Defective Lamellar Granule Secretion in Arthrogryposis, Renal Dysfunction, and Cholestasis Syndrome Caused by a Mutation in VPS33B

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Background: Arthrogryposis, renal dysfunction, and cholestasis (ARC) syndrome is a rare and usually fatal metabolic autosomal recessive disorder, which has recently been shown to result from mutations in VPS33B located on chromosome 15q26.1. Neurological signs and ichthyosis almost invariably accompany the disease.

Observations: We assessed a consanguineous family with 2 identical twins affected with ARC syndrome. Complete sequencing of the VPS33B gene revealed a homozygous missense mutation (D234H), which segregated with the disease in the affected family. The mutation causes aberrant splicing, resulting in the skipping of exon 9 or exons 9 and 10. VPS33B encodes a homologue of the class C yeast vacuolar protein-sorting molecule, Vps33, which regulates soluble N-ethylmaleimide–sensitive factor attachment protein receptor (SNARE) protein–mediated vesicle-to-target fusion, necessary for secretion to occur. Lamellar granules, forming a specialized vesicular system in the epidermal upper layers, are usually secreted at the boundary between granular and lower cornified cell layers. However, ultrastructural examination of the skin in ARC syndrome revealed many entombed lamellar granules in the cornified cells.

Conclusions: The present observations indicate that VPS33B deficiency results in abnormal secretion of lamellar granules, which underlies ichthyosis in ARC syndrome. These data underscore the importance of SNARE-mediated vesicle fusion during normal epidermal differentiation.

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ARTHROGRYPOSIS, RENAL dysfunction, and cholestasis (ARC) syndrome (OMIM 208085) is a rare autosomal recessive multisystem disorder. Most cases include neurogenic arthrogryposis multiplex congenita, renal tubular dysfunction of variable severity, neonatal cholestatic jaundice, and severe lamellar ichthyosis. Additional features reported in some patients include dysmorphism, recurrent infections, abnormal platelet function, failure to thrive, nephrogenic diabetes insipidus, neurogenic muscular atrophy, absent corpus callosum, and nerve deafness. Most patients die by the age of 7 months, and those who survive longer display severe developmental delay. Recently, loss-of-function mutations in the VPS33B gene (GenBank NC_000002.10) have been shown to cause ARC syndrome. VPS33B encodes a homologue of the yeast class C vacuolar protein-sorting protein Vps33, which regulates vesicle-to-target soluble N-ethylmaleimide–sensitive factor attachment protein receptor (SNARE) complex formation in the course of vesicle fusion. A number of ichthyosiform disorders have been shown to involve abnormal function of lamellar granules, a specialized vesicular system, most probably derived from the Golgi complex. Mutations in the ABCA12 gene (GenBank NC_000002.10), coding for a lipid-trafficking molecule, give rise to Harlequin ichthyosis, associated with defective secretion of lamellar granule lipid contents. Reduced expression of SNAP29, a SNARE protein involved in vesicle fusion, results in aberrant maturation and secretion of lamellar granules and causes a rare neurocutaneous disease, known as cerebral dysgenesis–neuropathy–ichthyosis–keratoderma (CEDNIK) syndrome (OMIM 609528). Taken altogether, these data suggest that deleterious VPS33B mutations may interfere with normal lamellar gran-
ule function, which may underlie cutaneous manifestations in ARC syndrome. In the present study, we assessed this possibility through the study of an affected consanguineous family.

**METHODS**

**PATIENTS AND BIOLOGICAL MATERIAL.**

We studied a consanguineous kindred of Druze ancestry, comprising 2 siblings affected with ARC syndrome and their parents. All participants or their legal guardian provided written informed consent in accordance with a protocol previously approved by the local Helsinki Committee and by the National Committee for Genetic Studies of the Israeli Ministry of Health. From each participant, 15 mL of blood was drawn, and DNA was extracted using a salt-chloroform extraction method. Skin biopsy samples were fixed in Karnovsky fixative for transmission electron microscopy. DNA from skin fibroblast cell cultures was extracted using a salt-chloroform extraction method. Proven by the local Helsinki Committee and by the National Committee for Genetic Studies of the Israeli Ministry of Health.

**MUTATION ANALYSIS**

Genomic DNA of affected individuals was amplified by polymerase chain reaction (PCR) using primer pairs covering the entire coding sequence as well as intron-exon boundaries of VPS33B. Genomic DNA of affected individuals was amplified by polymerase chain reaction (PCR) using primer pairs covering the entire coding sequence as well as intron-exon boundaries of VPS33B. Intron-crossing primers located in exons 8 and 11 (forward 5' CACCTTCTCAGCACTCTCAGTC-3' and reverse 5'-GATGAGTAGCACCTTCAGTC-3') and was subjected to digestion with MaelII.

**REVERSE TRANSCRIPTASE–PCR**

Total RNA was extracted from skin fibroblast cell cultures using the High Pure RNA Isolation Kit (Roche Diagnostics GmbH, Mannheim, Germany). Total complementary DNA (cDNA) was synthesized using the Reverse-iT 1st Strand Synthesis Kit (ABgene, Surrey, England) and amplified using Taq polymerase. Q solution (Qiagen), and intron-crossing primers located in VPS33B exons 8 and 11 (forward 5'-CACCTTCTCAGCACTCTCAGTC-3' and reverse 5’-GATGAGTAGCACCTTCAGTC-3'). Cycling conditions were 95°C for 5 minutes followed by 35 cycles at 95°C for 30 seconds, 57°C for 45 seconds, 72°C for 90 seconds, and a final extension step at 72°C for 7 minutes. As a control, we amplified total RNA as previously described with ACTB-specific primers (forward 5’-CCAAGGCCAACCGCGAGAAGACG-3' and reverse 5’-AGGGTATCTGGTGGTTGGCCCAGACG-3'; expected size, 387 bp).

Transmission electron microscopy was performed as described elsewhere.**

**RESULTS**

**CLINICAL FEATURES**

The 2 patients studied were identical twins. Their parents were second-degree cousins of Druze origin (Figure 1A). They were delivered by cesarean delivery at the end of the 36th gestational week, weighing 2010 g and 2212 g. At birth, they had normal pink skin, a head circumference of 31.4 cm and 31.5 cm, facial dysmorphism, and low-set ears. Both babies had multiple contractures of limbs or arthrogryposis, including the elbows and knees, talipes equinovarus, and rocker-bottom feet (Figure 1B). Arthrogryposis is part of many congenital disorders and often results from decreased le-
tal movements due to primary neurogenic, muscular, or connective tissue abnormalities. Chest radiography revealed narrow ribs and osteopenia. Widening of the collecting system was evident by renal ultrasonography in both infants, and renal calcification was found in one. Ultrasonographic findings of the brain were normal. Laboratory data were consistent with severe renal tubular dysfunction or renal Fanconi syndrome as previously described in most patients with ARC, which manifested with aminoaciduria, glycosuria, renal tubular acidosis, and nephrogenic diabetes insipidus. These abnormalities in turn caused chronic dehydration, and most probably significantly contributed to severe failure to thrive in these patients.

In addition, cholestatic jaundice was noticed and was associated with direct hyperbilirubinemia, almost normal serum γ-glutamyltranspeptidase (γ-GT) level, and markedly elevated alkaline phosphatase level (Table 2). Urinary organic acid analysis showed elevated levels of tyrosine metabolites but no succinylacetone, thus excluding tyrosinemia type I and suggesting nonspecific liver disease. At age 6 weeks, physical examination revealed clinical rickets, including rachitic rosary and craniotabes. At that stage, widespread lamellar ichthyosis appeared simultaneously in both patients involving the trunk, scalp, and extremities, with sparing of the face and of the palmoplantar surface (Figure 1C). Subsequent clinical course was dominated by recurrent infections, diarrhea, dehydration, severe failure to thrive, and metabolic acidosis. Despite treatment with medium-chain triglyceride-based formula, fat soluble vitamins, ursodecholic acid, and sodium bicarbonate, the patients did not gain weight and failed to achieve any developmental milestones. They died at home, a few days apart, at age 11 months. Of note, 3 siblings of the father had died during the neonatal period also. The small amount of clinical information available regarding these individuals suggests that they were affected by a rapidly progressing wasting disorder, associated with dry skin.

**MUTATIONAL ANALYSIS**

The combination of arthrogryposis, cholestatic jaundice with markedly elevated alkaline phosphatase and normal to minimally elevated γ-GT level, renal tubulopathy, ichthyosis, and severe failure to thrive with developmental retardation, suggested the diagnosis of ARC syndrome, which was recently shown to be caused by mutations in the VPS33B gene. We therefore sequenced the
entire coding region of this gene, including intron-exon boundaries. In both patients, we identified a homozygous G > C transition at cDNA nucleotide position 700 (VPS33B; GenBank NM_018668.2) (Figure 2A). The c.G700C mutation results in substitution of an aspartate residue for a histidine residue at amino acid posi-
tion 234 (p.D234H). Using direct sequencing, we showed that the mutation was present in a heterozygous state in both parents. Using a mutation-specific PCR-RFLP assay (Figure 2B), we excluded the mutation in a panel of 100 population-matched control subjects.

**CONSEQUENCES OF THE MUTATION IN VPS33B**

In silico simulation using the Splice Site Prediction by Neural Network software (http://www.fruitfly.org/seq_tools/splice.html) suggested that c.G700C may significantly disrupt the donor splice site of intron 9. We therefore used PCR amplification on cDNA derived from patient fibroblast cell cultures with primer pairs encompassing exons 8 through 11. Reverse transcriptase–PCR analysis demonstrated that wild-type mRNA species was absent in the patient's cDNA (Figure 2C). In contrast, 2 major aberrant splicing products were seen. Direct sequencing of these 205-bp and 127-bp splice variants demonstrated the skipping of exon 9 and of exons 9 and 10, respectively (Figure 2D), which was predicted in both cases to result in premature termination of translation and significant truncation of the VPS33B protein.

**DELETERIOUS MUTATIONS IN VPS33B AND LAMELLAR GRANULE SECRETION IN ARC**

Because VPS33B is thought to regulate SNARE-mediated vesicle secretion and because deficient SNARE protein function has previously been shown to result in ichthyosis due to defective lamellar granule function, we assessed by transmission electron microscopy the ultrastructural features of affected skin in ARC syndrome. Lamellar granule secretion was found to be abnormal, as attested by the formation of the cornified cell envelope before completion of lamellar granule secretion (Figure 3A-D). In contrast to previous observations in CEDNIK syndrome, lamellar granule internal structure was normal (Figure 3E and F). However, numerous lamellar granules were retained in cornified cells (Figure 3G-J).

**COMMENT**

Eukaryotic cells are highly compartmentalized into distinct membrane-bound organelles. Molecule transfer between these organelles involves membrane fusion between vesicles, a process that is tightly controlled owing to the unique composition of proteins and lipids in each cell compartment. Central to this fusion process is the function of the SNARE proteins, a family of integral membrane proteins present on both vesicle (v-SNARE) and target (t-SNARE) membranes. These molecules are presumed to determine the specificity as well as the rate of the fusion reaction. A second family of proteins called Sec1/Munc18 (SM) proteins also seems to be involved in determining the specificity of SNARE-mediated membrane fusion. However, owing to variability in their function in different systems, there is no consensus as to their exact role, site of action, and relation to SNARE proteins. Homozygous loss-of-function mutations in VPS33B encoding a member of the SM family was recently shown to cause ARC syndrome and abnormal trafficking of surface-expressed proteins in the liver. Despite the conspicuous presence of cutaneous abnormalities in ARC syndrome, little is currently known about the consequence of abnormal VPS33B function during skin differentiation. In the present study, a deleterious mutation in VPS33B was shown to result in impaired secretion of lamellar granules, which is likely to contribute to the development of ichthyosis in ARC syndrome. The yeast homologue of the VPS33B protein, Vps33p, a class C vps protein, is required for vacuolar biogenesis and plays a key role in protein trafficking from Golgi to vacuole. Mutations in class C vps proteins have been shown to result in intracellular acidification, amino acid pool deficiency, and temperature-sensitive growth failure in yeast. Interestingly, lamellar granules are considered as being part or at least being derived from the Golgi system, suggesting that cutaneous manifestations in ARC syndrome may be the result of abnormal intracellular organelle trafficking. Surprisingly, loss of function of VPS33B analogues in Drosophila and mouse models leads to relatively minor clinical symptoms. In Drosophila, the hypomorphic carnation (car) allele of the Vps33 homologue causes the carnation eye color phenotype. In mice, the Buff (bf) phenotype, characterized by coat-color hypopigmentation, mild platelet storage pool deficiency, and little if any effect on lysosomal function, is caused by mutations in the Vps33a gene. The existence of 2 homologues of the yeast Vps33p protein in multicellular organisms may explain the significant differences between the mouse and fly phenotypes and ARC syndrome. Several types of ichthyosis are associated with mutations in genes involved in lamellar granule function. In Harlequin and lamellar ichthyosis, mutations in ABCA12 cause defective lipid transport due to abnormal lipid loading in lamellar granules, and consequent abnormal cornification. Likewise, as often seen in ARC syndrome, severe lamellar ichthyosis developed late after birth in CEDNIK syndrome and is the result of impaired lamellar granule maturation and secretion. The present study indicates that ARC syndrome can be considered as part of this revealing group of cornification disorders. Despite the fact that both ARC and CEDNIK syndromes result from abnormal function of proteins involved in vesicle fusion, leading to impaired lamellar granule secretion, lamellar granule maturation was normal in ARC syndrome, whereas it is markedly abnormal in CEDNIK syndrome. Thus, these observations may be interpreted to suggest that in keratinocytes, VPS33B is involved mainly in regulating exocytosis, whereas SNAP29 is additionally involved in vesicle cargo loading in the Golgi system. In conclusion, we have demonstrated that lamellar granule secretion is impaired in ARC syndrome, underscoring the importance of SNARE-mediated vesicle fusion during normal epidermal differentiation.
Figure 3. Transmission electron microscopy. Examination of the granular cell layer (A–D) reveals formation of cornified cell envelopes (black arrows) before the completion of lamellar granule secretion in a patient with arthrogryposis, renal dysfunction, and cholestasis (ARC) syndrome. Parts A and C show low-magnification views from the upper stratum granulosum (Gr) to the stratum corneum (Co) of the patient’s (A) and normal (C) skin, respectively. Rectangular areas marked in A and C are shown at higher magnification in B and D, respectively. Some lamellar granules have been secreted in the patient’s as well as in normal skin (asterisk), but numerous intact granules are retained in the cytoplasm of the patient’s skin (white arrow). Normal lamellar internal structures (arrows) are seen in the lamellar granules of the patient (E) and normal control (F). Entombed lamellar granule-like structures are seen in the stratum corneum of the patient with ARC (G and H; arrows) compared with normal control skin (I and J). The rectangular areas marked in G and I are shown at higher magnification in H and J, respectively.
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Author Contributions: Dr Sprecher had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study concept and design: Mandel and Sprecher. Acquisition of data: Hershkovitz, Mandel, Ishida-Yamamoto, Chefetz, Hino, Ludger, Indelman, and Bergman. Analysis and interpretation of data: Hershkovitz, Mandel, Ishida-Yamamoto, Bergman, and Sprecher. Drafting of the manuscript: Mandel, Ishida-Yamamoto, Hino, Ludger, Bergman, and Sprecher. Critical revision of the manuscript for important intellectual content: Chefetz, Ludger, Indelman, Bergman, and Sprecher. Obtained funding: Sprecher. Administrative, technical, and material support: Hershkovitz, Chefetz, Hino, Ludger, Indelman, and Bergman. Study supervision: Mandel and Sprecher.

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