A new seipin-associated neurodegenerative syndrome

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ABSTRACT

Background: Seipin/*BSCL2* mutations can cause type 2 congenital generalized lipodystrophy (BSCL) or dominant motor neuron diseases. Type 2 BSCL is frequently associated to some degree of intellectual impairment, but not to fatal neurodegeneration. In order to unveil the etiology and pathogenetic mechanisms of a new neurodegenerative syndrome associated to a novel BSCL2 mutation, six children, four of them showing the BSCL features, were studied.

Methods: Mutational and splicing analyses of *BSCL2* were performed. The brain of two of these children was examined post-mortem. Relative expression of *BSCL2* transcripts was analyzed by real time RT-PCR in different tissues of the index case and controls. Overexpressed mutated seipin in HeLa cells was analyzed by immunofluorescence and western blotting.

Results: Two patients carried a novel homozygous c.985C>T mutation, which appeared in the other four patients in compound heterozygosity. Splicing analysis showed that the c.985C>T mutation causes an aberrant splicing site leading to skipping of exon 7. Expression of exon 7-skipping transcripts was very high with respect to that of the non-skipped transcripts in all of the analyzed tissues of the index case. Neuropathological studies showed severe neuron loss, astrogliosis and intranuclear ubiquitin(+) aggregates in neurons from multiple cortical regions and in the caudate nucleus.

Conclusions: Our results suggest that exon 7 skipping in the *BSCL2* gene due to the c.985C>T mutation is responsible for a novel early-onset, fatal neurodegenerative syndrome involving cerebral cortex and basal ganglia.

KEYWORDS: Neurodegeneration, BSCL2, seipin, aberrant splicing, Berardinelli-Seip syndrome, nuclear inclusions, protein misfolding.

INTRODUCTION

Phenotypes associated with mutations in the *BSCL2* gene are congenital generalized lipodystrophy type 2 (Berardinelli-Seip syndrome type 2, BSCL Type 2; OMIM: 269700) and *BSCL2*-related neurological disorders (OMIM 270685 and 600794) [1, 2].

BSCL type 2 is a rare autosomal recessive disorder characterized by marked adipose tissue paucity, muscle hypertrophy, acromegaloid features, insulin resistance, hypertriglyceridemia, early onset diabetes mellitus and hepatic steatosis [3]. Some degree of mental retardation is often present [4]. Most currently described mutations in the *BSCL2* gene causing BSCL type 2 are nonsense or frameshift mutations leading to premature stop codons [1]. On the other hand, *BSCL2*-related neurological disorders are dominant motor neuron diseases caused by specific missense mutations [5, 6].

BSCL2 encodes the protein seipin, highly expressed in the brain [1, 4]. Three principal transcripts exist, 462 (*BSCL2*-03), 398 (*BSCL2*-04, 05, 06) and 287 (*BSCL2*-08) amino acids long respectively [7]. Seipin is an integral membrane protein of the endoplasmic reticulum (ER) with two predicted transmembrane domains, an intraluminal loop and amino- and carboxy-terminal intracytoplasmic ends [8]. However, the short transcript has a completely different amino acid sequence from exon 6 onwards due to an alternative splicing which results in skipping of exon 7 and a reading frame shift.

The function of seipin is incompletely understood. Thus, some studies indicate a role for the protein in adipogenesis, lipid metabolism, and lipid droplet biogenesis [7, 9-14], while others have shown a potential neural involvement [7, 15, 16].

The diagnosis and follow-up of the index case, with BSCL phenotype and neurodegeneration, led us to review retrospectively the charts of other known patients with similar phenotype. Deceased patients´samples and their families were investigated. Here, we report six patients from Murcia, in southeastern Spain, from four apparently unrelated pedigrees, sharing the same c.985C>T novel mutation in the *BSCL2* gene. Homozygous patients suffered from progressive encephalopathy since age 2-3, with a fatal outcome at age 6-8, but showed mild BSCL clinical features. Three compound heterozygous subjects showed a typical BSCL phenotype, besides a neurological clinical course similar to that of the homozygous cases; a fourth case, still alive, currently shows, at 42 months, a psychomotor delay.

SUBJECTS AND METHODS

The Ethics Review Panel of Xunta de Galicia approved this study, conducted according to the ethical guidelines of the Helsinki Declaration. Patients' parents gave informed consent for participation in the study and publication of clinical and genetic information.

Subjects

All patients were born in three close towns within a 50 km radius from the city of Murcia.

The index case was a female, the only child born to a non-consanguineous couple in 2004. Physical examination was completely normal at birth. At 4 months of age the patient showed hepatomegaly, severe hypertriglyceridemia, coarse facies, and striking muscle induration of the limbs. At 6 months, she was placed on an animal fat-free diet, her liver size and plasma triglycerides became normal; her psychomotor development was within normal limits. Genetic

analysis of *BSCL2* revealed a novel nonsense mutation (*vide infra*). She walked independently at 16 months. By age 2 the patient spoke a few monosyllabic words and showed poor motor coordination, unsteady gait and difficulties in standing up, increased muscle tone and brisk deep tendon reflexes. The patient was thin, with normal nutritional status and Bichat's fat-pads (Figure 1A).

By age 3 the patient showed psychomotor regression. She lost all language and showed severe cognitive impairment. By age 4 she still walked albeit with ataxic gait. She showed widespread and generalized fine tremor, dystonia and sleep disturbances. Generalized tonic-clonic seizures appeared at this time.

At 5 years of age the patient was unable to walk or sit unsupported. She showed severe spasticity and suffered convulsive seizures of different morphology. At 6 years of age she had severe encephalopathy with tetraparesis, pyramidal and extrapyramidal signs and severe sleep disorder. All motor skills, social, language, and cognitive development were lost. Cardiac evaluation was always normal. She died at age 8 from an aspirative pneumonia.

Brain magnetic resonance imaging (MRI) at 21 months showed mild subcortical atrophy, which progressed to moderate atrophy three years later.

Electroencephalography showed multifocal spike-wave and sporadic generalized discharges associated or not with myoclonias. Electromyography and nerve conduction velocity studies were normal at 3 and 7 years of age. At 6 years of age nerve biopsy and muscle mitochondrial respiratory chain study were both normal. Creatine deficiency, congenital glycosylation defects, Niemann-Pick disease, GM1 and GM2 gangliosidosis, metachromatic leukodystrophy, and neuronal ceroid lipofuscinosis were excluded. Additional

genetic investigations including high resolution karyotype, DNA methylation analysis for the 15q11.2-q13 Angelman syndrome/Prader-Willi syndrome region, lipoprotein lipase gene analysis, and *MECP2* gene analysis were also normal.

Patient two was a boy born to non-consanguineous, healthy parents in 1986. At 4 months of age he showed a BSCL phenotype (Figure 1B). He walked independently at 12 months of age. At 3 years of age he had hyperactive behaviour, mild cognitive impairment and language delay and began to show tremor and myoclonic seizures. EEG showed generalized spike-wave discharges. At 4 years old, he had normal triglyceridemia, pyramidal signs, language loss and more severe epilepsy with myoclonic, partial and generalized seizure of difficult control. Progressive neurological deterioration continued with dystonia and difficult swallowing. He died at 8 years of age, maintaining his lipodystrophic phenotype, because of respiratory failure in a status epilecticus. Extensive metabolic work-up and brain MRI were normal.

Patient three was the first child born to a healthy, non-consanguineous couple in 1976. His development was normal in the first year, he walked independently at the 13th month. His parents noticed abnormal communication skills, language delay and hyperactivity during his second year of life. At age 2.5 he was admitted to the hospital due to complex partial seizures. He had BSCL phenotype, cognitive dysfunction, and ataxic gait (Figure 1C). No hepatomegaly or hypertriglyceridemia was present. At age 5 he showed severe cognitive impairment and progressive deterioration. At age 7 he had frequent seizures (myoclonic, partial and generalized tonic-clonic), and died from a severe respiratory infection.

Patient four was the younger brother of patient three, born in 1990. At 3 months of age he showed a lipodystrophic phenotype, hepatomegaly, and hypertriglyceridemia. He walked independently at 15 months of age. At age 3 he showed hyperactivity, severe language delay and myoclonic epileptic seizures. His neurological clinical course was similar to his brother's and he died at the age of 7 due to respiratory infection.

Patient five was the only female child born to a healthy, non-consanguineous couple. Her mother was the first cousin of patients 3 and 4 by paternal lineage. At two months of age, she showed the typical BSCL phenotype (Figure 1D) and she walked independently at 16 months of age. Currently, at three years and 6 months of age, she shows also a mild psychomotor delay, affecting the language acquisition and behaviour (irritability, hyperactivity and sociability), and brain hypometabolism (*vide infra*).

Patient six was the third child to healthy and consanguineous parents born in 1981. Her first referred sign was developmental delay without lipodystrophic phenotype. She walked independently at 19 months of age with ataxic gait. Myoclonic seizures were noted by 4 year of age. During her fifth year, a severe neurologic regression with marked irritability, dysphagia, sleep disorder and pyramidal signs was detected, and a neurological clinical course similar to that of the index case followed. The patient died at 6 years of age in another hospital. Death cause report is not available.

For more clinical information see Table 1S in Supplementary Data.

Clinical evaluation of patients' parents: Clinical exam, brain MRI, electromyography and nerve conduction velocity studies of 7 asymptomatic parents carrying the c.985C>T mutation (*vide infra*) were normal.

Methods

Genetic studies: For the mutational analyses, genomic DNA was isolated from peripheral leukocytes using standard procedures [17]. *BSCL2* exons 1-11 and the surrounding intronic sequences from the subjects were PCR amplified and sequenced as described [1]. *BSCL2* exons 4 and 7 were also analyzed in 322 volunteers from the three towns from which the cases originated, and in 50 control subjects from Galicia (Northwestern Spain).

Haplotype construction: Three biallelic markers within *BSCL2*, SNPs rs2850596, rs74388071, and rs2850597, plus the c.985C>T mutation were selected to construct haplotypes of 100 individuals. This group included the 13 carriers of the c.985C>T mutation, 14 relatives not carrying the mutation, and 72 controls from the same geographical region. The haplotypes were constructed by the Bayesian statistical method implemented in the PHASE v 2.0.2 program [18]. The markers were genotyped by direct sequencing of PCR products as described above.

Adipose tissue biopsies and cell culture: A small sample of subcutaneous adipose tissue of the abdominal area was obtained from the index patient at age 6. Control adipose tissue sample was obtained from a 6-year-old normal boy during cryptorchidism surgery in accordance with the current Spanish legislation. A small piece of tissue was placed on a 5 cm dish containing Dulbecco's modified Eagle's medium (DMEM) supplemented (Sigma, MO, USA) with 30% fetal bovine serum (FBS) (Gibco Invitrogen, NY, USA) and gentamicine. Preadipocytes were recognized by the presence of small lipid droplets within the fibroblast-like cells using a phase microscope.

Tissue samples: Brain, skeletal muscle, subcutaneous and visceral adipose tissue, liver, kidney, and vagal nerve were obtained from the index case during autopsy. Similar samples were obtained from three adult male adult decedents, 35, 50 and 79 years old respectively, all of which committed suicide and underwent autopsy in accordance with the Spanish legislation.

BSCL2 expression studies: Total RNA was extracted from tissue samples, lymphocytes, primary preadipocytes and fibroblasts and reverse-transcribed as previously reported [19]. BSCL2 cDNA was amplified with primers designed with the Primer3Plus software (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) (forward: 5'-CAGATGCTGGACACACTGGT-3', reverse: 5'-ATCACTGGCCTCAGGCTCTA-3'). PCR conditions are available upon request. Amplification fragments obtained were separated out by low-melting agarose gel electrophoresis (1%). The resulting bands were excised from agarose gel, and cDNA was extracted using the Qiaex II gel extraction kit (Qiagen, Chatsworth, CA, USA). cDNA was then amplified with the same primers and conditions as used for the first PCR; fragments were separated by agarose gel electrophoresis and directly sequenced.

Splicing analysis was as indicated in Figure 2.

Expression of *BSCL2* mRNA was quantified in a Light Cycler 2.0 (Roche Diagnostics, Sant Cugat del Vallès, Spain) using specific probes and oligonucleotide primers designed by Universal ProbeLibrary (Roche Diagnostics). Specific designed primers were used for *BSCL2* exon 7 transcripts and for *BSCL2* spliced transcript. Details are available upon request. Results were normalized to the RNA polymerase II and 18s genes, using the $2^{-\Delta\Delta CT}$ method [21].

Cell studies: Preadipocytes were cultured from adipose tissue biopsy samples and imaged by transmission electron microscopy (see Supplementary Data). ER stress marker, BiP, was analyzed by western blot (WB).

BSCL2 transfection into HeLa cells: A plasmid containing wild type seipin fused to a myc tag (6 myc-wt seipin pCS2+MT) was a kind gift from D. Ito, Keio University, Japan. A seipin R329X mutant was obtained by site-directed mutagenesis using the QuikChange® II Site-Directed Mutagenesis Kit following the manufacture instructions (Stratagene, Cedar Creek, TX, USA) with the oligonucleotide 5'-GGCATCTGGCCCTGACACCGCTTCTC-3' and its reverse complement on the wild type (wt) plasmid. A plasmid containing the sequence of skipped seipin fused to myc was created as follows: a 431 bp fragment without exon 7 was amplified from cDNA of the index patient, purified and sequenced as described above, and then it was inserted into the pGEM-T-easy vector following the manufacturer's instructions (Promega Corporation, USA). Then, a shorter fragment containing exons 6 and 8 was obtained by digestion with the restriction enzymes Eco0109I and BsiWI (New England Biolabs, Ipswich, MA, USA) from the cloned fragment and inserted at the corresponding site in the 6 myc-wt seipin pCS2+MT plasmid with the wt fragment previously removed using the same enzymes. Correct cloning was verified by sequencing.

HeLa cells were maintained in DMEM supplemented with 10% FBS, 1% penicillin/streptomycin (Sigma, MO, USA) and 1% L-glutamine (Gibco). Transfection was performed using Fugene 6 Transfection Reagent (Promega WI, USA), according to the manufacturer's instructions. After 48 hours the cells were lysed, using cold lysis buffer (20 mM HEPES, pH 7.4, 2 mM EGTA, Na_3VO_4, 1% Triton X100, 10% Glycerol, 50 mM β-Glycerophosphate, 1 mM

DTT, 2 μM leupeptin, 0.1% aprotinin and 400 μM PMSF). Cell lysates were separated by SDS-PAGE and transferred to a PVDF transfer membrane (Millipore MA, USA). The membrane was probed with 1:200 - 1:1000 anti c-Myc (Santa Cruz Biotechnology CA, USA) followed by 1:2000 ECL anti-mouse IgG horseradish peroxidase linked secondary antibody (GE Healthcare UK Limited, Buckinghamshire, UK).

Seipin subcellular fractionation analysis: The method of Yokoyama et al. [22] was followed. Briefly, HeLa cells were washed twice with PBS and harvested. Cells suspended in ice-cold buffer A (10 mM Hepes, pH 7.0, 5 mM MgCl2, 25 mM KCl, 1 mM Na3VO4, 1 mM PMSF, 10 μg/ml leupeptin, and 10 μg/ml aprotinin) were disrupted by repeated passage (10-times) through a 23 gauge-needle and then mixed immediately with an equal volume of ice-cold buffer A containing of 0.25 M sucrose. The mixture was centrifugated at 500 × g for 10 min. The pellet was resuspended in an equal volume of ice-cold buffer A containing 0.1 % of NP-40 and then homogenized again by passage through a 23-gauge needle ten times. Nuclei were isolated by centrifugation at 500 × g for 10 min, washed once with buffer A containing of 0.1 % of NP-40, and lysed in lysis buffer (10 mM Hepes, pH 7.0, 0.5 M KCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 1 mM DTT, 1 mM PMSF, 10 μg/ml leupeptin, and 10 μg/ml aprotinin). This is the subcellular fraction enriched in nuclei. EDTA was added to the supernatant from the initial low speed centrifugation (500 x g) to a final concentration of 10 mM and the mixture subjected to centrifugation at 16,000 × g for 15 min. The resultant pellet were washed once with buffer A containing of 0.25 M sucrose and lysed in a RIPA buffer (50 mM Tris-HCl, pH 7.2, 150 mM NaCl, 5 mM EDTA, 2 mM Na_3VO_4, 1% NP-40, 0.25 % sodium deoxycholate and 0.05 % SDS, 1

mM PMSF, 10 μg/ml leupeptin, and 10 μg/ml aprotinin). This fraction represents the standard subcellular fraction enriched in mitochondria + microsomes. The supernatant from the centrifugation at 16000 x g represents the subcellular fraction enriched in cytosol + plasma membrane. All centrifugations were carried out at 4ºC. A sample (100 μl) of the initial whole-cell suspension was lysed directly in RIPA buffer as control.

Each fraction was analyzed by WB using 9E10 anti-myc (Santa Cruz Biotechnology, CA, USA), anti-BiP (Cell Signalling, MA, USA), anti-Fibrillarin (H140) (Santa Cruz Biotechnology), anti-Grp94 (Cell Signaling) and anti-GAPDH (Sigma Aldrich, MO, USA) antibodies.

Immunofluorescence assay: HeLa cells grown on Milicell EZ_{slide} (Millipore, MA, USA) were transfected with the appropriate expression plasmid. After 48 h, the cells were fixed with 4% paraformaldehyde ice cooled for 10 min and then permeabilized in 0.5% Triton X-100 at room temperature for 5 min. After blocking of non-specific binding (5% BSA, 1 hour at room temperature), the slides were incubated with the primary antibody (9E10, 1:1000) at 4ºC overnight. The next day, after four washes, the slides were incubated with Texas Red conjugated anti-mouse secondary antibody and Hoechst 33258 stain (Sigma Aldrich, MO, USA) for 1 hour in the darkness and mounted. Immunofluorescence staining was examined using a Leica TCS SP5 confocal microscope and LAS AF Software (Leica, Mannheim, Germany).

Quantitative brain PET/MRI: Functional PET studies were performed on patient five at 2.8 years of age. Both visual inspection and quantitative analysis were carried out to identify brain areas with abnormal metabolism. These areas were

co-registered to a T1-weighted MRI-based atlas to obtain anatomical localization (see Supplementary data for details).

RESULTS

Mutational analysis of *BSCL2* gene: The index patient was homozygous for a novel nonsense mutation NM_001122955.3: c.985C>T, p.Arg329X in *BSCL2* (Figure 1E, 1F). Her parents were asymptomatic heterozygous mutation carriers. Patient five was a compound heterozygote for *BSCL2*: c.985C>T in the maternal allele and c.507_511del, (p.Tyr170CysfsX6) in the paternal allele (Figure 1G, 1H). DNA from patients three, four, and six, who died more than 15 years ago, was unavailable. The mother of the second case was a heterozygous carrier of the c.985C>T mutation, while his father was a heterozygous carrier of yet another novel nonsense *BSCL2* mutation: c.538G>T (p.Glu180X) (Figure 1I, 1J). Both parents were asymptomatic. The situation was similar for the third and fourth patients: their asymptomatic father carried the frameshift *BSCL2* mutation c.507_511del [1] and their asymptomatic mother the same mutation as the index patient (Figure 1G, 1H). The parents of the sixth case were asymptomatic heterozygous carriers of the c.985C>T mutation (Figure 1K).

The c.985C>T mutation appeared in heterozygosity in eight samples from the Murcia genetic study (allelic frequency: 0.012). Haplotype analysis suggests a founder effect (see Supplementary data for details). The other two mutations were not found in the 644 chromosomes from that area. None of these mutations was found in the 100 control chromosomes from Galicia.

Splicing analysis: The c.985C>T mutation causes an aberrant splicing site leading to skipping of exon 7 (Figure 2A). As seen in Figure 2B, samples from the index case exhibited, besides the 573 bp band corresponding to normal splicing, a second 431 bp band corresponding to the alternatively spliced product. Sequencing of this band confirmed skipping of exon 7 (Figure 2C), which would give rise to the mutated protein, p.Tyr289Leu*fs*X64.

Brain PET/MRI from patient #5 (2.8 years old): Visual inspection revealed bilateral temporal and occipital hypometabolism and also unilateral left thalamic hypometabolism (Figure 3). For quantitative values see the Supplementary data.

Autopsy study of the index case: This study revealed a severe lack of subcutaneous and visceral adipose tissue (see Supplementary data for details). On the other hand, postmortem examination revealed symmetrical moderate cortical atrophy of frontal, parietal and occipital lobes, intense atrophy of the caudate nucleus and moderate atrophy of the cerebellar vermis. On histological examination, atrophic areas displayed intense neuronal loss and astrogliosis. Immunohistochemical examination revealed intense immunoreactivity for phosphorylated neurofilaments in remaining neurons that frequently contained small proximal axonal spheroids. Occasionally these neurons displayed ubiquitin-positive intranuclear inclusions; either globular or granular (Figures 4A-C). Postmortem examination of archival fixed material from case 3 overall agreed with observations on material from the index case (Supplementary data).

ER hypertrophy and increased BiP expression in preadipocytes from the index case: In the index case, preadipocyte cytoplasm was rich in markedly dilated

rough ER, filled with medium dense granular material (Figure 4D). Expression of the reticulum stress marker BiP was increased in the index case compared to the control (Figure 4E).

BSCL2 transcript expression: The relative expression of the different seipin transcripts in both tissue samples and primary cultures is shown in Figures 5A-D (and in Tables 2-4S, Supplementary data). Expression of *BSCL2* transcripts containing exon 7 was reduced in all samples from the index case (to ≈9% of control values in the central nervous system (CNS) and ≈34% in the other tissues). Expression of the *BSCL2* transcript without exon 7 in the control samples was negligible (<0.5%) compared with the other transcripts. However, the exon 7-skipping transcript were highly expressed in all of the index case samples compared with their respective control samples (≈600% in CNS and ≈1300% in the other tissues).

Seipin overexpression in HeLa cells: Myc-tagged wild-type, exon 7 skipped, and R329X mutant seipin were overexpressed in HeLa cells (Figure 6A). Increased ER stress caused by exon 7 skipped seipin was confirmed by a clearly higher level of BiP expression (Figure 6B). To determine their subcellular location, wild-type, exon 7 skipped and R329X mutant seipin were analyzed by confocal microscopy. All three seipin forms presented a diffuse cytoplasmic localization, as previously described [23]. However, intense fluorescence rings were also detected surrounding cell nuclei, suggesting perinuclear/nuclear localization of a fraction of seipin (Figure 6C). Subcellular fractions were analyzed using an anti c-Myc antibody; nuclear localization of wild type and exon 7 skipped seipin was confirmed; immunoblots suggest a higher fraction of skipped seipin localized in the nucleus as compared to wt seipin (Figures 6D, E).

DISCUSSION

Here we report a new neurodegenerative syndrome associated with a novel mutation in the *BSCL2* gene, c.985C>T. This mutation gives rise to an aberrant splicing site which causes complete skipping of exon 7, change of reading frame and early termination which would result in the aberrant protein p.Tyr289Leu*fs*X64. This mutation, whether homozygous or in compounded heterozygosity with a second *BSCL2* "classic" mutation, results in an extremely severe neurological syndrome that leads to death during the first decade of life. The index patient, homozygous for c.985C>T, initially showed clinical features of BSCL that disappeared during the first months of life. Although the necropsy study of this patient showed a severe loss of adipose tissue, this contrasts with the phenotype of the patient, in whom adipose tissue was present (Figure 1A). On the other hand, the sixth case, also homozygous for c.985C>T, did not show any evidence of transient lipodystrophy. Thus, some degree of lipodystrophy might indeed exist in homozygous subjects albeit in a much lower degree than in compound heterozygous children.

Regarding the only alive child (patient 5), although, at present, she does not show any clinical evidence of neurodegeneration, given her genetic background, the presence of poor language acquisition and abnormal behaviour, and taking into account the natural history of her relatives (patients 3 and 4), it is reasonable to expect a neurological involution, although we must be very cautious about this.

So far, *BSCL2* mutations had been associated to a higher prevalence of intellectual impairment [4], but never before with such specific fatal neurodegenerative course. Strikingly, Van Maldergem et al. [4] reported a case

of severe psychomotor delay and pyramidal signs due to a splice site mutation resulting in exon 7 skipping. However, no information about the age and clinical evolution of this patient was provided.

Analysis of expression of *BSCL2* transcripts showed that in normal subjects the short transcript has a very low transcription rate (<0.5%), in agreement with published studies [7]. Further, total seipin expression in the index case was much reduced in all tissues; however, the expression of transcripts without exon 7 was very high compared with controls. This finding strongly suggests that this transcript plays a key role in the pathogenesis of this new neurological disease, and also allows us to speculate about a possible positive role it might have in the maintenance of adipogenesis.

The large RE dilatation observed in preadipocytes from the index case, and the increment of BiP in these and in transfected HeLa cells, suggest a possible accumulation of misfolded seipin within the ER, inducing ER stress, which might activate the unfolded protein response [24, 25], perhaps leading to neuron apoptosis, as in other neurodegenerative diseases [26-30]. The neuropathological studies showed a pattern of regional degeneration, with major involvement of cortical areas and basal ganglia. Strikingly, one of the most affected areas was the caudate-putamen nucleus; together with the presence of ubiquitin-immunoreactive intranuclear inclusions, this might suggest some pathogenic analogy with Huntington's disease and some frontotemporal dementias and hereditary ataxias [31, 32], although caution must be exercised given the non-specific nature of these findings. On the other hand, our studies with seipin-transfected HeLa cells show that seipin, besides localizing in the ER, also appears in the nucleus. In Huntington's disease, intranuclear inclusions are

the result of intranuclear accumulation of huntingtin fragments [33]. We have not yet been able to prove that the intranuclear inclusions seen in the brain of the index case are made up of seipin, as sufficiently specific seipin antibodies are not available. Efforts in that direction are under way. However, seipin/seipin fragment accumulation is the most parsimonious hypothesis. In this respect, our results showing nuclear localization of seipin are particularly relevant.

In summary, we describe a new fatal and early-onset neurodegenerative syndrome associated with exon 7 skipping in the *BSCL2* gene, affecting mainly cortical areas and basal ganglia, which should be considered in the differential diagnosis of the late infantile progressive genetic encephalopathies. This study highlights the importance of seipin, a mysterious protein, usually related with adipocyte biology, in brain function.

To Celia, *in memoriam*

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FIGURE LEGENDS

Figure 1. Images, pedigrees, and electropherograms of the studied subjects. Whole black symbols indicate homozygotes, half black or grey symbols indicate asymptomatic carriers, and black and grey symbols indicate compound heterozygotes. A) Index case (p.Arg329X homozygote) deceased at 8 years of age. This patient had a lipoatrophic appearance at 6 months of age, which regressed subsequently. Note full cheeks at 17 months, 2.5 and 5.5 years of age. B) Second case, deceased at 8 years of age. He was a compound heterozygote (Arg329X/Glu180X) with a mixed phenotype, both lipodystrophic and neurological. C) Third case, deceased at 7 years of age. He is the second uncle of the fifth case and was a compound heterozygote (Arg329X/ Tyr170CysfsX6) with a mixed phenotype, both lipodystrophic and neurological. D) Fifth case. She is a compound heterozygote (Arg329X/Tyr170CysfsX6) with typical features of Berardinelli-Seip syndrome and psychomotor delay. E) Pedigree of the index case and F) electropherogram of the c.985C>T mutation in homozygosis showing a C>T transition (CGA>TGA, arginine for premature stop codon). G) Pedigree of the third, fourth, and fifth cases and, H) electropherogram of c.507_511del mutation. I) Pedigree of the second case, and J) electropherogram of the c.538G>T mutation showing a G>T transversion (GAG>TAG, glutamic acid for premature stop codon). K) Pedigree of the sixth case.

Figure 2. Splicing analysis and amplification of the BSCL2 c.985C>T and wild-type transcripts. A) To confirm the hypothesis that the BSCL2 c.985C>T mutation results in a branch site (CCCCAG → CCCTAG) favoring splicing, the sequence of this gene in the region of the pathogenic mutation was analyzed in

silico with four splice site prediction programs [*NNSPLICE 0.9, #Alternative Splice Site Predictor (ASSP), §NetGene2 v2.4 and †Human Splicing Finder v2.4.1]. Human Splicing Finder v2.4.1, used to determine potential branch points, confirmed that the wild-type sequence (CCCCAG) has a consensus value of 86.03, whereas once mutated, the value of the branch point motif (CCCTAG) increases to 95.07. According to this computational tool, which is also able to establish potential splice sites, the mutated *BSCL2* sequence analyzed is predicted to have two splicing sites: a donor site at the beginning of intron 6-7 (CTGGGCTCAGgtgaggggcc) with a score of 96.91†, and an acceptor site at the end of intron 7-8 (tcctccacagGTTAACATCC) with a score of 96.95†. The same analysis was performed using NetGene2 v2.4§ and NNSPLICE 0.9*, which corroborate the presence of these splice sites: 0.96§* for the donor site and 0.96§ - 0.98* for the acceptor site. The ASSP tool was used to determine the constitutive or cryptic nature of the splice site: both donor and acceptor sites are constitutive, with high splice site scores of 14.346# and 13.11#, respectively [20]. B) Results of the amplification of a 573 bp region of cDNA from lymphocytes, fibroblasts and preadipocytes from the index case and different control subjects. Only samples from the index case show the presence of a 431 base pair-long additional band (bands 2, 5, and 8). C) Sequencing of the 431 base pair band showed complete skipping of exon 7.

Figure 3. Brain PET/MRI from patient 5 (2.8 years old). A 18F-FDG PET study was performed based on the EANM standard clinical protocol for paediatric examinations. Co-registration of T1-weighted MRI and functional PET images was required in order to combine functional and anatomical information in a common reference image. A fused PET/MR image of the patient was obtained

by using a mutual information approach from Statistical Parametric Mapping (Welcome Department of Cognitive Neurology, London, UK). PET data were then resampled along the planes of the MRI. In this picture we show the representation of the hypometabolic areas (from green to purple) co-registered to the brain MNI MRI atlas, which were obtained from the quantitative analysis. The bilateral hypometabolism related to the temporal and occipital areas, and the unilateral hypometabolism in the thalamic area, are shown.

Figure 4: A) Serial coronal sections of the left brain hemisphere from the level of the head of the caudate nucleus (left) to the level of thalamus (right). Note the extreme atrophy of the caudate nucleus (arrow, left) and the atrophy of the posterior corpus callosum and parasagital parietal cortex (arrow, right). B) High power image of involved occipital cortex immunostained for phosphorylated neurofilaments (SMI 213) showing multiple small immunoreactive axonal spheroids (arrows). C) High power image of involved parietal cortex immunostained for ubiquitin showing two pyramidal neurones containing conspicuous reactive intranuclear inclusions (arrows). D) Ultraestructural analysis of preadipocytes in primary culture from two control subjects and the index case: white lines indicate width of the rough ER. E) The expression of the reticulum stress marker BiP in primary preadipocytes was increased in the index case as compared to the control. The immunoblots shown are representative of two independent experiments.

Figure 5. A) Relative expression of exon 7-skipping *BSCL2* transcripts in different tissues normalised to the 18s gene. B) Percentage of change referred to the control pituitary in the expression of exon 7-skipping *BSCL2* transcripts. C) Relative expression of *BSCL2* transcripts containing exon 7 in different

tissues. D) Percentage of change referred to the control pituitary in the expression of the exon 7-containing *BSCL2* transcripts; white bar: controls; black bar: index case. Control subjects, n=3. All samples were analyzed in quadruplicate.

Figure 6. A) Immunoblot analysis of wild-type, exon 7-skipping and R329X seipin. Lysates from transfected HeLa cells were analyzed by Western blot with c-Myc (9E10) antibody. The membrane was reprobed with anti GAPDH as an internal loading control. Wild-type seipin exhibited a MW of ≈90 kDa whereas exon 7 skipped seipin appeared to have a MW of ≈60 kDa, in agreement with previously published results [23] and taking into account the theoretical mass decrease resulting from loss of exon 7. Moreover, R329X seipin showed a MW of ≈45 kDa, consistent with the stop codon produced by the mutation. High MW polymers and lower MW N-terminal fragments were detected in all cases, also in agreement with the literature [23]. B) Immunoblot analysis of BiP expression in lysates from transfected HeLa cells. GAPDH was probed as internal loading control. C) Subcellular localization of wild-type, exon 7 skipped and R329X seipin. Transfected HeLa cells were incubated with anti c-Myc (9E10) followed by Texas Red conjugated anti-mouse secondary antibody and Hoechst 33258 staining. Cells transfected with an empty vector was used as a control. All images were acquired by confocal microscopy under the same conditions. D and E) Subcellular fractionation analysis. HeLa cells overexpressing wild-type or exon 7-skipped seipin were subjected to subcellular fractionation. Fractions were analyzed by Western blot using c-Myc (9E10), GAPDH (cytoplasmic marker), Fibrillarin (nuclear marker) and Grp94 (ER marker) antibodies. Cells

transfected with an empty vector were used as a control. The immunoblots and confocal pictures shown are representative of two independent experiments.