M.E. Milner, W.M. O'Guin, K.A. Holbrook, B.A. Dale, Abnormal lamellar granules in harlequin ichthyosis, J. Invest. Dermatol. 99 (1992) 824–829.
[36] M. Akiyama, D.-K. Kim, D.M. Main, C.E. Otto, K.A. Holbrook, Characteristic morphologic abnormality of harlequin ichthyosis detected in amniotic fluid cells, J. Invest. Dermatol. 102 (1994) 210–213.
[37] M. Akiyama, B.A. Dale, L.T. Smith, H. Shimizu, K.A. Holbrook, Regional difference in expression of characteristic abnormality of harlequin ichthyosis in affected fetuses, Prenat. Diagn. 18 (1998) 425–436.
[38] M. Akiyama, K. Sakai, Y. Sugiyama-Nakagiri, Y. Yamanaka, J.R. McMillan, D. Sawamura, H. Niizeki, S. Miyagawa, H. Shimizu, Compound heterozygous mutations including a de novo missense mutation in ABCA12 led to a case of harlequin ichthyosis with moderate clinical severity, J. Invest. Dermatol. 126 (2006) 1518–1523.
[39] K. Natsuga, M. Akiyama, N. Kato, K. Sakai, Y. Sugiyama-Nakagiri, M. Nishimura, H. Hata, M. Abe, K. Arita, Y. Tsuji-Abe, T. Onozuka, S. Aoyagi, K. Kodama, H. Ujiie, Y. Tomita, H. Shimizu, Novel ABCA12 mutations identified in two cases of non-bullous congenital ichthyosiform erythroderma associated with multiple skin malignant neoplasia, J. Invest. Dermatol. 127 (2007) 2669–2673.
[40] M. Akiyama, Harlequin ichthyosis and other autosomal recessive congenital ichthyoses: the underlying genetic defects and pathomechanisms, J. Dermatol. Sci. 42 (2006) 83–89, (42).
[41] T. Yanagi, M. Akiyama, H. Nishihara, J. Ishikawa, K. Sakai, Y. Miyamura, A. Naoe, T. Kitahara, S. Tanaka, H. Shimizu, Self-improvement of keratinocyte differentiation defects during skin maturation in ABCA12 deficient harlequin ichthyosis model mice, Am. J. Pathol. 177 (2010) 106–118.
[42] H.K. Lee, G.W. Nam, S.H. Kim, S.H. Lee, Phytocomponents of triterpenoids, oleanolic acid and ursolic acid, regulated differently the processing of epidermal keratinocytes via PPAR-alpha pathway, Exp. Dermatol. 15 (2006) 66–73.
[43] S. Mitsutake, C. Suzuki, M. Akiyama, K. Tsuji, T. Yanagi, H. Shimizu, Y. Igarashi, ABCA12 dysfunction causes a disorder in glucosylceramide accumulation during keratinocyte differentiation, J. Dermatol. Sci. 60 (2010) 128–129.
[44] Y.J. Jiang, Y. Uchida, B. Lu, P. Kim, C. Mao, M. Akiyama, P.M. Elias, W.M. Holleran, C. Grunfeld, K.R. Feingold, Ceramide stimulates ABCA12 expression via peroxisome proliferator-associated receptor delta in human keratinocytes, J. Biol. Chem. 284 (2009) 18942–18952.
[45] K.R. Feingold, Y.J. Jiang, The mechanisms by which lipids coordinately regulate the formation of the protein and lipid domains of the stratum corneum: role of fatty acids, oxysterols, cholesterol sulfate and ceramides as signaling molecules, Dermatoendocrinol 3 (2011) 113–118.
[46] Y.J. Jiang, B. Lu, P. Kim, G. Paragh, G. Schmitz, P.M. Elias, K.R. Feingold, PPAR and LXR activators regulate ABCA12 expression in human keratinocytes, J. Invest. Dermatol. 128 (2008) 104–109.
[47] C. Zhang, I. Gurevich, B.J. Aneskievich, Organotypic modeling of human keratinocyte response to peroxisome proliferators, Cells Tissues Organs 196 (2012) 431–441.
[48] M. Kretz, Z. Siprashvili, C. Chu, D.E. Webster, A. Zehnder, K. Qu, C.S. Lee, R.J. Flockhart, A.F. Groff, J. Chow, D. Johnston, G.E. Kim, R.C. Spitale, R.A. Flynn, G.X. Zheng, S. Aiyer, A. Raj, J.L. Rinn, H.Y. Chang, P.A. Khavari, Control of somatic tissue differentiation by the long non-coding RNA TINCR, Nature 493 (2013) 231–235.
[49] A.W. Borkowski, K. Park, Y. Uchida, R.L. Gallo, Activation of TLR3 in keratinocytes increases expression of genes involved in formation of the epidermis, lipid accumulation and epidermal organelles, J. Invest. Dermatol. 133 (2013) 2031–2040.
[50] P. Fleckman, B. Hager, B.A. Dale, Harlequin ichthyosis keratinocytes in lifted culture differentiate poorly by morphologic and biochemical criteria, J. Invest. Dermatol. 109 (1997) 36–38.
[51] A.C. Thomas, D. Tattersall, E.E. Norgett, E.A. O'Toole, D.P. Kelsell, Premature terminal differentiation and a reduction in specific proteases associated with loss of ABCA12 in harlequin ichthyosis, Am. J. Pathol. 174 (2009) 970–978.
[52] C.A. Scott, S. Rajpopat, W.L. Di, Harlequin ichthyosis: ABCA12 mutations underlie defective lipid transport, reduced protease regulation and skin-barrier dysfunction, Cell Tissue Res. 351 (2013) 281–288.
[53] T. Yanagi, M. Akiyama, H. Nishihara, Y. Miyamura, K. Sakai, S. Tanaka, H. Shimizu, AKT has an anti-apoptotic role in ABCA12-deficient keratinocytes, J. Invest. Dermatol. 131 (2011) 1942–1945.
[54] X.Q. Wang, P. Sun, A.S. Paller, Inhibition of integrin-linked kinase/protein kinase B/Akt signaling: mechanism for ganglioside-induced apoptosis, J. Biol. Chem. 276 (2001) 44504–44511.
[55] P. Sun, X.Q. Wang, K. Lopatka, S. Bangash, A.S. Paller, Ganglioside loss promotes survival primarily by activating integrin-linked kinase/Akt without phosphoinositide 3-OH kinase signaling, J. Invest. Dermatol. 119 (2002) 107–117.

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The novel *GJB3* mutation p.Thr202Asn in the M4 transmembrane domain underlies erythrokeratodermia variabilis

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SIR,

Erythrokeratodermia variabilis (EKV; OMIM 133200) is a rare autosomal dominant disorder characterized by migratory erythematous areas and fixed keratotic plaques. *GJB3* and *GJB4*, which encode connexin 31 (Cx31) and Cx30.3, respectively, are causative genes for EKV[1,2] and nonsyndromic hearing loss. Here, we report a single novel mutation of *GJB3*, p.Thr202Asn, in the M4 transmembrane domain in a Japanese family suffering from EKV. In addition, we review the literature and characterize the possible genotype–phenotype correlations for *GJB3* mutations. Specifically, missense mutations, other than those in the second extracellular loop (E2) domain, are associated with EKV, and those in the E2 domain underlie autosomal dominant nonsyndromic hearing loss (ADNSHL).

A 17-year-old girl presented with palmoplantar keratoderma (PPK), which has been noted since infancy. Physical examination showed variably sized and irregularly shaped erythema with scaling on the trunk and the extremities (Fig. 1a). These lesions were transient, but reappeared repeatedly. Notable PPK was also observed (Fig. 1b). She had no hearing loss and refused to undergo hearing tests. Histological examination showed hyperkeratosis with intact granular layers. Her paternal grandfather, father, and sister showed similar clinical features (Fig. 1c). Clinical and histological features supported the diagnosis of EKV.

After ethical approval was granted, written informed consent was obtained from the participants in compliance with the Declaration of Helsinki. The coding regions, including the exon–intron boundaries of *GJB3* and *GJB4*, were amplified by PCR from genomic DNA obtained from the participants, as described previously[1,2]. The mutation

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analysis revealed that the proband, her father, and her sister harboured the heterozygous mutation p.Thr202Asn (c.605C>A) in *GJB3*, while her mother did not (Fig. 1d). MutationTaster (http://www.mutationtaster.org/) predicted that p.Thr202Asn was a disease-causing allele. p.Thr202Asn is not reported in the Exome Sequencing Project of the Human Genetic Variation Browser (http://www.genome.med.kyoto-u.ac.jp/SnpDB/), which is a database of genetic variants in more than 1,400 Japanese individuals. Thus, the proband and the affected family members were diagnosed as having EKV with the heterozygous *GJB3* mutation.

Connexins (Cxs) are highly homologous and comprise a large gene family encoding plasma membrane proteins[3]. Cxs contain four transmembrane domains linked by one cytoplasmic and two extracellular loops; N- and C-termini are located on the cytoplasmic side (Fig. 2). The transmembrane domains bear conserved amino acids, whereas the cytoplasmic loop and the C-terminal region are highly variable among Cxs. Cxs are assembled in groups of six to form hemichannels in the plasma membrane, and two hemichannels of adjacent cells then combine to form a gap junction. Various Cxs combine into homomeric and heteromeric gap junctions.

Cx31 is expressed in the upper differentiating epidermal layers[4]. In addition to the epidermis, it is expressed in the cochlea, and some mutations are involved in hearing loss without obvious cutaneous phenotypes[5]. Figure 2 summarizes the *GJB3* missense mutant alleles reported thus far, including the present case, and their associations with EKV or ADNSHL, according to the Human Gene Mutation Database (http://www.hgmd.cf.ac.uk/ac/index.php) of the Institute of Medical Genetics in Cardiff,

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UK. p.Thr202Asn is the first autosomal dominant missense mutation reported in the M4 domain; it was the only remaining domain of Cx31 for which no pathogenic autosomal dominant mutations were reported (Fig. 2). Interestingly, all missense mutations in any domain other than E2 were associated with EKV. In contrast, all missense mutations in the E2 domain were associated with ADNSHL. Supplementary Figure S1 shows the sequence alignment of the E2 domain for diverse vertebrate species. p.174Val, p.177Tyr, and p.183Glu are conserved among vertebrate species, but p.166Asn is not. Based on a review of *GJB3*-associated autosomal recessive nonsyndromic hearing loss, *GJB3* missense mutations have been reported that are not located in the E2 domain (http://www.hgmd.cf.ac.uk/ac/index.php).

We assume different pathogenic mechanisms determine the phenotypes associated with *GJB3* dominant missense mutations in the E2 domain and those outside the E2 domain. The E1 domain is important for the formation of the gap junction channel and the E2 domain is important for docking compatibility in heterotypic channels in many members of the connexin family[3]. Indeed, one of the missense mutations in the E2 domain of Cx31 reportedly has no effect on the function of wild-type Cx31, but interferes with Cx26 function[6]. Of all Cxs, malfunction of Cx26 results in hearing loss most frequently[7]. In this context, we suspect that *GJB3* dominant missense mutations in the E2 domain might cause hearing loss via dysfunction of Cx26.

In summary, to our knowledge, we have identified the first *GJB3* dominant pathogenic missense mutation in the M4 transmembrane domain. As was the case for other *GJB3* dominant pathogenic missense mutations outside the E2 domain, the mutation led to the EKV phenotype in the patient's family. Our results, combined with a literature review,

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suggested that *GJB3* dominant missense mutations outside the E2 domain are associated with EKV and those within the E2 domain cause ADNSHL.

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References

1 Richard G, Smith LE, Bailey RA *et al.* Mutations in the human connexin gene GJB3 cause erythrokeratodermia variabilis. *Nat Genet* 1998; **20**: 366-9.

2 Macari F, Landau M, Cousin P *et al.* Mutation in the gene for connexin 30.3 in a family with erythrokeratodermia variabilis. *Am J Hum Genet* 2000; **67**: 1296-301.

3 Bai D, Wang AH. Extracellular domains play different roles in gap junction formation and docking compatibility. *Biochem J* 2014; **458**: 1-10.

4 Di WL, Rugg EL, Leigh IM *et al.* Multiple epidermal connexins are expressed in different keratinocyte subpopulations including connexin 31. *J Invest Dermatol* 2001; **117**: 958-64.

5 Kelsell DP, Di WL, Houseman MJ. Connexin mutations in skin disease and hearing loss. *Am J Hum Genet* 2001; **68**: 559-68.

6 Li TC, Kuan YH, Ko TY *et al.* Mechanism of a novel missense mutation, p.V174M, of the human connexin31 (GJB3) in causing nonsyndromic hearing loss. *Biochem Cell Biol* 2014: 1-7.

7 Xu J, Nicholson BJ. The role of connexins in ear and skin physiology - functional insights from disease-associated mutations. *Biochim Biophys Acta* 2013; **1828**: 167-78.

Figure legends

Fig. 1 Clinical features of the proband, pedigree of the family, sequence data for *GJB3*, and conservation analysis of the mutated amino acid of Cx31

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(a) Persistent indurated erythematous plaques on the lower limbs. (b) Hyperkeratotic skin on the soles. (c) Pedigree of the proband's family. (d) Sequence of *GJB3* for the proband and control. Prob. and Cont. indicate proband and control, respectively.

Fig. 2 Reported causative dominant pathogenic missense mutations for EKV and ADNSHL in Cx31.

Only dominant missense mutations in the E2 domain underlie ADNSHL (blue circles), and all dominant pathogenic missense mutations outside the E2 domain cause EKV (red circles and asterisk). Residues at the boundaries between the functional domains are indicated by green numbers.

M1–M4, transmembrane domains 1–4; E1 and E2, extracellular domains 1 and 2, respectively; CL, cytoplasmic loop; red circles, causative mutations of EKV; red asterisk, the causative mutation of EKV from the current study; blue circles, causative mutations of ADNSHL.