A Patient with Congenital Generalized Lipodystrophy Due To a Novel Mutation in *BSCL2*: Indications for Secondary Mitochondrial Dysfunction

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Abstract *Background:* Congenital generalized lipodystrophy (CGL) results from mutations in *AGPAT2*, encoding 1-acyl-glycerol-3-phosphate-acyltransferase 2 (CGL1;

MIM 608594), *BSCL2*, encoding seipin (CGL2; MIM 269700), *CAV1*, encoding caveolin1 (CGL3; MIM 612526) or *PTRF*, encoding polymerase I and transcript release factor (CGL4; MIM 613327). This study aims to investigate the genotype/phenotype relationship and search for a possible pathogenic mechanism in a patient with CGL.

Design: Case report.

Patients and Setting: A 7-day-old child of consanguineous Turkish parents presented with a generalized loss of subcutaneous fat. He had a strikingly enlarged liver, high serum triglycerides, and hyperglycaemia, suggestive for CGL.

Results: A novel homozygous mutation in the acceptor splice site of exon 5 of the *BSCL2* gene was found in the genome of the proband. This mutation causes a complex RNA splicing defect and results in two different aberrant seipin proteins, which were normally expressed and localized to the endoplasmic reticulum like wild type protein. Analysis of the patient's urine showed intermittent elevations of citric acid intermediates and persistently high concentrations of ethylmalonic acid, suggestive of a disturbance of the mitochondrial respiratory chain.

Conclusion: Here we report abnormal urinary organic acid levels, indicative of mitochondrial dysfunction, in a patient with CGL resulting from a novel mutation in *BSCL2*. Our findings suggest for the first time an association between CGL and secondary mitochondrial dysfunction.

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Introduction

Adipose tissue plays an essential role in whole body metabolism as illustrated by lipodystrophies in which the lack of adipose tissue results in many metabolic complications

such as severe insulin resistance, hypertriglyceridemia, and hepatic steatosis (Hegele et al. 2007). Lipodystrophies can be classified as acquired or genetic and subclassified as partial or generalized. Genetic lipodystrophies are the most severe and either autosomal dominant or recessive. Congenital generalized lipodystrophy (CGL), also known as Berardinelli–Seip syndrome is an autosomal recessive disorder and is characterized by a near complete absence of adipose tissue, already present at birth or in early infancy. Lipids are stored aberrantly in muscle and liver resulting in extreme insulin resistance. In addition to hepatomegaly and generalized muscular hypertrophy, patients have acromegaloid features such as enlarged hands and feet, acanthosis nigricans, and excessive body hair (Garg 2004; Van Maldergem et al. 2002). A significant number of patients develop hypertrophic cardiomyopathy (HCM) (Van Maldergem et al. 2002; Agarwal et al. 2003). To date CGL has been associated with mutations in four different genes (Jeninga and Kalkhoven 2010): *AGPAT2* (Agarwal et al. 2002), *BSCL2* (Magre et al. 2001), *CAVI* (Kim et al. 2008), and *PTRF* (Hayashi et al. 2009). *AGPAT2* encodes 1-acyl-glycerol-3-phosphate-acyltransferase 2, an enzyme involved in the synthesis of triglycerides and predominantly expressed in adipose tissue (Agarwal et al. 2004). Patients with *AGPAT2* mutations therefore have lipodystrophy, known as CGL type 1. In CGL type 3 mutations are present in *CAVI*, which encodes caveolin1, a highly conserved protein which is the key component of membrane invaginations known as caveolae (Parton and Simons 2007). Caveolae are abundant in adipocytes and involved in lipid metabolism and insulin signaling (Ortengren et al. 2007). Mutations in the *PTRF* gene cause CGL type 4. This gene encodes PTRF (polymerase 1 and transcript release factor), a protein that stabilizes caveolin 1–3 and is required for the formation of caveolae (Hayashi et al. 2009). Type 2 CGL is caused by mutations in the *BSCL2* gene, as first reported by Magre et al. (2001). They described nonsense, frameshift, deletion, insertion, and missense mutations associated with CGL in *BSCL2* and called the protein “seipin” (Magre et al. 2001). Seipin is an integral ER membrane protein and mainly expressed in adipose tissue, brain, and testis (Payne et al. 2008; Magre et al. 2001). Lundin et al. determined the membrane topology of seipin showing that this protein spans the ER membrane twice with the C- and N-termini facing the cytoplasm and a glycosylation site in the luminal segment (Lundin et al. 2006). Here, we report a novel mutation in *BSCL2* and show for the first time abnormalities in urinary organic acids suggestive for secondary mitochondrial dysfunction.

Subject and Methods

Study Subject

The patient had been referred to the University Medical Centre Utrecht for clinical evaluation, and informed consent for this study was obtained from the parents of the patient.

Isolation of Genomic DNA and RNA, RT-PCR and Mutation Analysis

Genomic DNA was isolated from EDTA containing blood samples. Exons, including the intron boundaries of *BSCL2*, were amplified by PCR. RNA was isolated from EDTA containing blood samples and used for cDNA synthesis using oligo (dT) primers and Superscript II (Invitrogen). RT-PCR was performed using primers located in exon 4 (forward) and exon 6 (reverse). PCR products were purified and sequenced using a BigDye Terminator v1.1 cycle sequencing kit (Applied Biosystems). The sequence products were purified and analyzed on an ABI Prsim 3730 DNA analyzer (Applied Biosystems). Primer sequences are available upon request.

Plasmids

All recombinant DNA work was performed according to standard procedures (Ausubel et al. 1993). The pLPS-seipin-EGFP expression construct (Windpassinger et al. 2004) was a kind gift of Dr. K. Wagner (Medical University, Graz, Austria). The pEBB-Flag-seipin wild-type construct was generated by PCR amplification of full length seipin from this vector using primers containing *BamHI* and *NotI* restriction sites and cloned into the respective sites of pEBB-Flag. The pEBB-Flag-seipin Δ Exon 5, and FS constructs were generated by Quickchange mutagenesis (Stratagene) and the sequences were verified by sequencing. pEBB-HA-seipin wild type, Δ Exon 5 and FS were generated using the same procedures as described above, but cloned in the pEBB-HA vector.

Western Blot Analysis

The human osteosarcoma cell line U2OS was maintained in DMEM Glutamax (Dulbecco) containing 10% fetal calf serum (Gibco Life Technologies), 100 μ g of penicillin/ml and 100 μ g of streptomycin/ml (Gibco Life Technologies). U2OS cells were transiently transfected with Seipin expression constructs using PEI transfection reagent. Cells were lysed in 2 $\times$ Laemli sample buffer and subjected to SDS-PAGE, and then transferred to Immobilon membranes

(Millipore). Anti-Flag M2 antibody (F3165, Sigma) was used to probe for the respective proteins. ECL Plus (Perkin Elmer) was used for detection.

Immunofluorescence Microscopy

Immunofluorescence microscopy was performed as described earlier (van de Graaf et al. 2006). Briefly, U2OS cells were grown on coverslips and transiently transfected with seipin expression constructs using PEI transfection reagent. Cells were washed, fixed, permeabilized, blocked in BSA, and subsequently incubated with mouse-anti-HA (H9658, Sigma) and rabbit-anti-calreticulin (ALX-210-126-R100, Alexis Biochemicals) antibodies. As secondary antibodies Alexa Fluor 568 anti-mouse (A11031, Invitrogen) and Alexa Fluor 488 anti-rabbit (A11034, Invitrogen) were used. The coverslips were washed and mounted with FlourSave reagent (Calbiochem). Images were analyzed on a confocal laserscanning microscope (Zeiss LSM 710 Meta).

Organic Acid Analysis in Urine

Organic acid profiles in urine were analyzed by gas chromatography and flame ionization detection after ethoximation and formation of TMS ethers. The identities of the increased components were confirmed by mass spectrometry.

Results

Patient Medical History and Clinical Evaluation

The patient is the third child of consanguineous Turkish parents. Family history is uneventful. He was born after a normal pregnancy with a birth weight of 3,360 g. He was first admitted at the age of 7 days for feeding problems, weight loss, and hyperglycemia. Physical examination was remarkable for the generalized absence of subcutaneous fat and a strikingly enlarged liver (Fig. 1). Laboratory examination demonstrated severe hypertriglyceridemia (7.4 mmol/l), low HLD cholesterol (0.5 mmol/l), impaired liver function, and hyperglycaemia (Supplemental Table 1). Liver biopsy showed micro- and macrovesicular steatosis. Serum leptin concentration was undetectable. At the age of 4 weeks, he was started on a low fat diet supplemented with medium chain triglycerides, protein, essential fatty acids, and vitamins. Insulin was administered intravenously and later on subcutaneously in doses exceeding 2 U/kg and was discontinued after 1 month. Subsequently two short courses of insulin were necessary during upper respiratory tract infection. During the first months, the diet resulted in an

impressive decrease in serum lipid concentration and improvement of liver function (Supplemental Table 1), but at the age of 10 months, liver function again deteriorated and low dose metformin was added. Subsequently serum insulin levels decreased dramatically and normalized, whereas liver function and serum lipids improved but remained slightly elevated (Supplemental Table 1).

At the age of 4 months, routine echocardiographic examination disclosed HCM in an otherwise asymptomatic child. The HCM worsened significantly over time with intraventricular septum diameter of 15 mm ($p_{95} = 6.4$ mm) and a left ventricular posterior wall dimension of 12 mm ($p_{95} = 6$ mm) at 6.5 months of age (Fig. 1e, left panel). In spite of massive HCM, left ventricular outflow tract obstruction never occurred. Propranolol was initiated to enhance ventricular filling (maximum dose 4 mg/Kg/day). Twenty four-hour ambulatory ECG monitoring was unremarkable. MRI showed biventricular hypertrophy without intracardiac fatty accumulation (data not shown). Over time, HCM stabilized and resolved with normalization of cardiac dimensions at 16 months of age (Fig. 1e, right panel). Propranolol was successfully tapered.

At present, he is 2 years old and has normal height and weight for age. His physical examination, in addition to the lipodystrophy, is remarkable for muscle hypertrophy, acanthosis nigricans, large hands and feet, and enlarged external genitalia (Fig. 1). He is mildly retarded.

Identification of a Homozygous Mutation in the Acceptor Splice Site of Exon 5 of BSCL2

In the genome of the proband, we found a homozygous nucleotide substitution in the splice site of intron–exon boundary 5 (NM_032667.5:c.439-2A > C) of *BSCL2* (Fig. 2b). The clinically unaffected grandfather, father, mother, and an older brother (I.1; II.1; II.2 and III.2 respectively) were heterozygous for the same nucleotide substitution, and the clinically unaffected grandmother and the oldest brother (I.2 and III.1 respectively) had two wild-type alleles (Fig. 2a). All other regions analyzed were devoid of DNA sequence changes.

Since the mutation is located in a splice site, we next examined whether this mutation affects RNA splicing. RT-PCR analysis resulted in a single PCR product with homozygous wild-type sample (I.2), while RNA from the proband resulted in two smaller bands, suggesting that two aberrant mRNA products are formed. RNA from the heterozygous mother (II.2) displayed in addition to the two aberrant bands a band similar to the wild type band, albeit to a lesser intensity. Sequencing of the two aberrant mRNA products revealed that the smaller band is the result of an exon 5 skipping, encoding a protein with an internal deletion (p.V147_S191del; hereafter termed Δ Exon5).

Fig. 1 Clinical aspects of the proband. Photographs of the proband showing the absence of subcutaneous fat, muscular hypertrophy (**a, b, d**), enlarged hands and feet (**b, d**), hirsutism and acanthosis nigricans (**d**). (**e**) Echocardiographic end-diastolic parasternal long axis view

showing massive biventricular hypertrophy at 6.5 months (*left panel*) and normalization of cardiac dimensions at 16 months of age (*right panel*)

The other product is the result of a cryptic acceptor site in exon 5 resulting in a partial deletion and frameshift ultimately leading to a premature stopcodon in exon 6, encoding a truncated seipin protein (p.V147fsX59; hereafter termed Fs).

Expression and Cellular Localization of Aberrant Seipin Proteins

To investigate the subcellular localization of the aberrant seipin proteins, U2OS cells transfected with expression

vectors encoding wild-type (FL) and mutant (Δ Exon5 and Fs) seipin proteins were examined by confocal immunofluorescence microscopy. Like the wild-type protein, seipin- Δ Exon5 and seipin-Fs both localized to the endoplasmic reticulum as it colocalized with the ER marker calreticuline (Fig. 3a). Furthermore, this colocalization pattern was absent in the empty vector negative control (Fig. 3a). In parallel, protein expression levels were determined by Western blot analysis and showed similar expression levels for the different seipin proteins (Fig. 3b). In contrast to wild-type and Δ Exon5 seipin protein, a high molecular

Fig. 2 A novel homozygous mutation at the intron–exon boundary of exon 5 of the *BSCL2* gene affects RNA splicing. (a) Family pedigree with the affected proband indicated as a filled symbol and marked with an arrow. Half-filled symbols are clinically unaffected subjects harboring a heterozygous mutation; unfilled symbols are clinically unaffected subjects harboring the wild-type sequence. (b) DNA

sequencing chromatograms of the intron–exon boundary of exon 5 of the *BSCL2* gene illustrating the A→C nucleotide substitution at position –2 of exon 5 (NM_032667.5:c.439–2A>C). (c) DNA gel analysis and DNA sequencing chromatograms of PCR products after PCR amplification of cDNA synthesized from patients RNA using primers encompassing exon 5, showing that the mutation affects RNA splicing

weight complex could not be detected for seipin-Fs, which is consistent with its lack of transmembrane domain 2 (Ito et al. 2008).

In conclusion, the mutant seipin proteins (seipin-ΔExon5 and seipin-Fs) are normally expressed and localize to the ER like wild-type protein.

Abnormal Organic Acid Levels in the Urine of the Proband

Before the diagnosis, organic acids in urine were analyzed as part of a metabolic screening. In this urine sample, collected at the age of 3 weeks, ethylmalonic acid, fumaric acid, and 2-ketoglutaric acid were increased. The concentration of lactic acid was normal. Subsequently organic acids were analyzed in six different urine samples in the first 2 years of life (Table 1). In all samples, the concentration of ethylmalonic acid was increased. Fumaric acid, citric acid, and 2-ketoglutaric acid concentrations

were elevated in some, but not all samples. Lactic acid was normal in all samples. No consistent effect of the diet adaptations (started at week 4) or metformin treatment (started at 10 months) was observed. In addition, the clinically unaffected parents displayed normal organic acid profiles, except for mildly elevated citric acid levels in the urine of the father (Table 2). These findings suggest that the abnormal organic acid levels measured in the urine of the patient are associated with CGL2.

Discussion

Rare genetic defects in adipose tissue development or function, like congenital generalized lipodystrophies (CGLs), provide important insights into the regulation of whole body energy homeostasis and the metabolic complications associated with common obesity (Huang-Doran

Fig. 3 Aberrant seipin proteins are expressed and colocalize with the ER. **(a)** U2OS cells were transiently transfected with expression vectors encoding wild-type (wt) or mutant HA-seipin proteins p.V147_S191del (Δ Exon5) and p.V147fsX59 (Fs). Cells were immunostained with HA-antibody (seipin) and calreticulum-antibody (ER-marker) and proteins were visualized by confocal immunofluorescence microscopy. The yellow pattern as observed in the merged images is suggestive for colocalization of the seipin proteins (wild type and mutants) with the ER. Bar, 10 μ m. **B**, U2OS cells were transiently transfected with expression vectors encoding empty vector (ev), wild-type (wt) or mutant Flag-seipin proteins (Δ Exon5 and Fs, respectively), and protein expression was assessed by Western blot analysis using anti-Flag antibody

et al. 2010). Our characterization of the genotype and phenotype of a patient with CGL type 2 resulted in three novel findings.

First, the genome of the patient harbors a novel homozygous mutation in an acceptor splice site of the *BSCL2* gene, encoding the integral ER membrane protein seipin. We show that this mutation causes a complex RNA splicing defect, resulting in the expression of two different aberrant seipin proteins. Consistent with the preservation of transmembrane domain 1, which has been shown to be critical for ER retention (Ito et al. 2008), both aberrant proteins localized to the ER like wild-type protein. While its exact function remains to be established, recent studies in yeast and human cells have shown that lack of seipin results in altered lipid droplet morphology and number, strongly implicating this protein in lipid storage (Szymanski et al. 2007; Fei et al. 2008; Boutet et al. 2009). In addition, seipin plays a role in lipid biosynthesis, as lymphoblastoid cells from CGL2 patients displayed a defect in Δ 9-desaturase (stearoyl-CoA desaturase; SCD) activity (Boutet et al. 2009). Finally, seipin is essential for adipocyte differentiation, as its expression strongly increases during adipogenesis (Payne et al. 2008). Moreover, RNAi-mediated knockdown in mesenchymal cells impaired adipocyte differentiation (Payne et al. 2008). Although the highest expression levels of seipin are observed in brain (Magre et al. 2001), these studies clearly demonstrate that seipin is critically important in the differentiation of adipocytes and their lipid storage function.

Second, the patient's clinical course is remarkable. While HCM is described in CGL type 2 (Agarwal et al. 2003; Van Maldergem et al. 2002), it is rarely present in the first months of life (Friguls et al. 2009) as observed here. In addition, our patient is unusual since cardiac dimensions improved and even normalized. This occurred after the administration of metformin, which clearly ameliorated insulin resistance. The origin of the cardiomyopathy is presumably the hyperinsulinemia (Klar et al. 1993; Bhayana et al. 2002), and it would be tentative to relate the cardiac diameter normalization to the metformin treatment. In the largest series of cardiomyopathy cases in congenital and acquired generalized lipodystrophy reported, cardiac dimension and function stabilized or worsened (Lupsa et al. 2010).

Finally, our patient had urinary findings never reported before in CGL: urinary organic acid screening showed intermittently elevated levels of several organic acids, including intermediates of the citric acid cycle (fumaric acid, citric acid and 2-ketoglutaric acid as well as consistently elevated concentrations of ethylmalonic acid), suggestive for mitochondrial dysfunction. Ethylmalonic acid elevations are, in combination with increased butyrylcarnitine in blood, also found in patients affected with a deficiency of short chain acyl-CoA dehydrogenase (Blau et al. 2003). In our patient, butyrylcarnitine was within the normal range, making SCAD deficiency as a cause of

Table 1 Concentrations of organic acids in urine of the proband

	3 weeks	6 weeks	16 weeks	49 weeks	18 months	21 months
Lactic acid	292 (46–348)	151(57–346)	53(57–346)	254 (22–258)	37 (22–258)	72 (22–258)
Ethylmalonic acid	60 (0.4–17)	123 (0.4–17)	76 (0.4–17)	54 (0.4–17)	43 (0.4–17)	50 (0.4–17)
Fumaric acid	91 (10–45)	55 (4–45)	12 (4–45)	59 (1–27)	16 (1–27)	15 (1–27)
Citric acid	1,164 (0–1,045)	1,086 (104–268)	850 (104–268)	2,846 (0–656)	1,213 (0–656)	1,765 (0–656)
2-Ketoglutaric acid	875 (22–567)	1,064 (63–552)	282 (63–552)	1,472 (36–103)	152 (36–103)	450 (36–103)

Levels of lactic acid, ethylmalonic acid, fumaric acid, citric acid and 2-ketoglutaric levels in urine samples of the patient at ages 3, 6, 49 weeks and 18 and 21 months expressed in mmol/mol creatinine. Age-dependent reference values are indicated between brackets (Blau et al. 2003; Rinaldo 2008)

Table 2 Concentration of organic acids in urine of the parents of the proband

	Father	Mother
Lactic acid	10 (13–46)	12 (<46)
Ethylmalonic acid	9 (<10)	6 (<10)
Fumaric acid	n.d. (<1)	n.d.(<1)
Citric acid	405 (70–226)	212 (70–226)
2-Ketoglutaric acid	6 (4–74)	16 (4–74)

Levels of lactic acid, ethylmalonic acid, fumaric acid, citric acid and 2-ketoglutaric levels in urine samples of the father and mother of the patient expressed in mmol/mol creatinine. Age-dependent reference values are indicated between brackets (Blau et al. 2003; Rinaldo 2008)

increased urinary organic acids unlikely (data not shown). Likewise, glutaric aciduria type II (also called multiple acyl-CoA dehydrogenase defect (MADD)) is not very likely to be the cause of the elevated ethylmalonic acid levels (Blau et al. 2003), as the glutaric acid and dicarboxylic acid levels in our patient were normal (data not shown). As the parents, who were both heterozygous for the *BSCL2* mutation, showed normal urinary organic acid levels, the abnormal levels of urinary organic acids in the CGL2 patient are most likely a secondary effect of the homozygous *BSCL2* mutation. It should be noted, however, that we cannot exclude an additional autosomal recessive mutation in another gene at this moment, particularly since the parents are consanguineous. Interestingly, mitochondrial dysfunction, as suggested here for CGL2, has also been reported in obesity and type II diabetes (Kelley et al. 2002; Lowell and Shulman 2005). A commonality in obesity and lipodystrophy is the lack of sufficient lipid storage capacity in adipose tissue resulting in ectopic accumulation of lipids. Ectopic fat accumulation in liver and skeletal muscle is strongly associated with insulin resistance in these tissues (Savage et al. 2007) and might therefore be the cause of mitochondrial dysfunction. In line with this, patients with primary congenital insulin resistance due to mutations in the insulin receptor gene (*INSR*) were recently reported to

display mitochondrial dysfunction (Sleigh et al. 2011). Based on these findings, we hypothesize that due to the lack of lipid storage capacity in CGL2 patients excessive lipids accumulate in liver and muscle, causing insulin resistance and subsequent mitochondrial dysfunction. Thus the association between CGL2 and mitochondrial dysfunction observed here is most likely secondary. In summary, here we show for the first time elevated urinary organic acid levels in a patient with CGL due to a novel mutation in *BSCL2*, suggestive for secondary mitochondrial dysfunction.

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Synopsis

Secondary mitochondrial dysfunction in Congenital Generalized Lipodystrophy type 2 (CGL2).

Author Contributions

EHJ and NH conducted experiments, MV and JMPJB performed clinical assessments of the patient, NMVD supervised organic acid analyses, MV, RB, and EK supervised the study, and EHJ, MV, and EK wrote the manuscript.

Guarantor

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Competing Interest Statement

The authors have nothing to declare.

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Details of Ethical Approval

No ethical approval was required for this research study.

Patient Consent Statement

Informed consent for this study was obtained from the parents of the patient.

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