Molecular Genetics and Metabolism 110 (2013) 162–169

Contents lists available at ScienceDirect

Molecular Genetics and Metabolism

journal homepage: www.elsevier.com/locate/ymgme

SERCA1 protein expression in muscle of patients with Brody disease and Brody syndrome and in cultured human muscle fibers

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ARTICLE INFO

Article history:
Received 10 June 2013
Received in revised form 14 July 2013
Accepted 15 July 2013
Available online 20 July 2013

Keywords:
Brody disease
Sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase 1 (SERCA1)
Brody syndrome
ATP2A1 gene

ABSTRACT

Brody disease is an inherited myopathy associated with a defective function of sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase 1 (SERCA1) protein. Mutations in the *ATP2A1* gene have been reported only in some patients. Therefore it has been proposed to distinguish patients with *ATP2A1* mutations, Brody disease (BD), from patients without mutations, Brody syndrome (BS). We performed a detailed study of SERCA1 protein expression in muscle of patients with BD and BS, and evaluated the alternative splicing of SERCA1 in primary cultures of normal human muscle and in infant muscle. SERCA1 reactivity was observed in type 2 muscle fibers of patients with and without *ATP2A1* mutations and staining intensity was similar in patients and controls. Immunoblot analysis showed a significant reduction of SERCA1 band in muscle of BD patients. In addition we demonstrated that the *wild type* and mutated protein exhibits similar solubility properties and that RIPA buffer improves the recovery of the *wild type* and mutated SERCA1 protein. We found that SERCA1b, the SERCA1 neonatal form, is the main protein isoform expressed in cultured human muscle fibers and infant muscle. Finally, we identified two novel heterozygous mutations within exon 3 of the *ATP2A1* gene from a previously described patient with BD.
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1. Introduction

Brody disease (BD) is a rare skeletal muscle disorder transmitted as an autosomal recessive or dominant trait [1]. The recessive inheritance is associated to mutation of *ATP2A1* gene encoding the sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase 1 (SERCA1), a protein that catalyzes the ATP dependent Ca^{2+} uptake from the cytosol to the lumen of sarcoplasmic reticulum [2]. The removal of calcium from the cytoplasm after muscle contraction is required to restore the tropomyosin mediated inhibition of actin–myosin interaction and, therefore, to allow muscle relaxation [3]. However mutations in the *ATP2A1* gene are missed in some patients with recessive inheritance and have never been found in patients with an autosomal dominant pattern suggesting the genetic heterogeneity of the diseases [4–7].

The main clinical feature is the exercise-induced delay in muscle relaxation which causes painless muscle stiffness following contraction, these symptoms usually resolve after a few minutes of rest [4,5,8,9]. Serum creatine kinase (CK) is normal or slightly increased, needle electromyography (EMG) records no myotonic and pseudomyotonic discharges during the exercise-induced muscle stiffness ("silent cramps") and percussion myotonia is absent [5,8–11]. At present, no treatment is available for patients even though dantrolene and verapamil turned out to be useful for reduction of muscle stiffness and thus for improving exercise tolerance [6,10].

Conventional histochemical studies of muscle tissue may reveal variability of fiber diameter and increased internal nuclei [5,7,12]. Ultrastructural abnormalities include increased number of membranous bodies within sarcoplasmic reticulum (SR) vesicles, enlargement of

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http://dx.doi.org/10.1016/j.ymgme.2013.07.015

lateral cisternae with proliferation of the tubular SR elements and presence of swollen mitochondria [11,12].

Reduction of SR Ca^{2+} ATPase activity has been reported in all described cases, independently from the association with *ATP2A1* mutation [5,6,8–10]. Conversely, data on SERCA1 protein expression are very few and still under debate [5,6,9].

In 2002, MacLennan proposed the term Brody syndrome (BS) to describe the patients with reduced SERCA activity without *ATP2A1* mutations and recently Voermans, by comparing patients with and without gene mutations, defined clinical features which may help to distinguish BD from BS [7,13]. Up to now, a total of 45 patients with reduced SERCA activity have been reported in the literature, of which only 11 with mutation in *ATP2A1* gene [7].

In the present study we evaluated in depth SERCA1 expression in muscle of seven patients with reduced SERCA activity and the solubility properties of the mutated and *wild type* protein. In addition, we report on a new patient with BS and document two novel mutations in the *ATP2A1* gene of a previously described BD patient. Finally, we provide data on SERCA1 isoforms expression in cultured human muscle fibers and in infant muscle.

2. Materials and methods

2.1. Clinical data

2.1.1. BS1

A 30-year-old man reported muscle cramps after strenuous exercise since childhood. He was never able to walk or run for long distances and never played a football match up to the end. For this reason he gave up with sports characterized by prolonged and sustained exertional activity and, instead, preferred sports characterized by sudden rapid movements such as shot put. As he was able to carry out the daily activities without problems, and since the muscle cramps were ascribed to insufficient physical preparation, he did not come to medical attention. At age 22 the patient complained of prolonged and painful exercise-induced muscle stiffness at the upper limbs, particularly at the right arm. Symptoms gradually involved also the lower limbs and trunk, and became as severe as to prevent him from working as a builder. He was the first son of two unrelated healthy parents and had a sister affected with Crohn's disease. A brother died at young age for unknown causes. Neurological examination showed muscle hypertrophy, normal strength, but no percussion or grip myotonia; deep tendon reflexes, sensation, cerebellar function and cranial nerves were normal. Serum CK ranged from 300 to 700 U/L. Liver function tests were mildly elevated. Abdominal ultrasound showed liver steatosis. Nerve conduction studies were normal. EMG recorded some low amplitude and short duration motor unit potentials with a full interference pattern at the biceps brachii and tibialis anterior muscles. No myotonic or pseudomyotonic discharges were present. The patient was treated with dantrolene sodium. The dosage was slowly built up to 100 mg a day. Exercise tolerance improved and serum CK decreased to normal values. Unfortunately dantrolene sodium caused liver toxicity and was therefore stopped. Without treatment all muscle symptoms returned.

2.1.2. BD1

Clinical data have been previously reported [14]. Briefly, this 38-year-old man was referred for muscle rigidity with neonatal onset. Motor milestones were delayed and the patient walked unassisted at 2 years of age. The patient complained of muscle cramps and exercise-induced muscle stiffness from childhood. A muscle biopsy performed at the age of 18 documented mild myopathic changes. Four years later, after an anesthesiological procedure with succinylcholine, the patient developed rhabdomyolysis (CK up to 53.000 U/L), muscle contractures, fever and myoglobinuria. EMG showed absence of myotonic discharges at rest and after muscle exercise, and nerve conduction studies were normal. A second muscle biopsy was performed and tested for in vitro contracture with halothane and caffeine, and resulted positive. A tentative diagnosis of malignant hyperthermia (MH) was done but *RYR1* gene analysis was negative. At the age of 23 another episode of myoglobinuria occurred after muscle exercise. At our last evaluation, at age 38, neurological examination was normal except for mild proximal and distal leg hypotrophy.

2.2. Muscle biopsies

Available muscle biopsy specimens from seven unrelated affected patients were evaluated. Five of these patients (BD2 and BS2–BS5) have been previously described at clinical, morphological, biochemical and genetic level; one (BD2) was a compound heterozygous with two in-frame deletions occurring in exon 3 (Leu65) and in exon 15 (Glu606) of *ATP2A1* [12] while in the remaining four patients (BS2–BS5) no pathogenic mutations were detected in the gene (S3, S2, S8 and S5 in Voermans et al. [7]).

Post mortem muscle biopsy was taken from the vastus lateralis of a two months old infant born as “A” part of a Gemini at the 24th gestational week with 700 g birth weight, Apgar 1–4–5, history: respiratory distress syndrome, cerebral hemorrhage, necrotizing enterocolitis, dobutamine therapy because of unstable circulation and no apparent muscle disease.

2.3. Histology and histochemistry

Muscle biopsy of vastus lateralis was performed in six out of seven patients. BS1 underwent an open biopsy of deltoid muscle. Serial 8-μm-thick cryosections were stained with hematoxylin and eosin, modified Gomori trichrome, ATPase (pH 4.3, 4.6 and 10.4), succinate dehydrogenase (SDH), cytochrome c oxidase (COX), nicotinamide adenine dinucleotide-tetrazolium reductase (NADH-TR), Periodic acid Schiff, Sudan black and acid phosphatase.

2.4. Tissue cultures

Primary cultures of normal human muscle were established as routinely performed in our laboratory [15]. Experiments were done on three culture sets which were established from satellite cells derived from a portion of diagnostic muscle biopsy of three different patients who, after all tests had been performed, were considered free of muscle diseases.

For immunofluorescence analysis myoblasts and myotubes, obtained from about 7–10 days after fusion of myoblasts, were fixed in 0.1% paraformaldehyde, 0.01% glutaraldehyde and permeabilized in 0.1% Triton X-100.

2.5. Immunohistochemistry and confocal immunofluorescence microscopy

Immunohistochemistry was performed on (1) 8-μm-thick transverse muscle sections with antibodies against SERCA1 (Santa Cruz Biotechnology, dilution 1:500) and SERCA2 (Santa Cruz Biotechnology, dilution 1:100) and (2) on myoblasts and myotubes grown in vitro with antibodies against SERCA1 (Santa Cruz Biotechnology, dilution 1:100), SERCA2 (Santa Cruz Biotechnology, dilution 1:50) and with a rabbit antiserum against SERCA1b (dilution 1:50) [16].

Briefly, sections and cultured human muscle fibers were blocked with normal goat serum for 60 min at room temperature and then probed overnight at 4 °C with specific antibodies. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI). The reactions were revealed by immunofluorescence methods. Confocal images were acquired with the Leica TCS-SP5 confocal microscope (Leica-Microsystem, Wezlar, Germany) using the same acquisition setting.

Control muscles were biopsies from subjects who were ultimately deemed to be free of muscle diseases. To control staining specificity the primary antibody was omitted or replaced with non-immune sera at the same concentration.

2.6. SDS-PAGE, 2D-PAGE and immunoblot analysis

Frozen muscle sections were homogenized using three different solubilizing solutions. RIPA buffer was 50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% NP-40, 0.1% SDS, 0.5% deoxycholic acid. 2D lysis buffers were prepared with 7 M urea, 2 M thiourea, 40 M Tris-base, 65 mM dithioerythritol using two different detergent compositions. In particular, 2D lysis buffers contained the zwitterionic detergent 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonic acid (CHAPS, 0.4% w/v) and the nonionic detergent Triton X-100 (0.5% v/v) or the more polar zwitterionic detergent ASB-14 (2% w/v). Homogenization of myoblasts and myotubes grown in vitro was performed in RIPA buffer. Protease inhibitor cocktail was added to solubilizing solutions immediately before the experiments. Homogenates were clarified by centrifugation at 1300 *g* for 10 min obtaining total crude extracts and protein concentration was determined with the Bradford method. Samples were separated by SDS-PAGE on 10%T polyacrilamide gel and proteins were transferred to nitrocellulose membranes.

2D-PAGE was performed using the immobiline-polyacrylamide system. The isoelectric focusing (first dimension) was carried out on nonlinear wide-range immobilized pH gradients (pH 3–10; 18 cm long IPG strips). The second dimension was on 10%T polyacrylamide gels at 40 mA/gel and the gels were blotted overnight at 10 V on 0.2 μm nitrocellulose membranes.

For immunoblotting, membranes were blocked with 10% non-fat dried milk for 1 h at RT and then incubated with the specific antibodies (SERCA1, diluted 1:1000; SERCA2, diluted 1:500; rabbit antiserum against SERCA1b, diluted 1:1000 [16]) overnight at 4 °C. Bands and spots were visualized with the ECL Advance Western Blotting Detection Kit (Amersham Pharmacia Biotech, Buckinghamshire, UK). Protein loading was confirmed by Coomassie Brilliant Blue G-250 staining or by immunoblot with actin antibody (Sigma-Aldrich, diluted 1:60000).

SR proteins were isolated from the post mortem infant muscle biopsy, loaded onto SDS-PAGE and analyzed on immunoblot using ECF detection kit (Amersham Pharmacia Biotech) as described in details previously [16].

2.7. Biochemistry

Sarcoplasmic reticulum Ca^{2+}-ATPase activity was measured in whole muscle homogenates as Ca^{2+}-dependent ATPase activity as previously described [12].

2.8. ATP2A1 genomic analysis

In BS1 and BD1 sequence analysis was performed on the full coding region of the *ATP2A1* gene (from exon 1 to exon 23), including 3′ and 5′ intronic boundaries, according to standard procedures of direct sequencing. Utilized primers are available upon request. For Southern blot analysis in BS1, genomic DNA was digested with BamHI, EcoRI and Hind III restriction enzymes and hybridized with a mixed cDNA probes covering exons 1 to 6 and exons 16 to 23. Probes were labeled with 32P by random primer extension and hybridization was carried out according to standard techniques. Real-Time PCR assays for ATP2A1 exon 8 was performed on BS1. Utilized primers and MGB-probes (Applied Biosystems) sequences are as follows: ATP2A1ex8F: 5-′CTG CCA CAG AAC AGG ACA AGA C-3′; ATP2A1ex8R: 5′-TGC TCC CCA AAC TCA TCC A-3′: Probe: 6-FAM-CCC TTG CAG CAG AAG-MGB; DYS43F: 5′-GCA GCA TTG CAA AGT GCA A-3′; DYS43R: 5′-GCT GGG AGA GAG CTT CCT GTA G-3′: Probe: 6-FAM-CCT GTG GAA AGG GTG AA-MGB. Real-Time PCR was performed in triplicate in 96-well plate; each 25 μl reaction consisted of 1× Taqman Master Mix (Applied Biosystems), 300 nM forward and reverse primers, 100 nM Taqman probes and 50 ng genomic DNA. The Real-Time PCR analysis was performed on an Applied Biosystems Prism 7900HT Sequence detection system. The relative quantification of the target sequence (*ATP2A1* exon 8) with respect to the reference sequence (dystrophin exon 43) and normalized to a calibrator (3 control males) was performed by the comparative CT method (ΔΔCt method) (Applied Biosystems User Bulletin #2).

2.9. ATP2A1 RT-PCR analysis on muscle biopsies and cultured human muscle fibers

Total RNA was isolated from muscle biopsy of BS1 and BD1 and from myoblasts and myotubes using the RNeasy Kit (Qiagen) following manufacturer's instructions. RNA was treated with RNase-free Dnase set (Qiagen) and checked for DNA contamination by PCR. Reverse transcription (RT) for cDNA synthesis was performed both on samples and controls (commercial SKM RNA from Roche) with High Capacity cDNA Reverse Transcription kit (Applied Biosystem) according to manufacturer's instructions. In BS1, the entire *ATP2A1* transcript was analyzed by using different couples of oligonucleotides (sequence available upon request). All the PCR fragments were purified by using QIAquick purification kit (QIAGEN) and sequenced on ABI 3130 automated sequencer. For *ATP2A1* isoforms analysis on cultured myoblasts and myotubes, PCR was performed according to standard chemical and cycling parameters using a specific primers pair within exon 20 and 23 respectively (forward: 5′-TGTCCGAGAACCAGTCCCTGC-3′; reverse: 5′-TACAAGGTTGAGAGGGAGGCGGG-3′). Amplification products were analyzed by Agilent 2100 Bioanalyzer with High Sensitivity DNA chip, according to manufacturer's instructions.

3. Results

3.1. Morphological, biochemical and molecular characterization of patients

3.1.1. Light microscopy on frozen sections

Muscle biopsy of BS1 showed increased variation in fiber diameter. Atrophic fibers belonged to both type 1 and 2 and a mild hypertrophy of type 2 fibers was also present. Type 2 fibers represented 53% and type 1 fibers 47% of the total fiber population. Neither necrotic nor regenerating muscle fibers were observed. All other histochemical stainings were normal.

Increased fiber size variability was observed in muscle biopsy from BD1. Prevalence of type 2 fibers (80% of the total fibers), increased number of central nuclei (5–10% of muscle fibers), scattered fibers with areas strongly reacting with NADH-TR staining and fibers with central or peripheral areas avoided of reaction with oxidative stainings were also documented. Neither increase in perimysial nor endomysial connective tissue nor necrotic or regenerating fibers were present.

The histological features of BD2 and BS2–BS5 have been reported in previous publications [7,12].

3.1.2. Immunohistochemistry and confocal immunofluorescence microscopy

In muscle of patients and control subjects SERCA1 and SERCA2 immunoreactivity was observed in type 2 and type 1 fibers, respectively. SERCA1staining was comparable in patients and controls (Fig. 1A). None fiber reacted with both antibodies (Fig. 1B). SERCAs staining of the BD2 and BS2–BS5 has already been reported [7].

3.1.3. SDS-PAGE and immunoblot analysis

In normal and disease muscle biopsies SERCA1 migrated as a band at a molecular weight of approximately 110 kDa (Fig. 2A). SERCA1 migration during SDS-PAGE decreased in samples homogenized in 2D buffers compared to samples lysates in RIPA buffer (Fig. 2B). Immunoblot analysis showed that the level of SERCA1 was markedly reduced in patients with *ATP2A1* mutation compared to normal controls and patients without *ATP2A1* mutation (Fig. 2A). In particular by using muscle tissue lysates in RIPA and 2D buffers the intensity of SERCA1 band detected in control subjects and in patients without *ATP2A1* mutation after loading 2 μg of total proteins was similar to that detected in muscle of patients

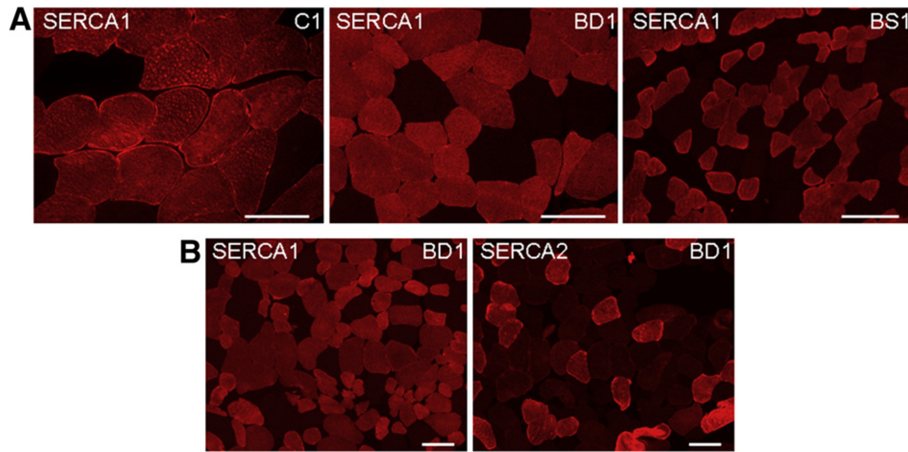

Fig. 1. Immunohistochemistry for SERCA1 and SERCA2 on muscle sections. (A) SERCA1 immunostaining is comparable in control (C1) and in patients with (BD1) and without (BS1) *ATP2A1* mutation. (B) Serial sections from muscle biopsy specimen of patient with *ATP2A1* mutation (BD1) show that SERCA1 and SERCA2 antibodies stain type 2 and type 1 fibers, respectively. Bars, 100 μm.

with *ATP2A1* mutation after loading an amount of proteins five times greater (Fig. 2B). In all analyzed muscles SERCA1 recovery was higher by using muscle tissue lysates in RIPA buffer instead of 2D buffers (Fig. 2B). Otherwise the 2D buffer was more effective for SERCA1 extraction when supplemented with ASB-14 than with CHAPS and Triton X-100 (Fig. 2B).

SERCA2 was detected as a band of nearly 110 kDa whose intensity was similar in muscle of patients with and without *ATP2A1* mutation and of controls subjects (Fig. 2A).

3.1.4. 2D-PAGE and immunoblot analysis

SERCA1 immunoblotting after 2D-PAGE of total protein extracts showed a spot around pI 5.0 with apparent molecular weight of 110 kDa in muscle of controls and of patients with and without *ATP2A1* mutation. The total amount of labeled SERCA1 was significantly reduced in patients with *ATP2A1* mutation compared to control subjects and patients without *ATP2A1* mutation (Fig. 3).

SERCA2 revealed a single spot at the isoelectric point around pH 5.0 and molecular mass approximately 110 kDa. Analysis of spot intensity showed that the level of SERCA2 was similar in controls and patients with and without *ATP2A1* mutation (Fig. 3).

3.1.5. Biochemistry

The activity of SR Ca^{2+}-ATPase was 35.2 mU/mg proteins in the muscle from BS1. This activity was reduced to about 40% as

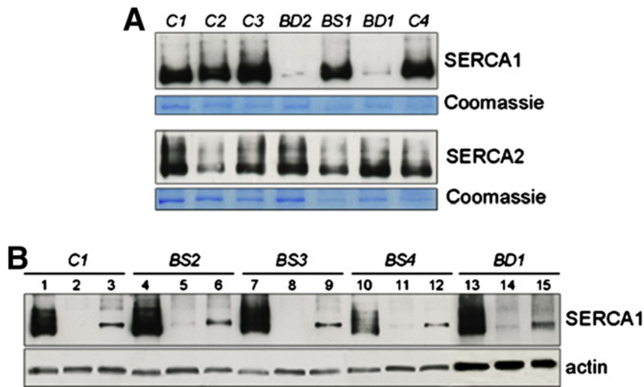

Fig. 2. Immunoblot of SERCA1 and SERCA2 on muscle protein extracts after SDS-PAGE. (A) SERCA1 migrates as a band at a molecular weight of approximately 110 kDa. The intensity of protein band is strongly decreased in both patients with *ATP2A1* mutation (BD1 and BD2) compared to controls (C1–C4) and patient without *ATP2A1* mutation (BS1). Muscle tissue lysates in RIPA buffer (1 μg of total proteins). SERCA2 is detected as a band of nearly 110 kDa whose intensity was similar in muscle of controls (C1–C4) and of patients with (BD1 and BD2) and without (BS1) *ATP2A1* mutation. Muscle tissue lysates in RIPA buffer (2 μg of total proteins). (B) SERCA1 migration during SDS-PAGE decreases in samples homogenized in 2D buffers compared to samples lysates in RIPA buffer. In muscle of control (C1) and of patients with (BD1) and without (BS2–BS4) *ATP2A1* mutation, SERCA1 recovery is higher by using RIPA buffer instead of 2D buffers and in 2D lysis buffer with ASB-14 instead of 2D lysis buffer with CHAPS and Triton X-100. Lanes 1, 4, 7, 10 and 13: RIPA buffer; lanes 2, 5, 8, 11 and 14: 2D buffer with CHAPS and Triton-X100; lanes 3, 6, 9, 12 and 15: 2D buffer with ASB-14. Loading: 2 μg from lanes 1 to 12; 10 μg from lanes 13 to 15.

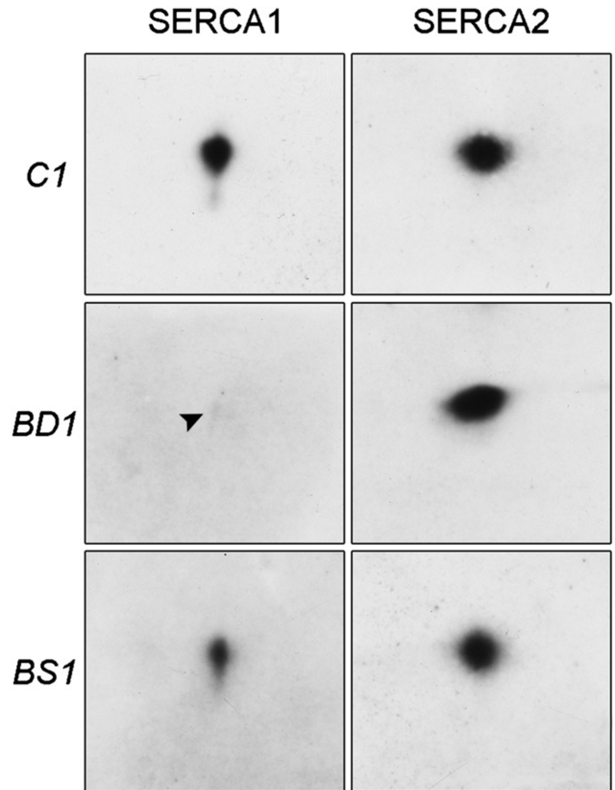

Fig. 3. Immunoblot of SERCA1 and SERCA2 on muscle protein extracts after 2D-PAGE. Both SERCA1 and SERCA2 are detected as a spot around pI 5.0 and ~110 kDa in control (C1) and in patient without *ATP2A1* mutation (BS1). SERCA1 spot is barely detectable (arrowhead) in muscle of patient with *ATP2A1* mutation (BD1).

compared with the mean of age-matched controls. The protein content was142 mg (g wet weight)$^{-1}$ and therefore comparable with controls (150 ± 35 mg (g wet weight)$^{-1}$).

The compound SERCA activity of BD2 and BS2–BS5 has been previously reported while SR Ca^{2+}-ATPase activity was not determined in the muscle from BD1 because the left material was not in sufficient amount [7,12].

3.1.6. Molecular characterization

3.1.6.1. BS1. An extensive genomic and RNA analysis of *ATP2A1* gene was performed in BS1 and no mutations were identified. Sequence analysis of the whole *ATP2A1* coding region and intronic boundaries failed to identify any pathogenic change. A previously reported C/T polymorphic variation in exon 8 (nt. 20.025, accession number: AC109460) was detected in homozygosis [4].

In order to investigate the presence of gross rearrangements, Southern blot analysis was performed. No abnormal restriction fragments, suggesting a partial deletion of the *ATP2A1* gene, were identified in the patient compared to a control subject. Furthermore, the occurrence of a gross deletion event was excluded by a semiquantitative approach using Real-Time PCR. By using the comparative ΔΔCt method the copy number of *ATP2A1* exon 8 was analyzed and normalized to three control samples. This analysis excluded the presence in the proband of single copy of *ATP2A1* exon 8. Finally, the occurrence of deep intronic mutations (not investigated at the genomic level) affecting *ATP2A1* RNA processing was excluded by transcription analysis on RNA from patient's muscle biopsy sample. No abnormal PCR fragments were detectable, suggesting splicing abnormalities.

3.1.6.2. BD1. Sequence analysis of the *ATP2A1* gene coding region in BD1 allowed to identify a compound heterozygous genotype for two novel *ATP2A1* mutations, both located within exon 3 (accession Number NM_004320: c.T200G – p.Leu67Arg; c.178delC – p.Leu59Ser fs*37) (Fig. 4). RNA analysis on muscular biopsy showed monoallelic expression of the *ATP2A1* messenger, with pseudo-homozygosity for the Leu67Arg mutation (Fig. 4). This behavior suggests the occurrence of non-sense mediated decay of the transcript containing the frameshift mutation.

3.2. ATP2A1 RNA and protein isoforms analysis on cultured human muscle fibers and infant muscle

SERCA1 transcript undergoes alternative splicing, giving rise to the adult isoform SERCA1a (including exon 22) and to the neonatal isoform SERCA1b (lacking exon 22) adult skeletal muscle only expressed the adult SERCA1a isoform (Fig. 5). Differently, an almost exclusive expression of the neonatal isoform SERCA1b was detectable both in myoblasts and myotubes (Fig. 5).

Immunostaining for SERCA1, SERCA1b and SERCA2 was observed in myoblasts and myotubes grown in vitro (Fig. 6).

By immunoblotting after SDS-PAGE SERCA1b and SERCA2 displayed a band of about 110 kDa in both myoblasts and myotubes (Fig. 7). SERCA2 protein expression was slightly increased in myotubes as

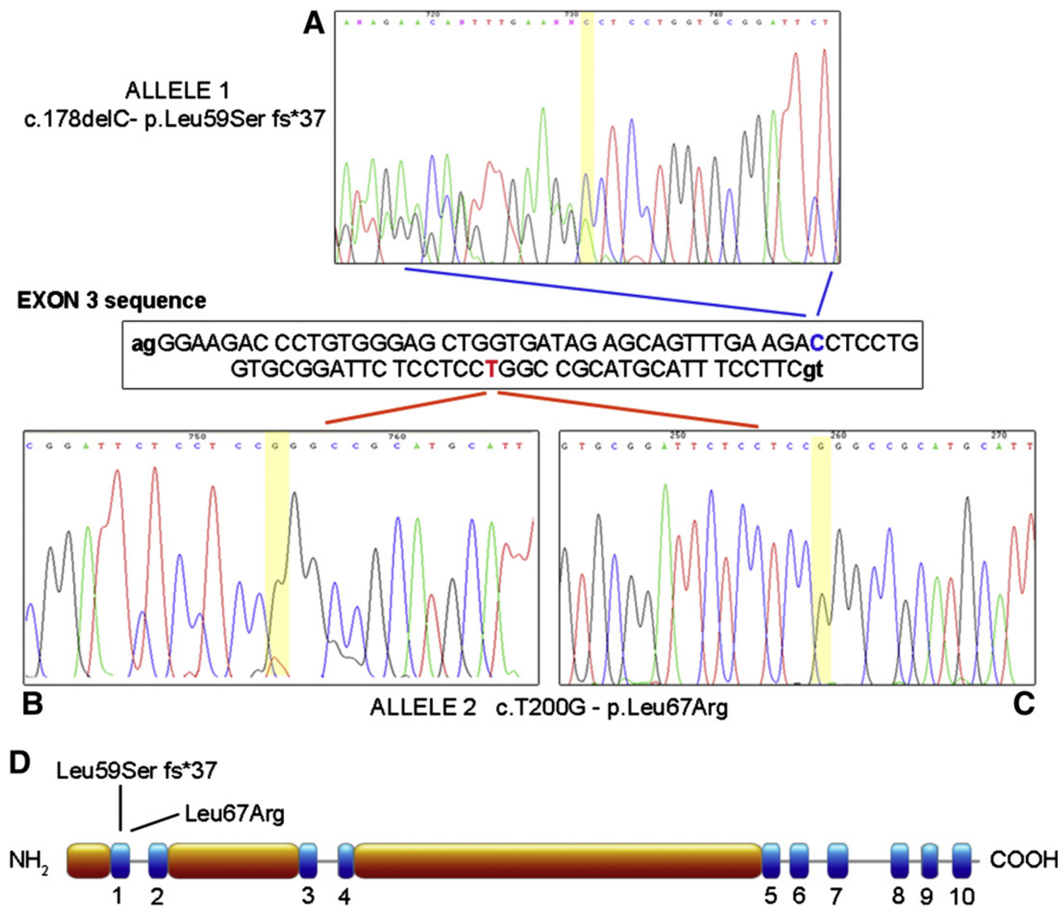

Fig. 4. Schematic representation of *ATP2A1* compound heterozygous genotype identified in BD1. (A) Allele 1 harbors the heterozygous c.178delC (p.Leu59Ser fs*37) frame shift mutation. Allele 2 shows at genomic level (B) the heterozygous c.T200G mutation (p.Leu67Arg) which is at pseudo-homozygous at cDNA level (C). The *ATP2A1* transcription is monoallelic. (D) Schematic representation of SERCA1 protein. The blue boxes represent SERCA1 transmembrane helices which are numbered from 1 to 10 and red boxes indicate the cytosolic regions of the protein including the actuator domain, the phosphorylation domain and the nucleotide-binding domain. The mutations identified in BD1 are located in the transmembrane helix 1.

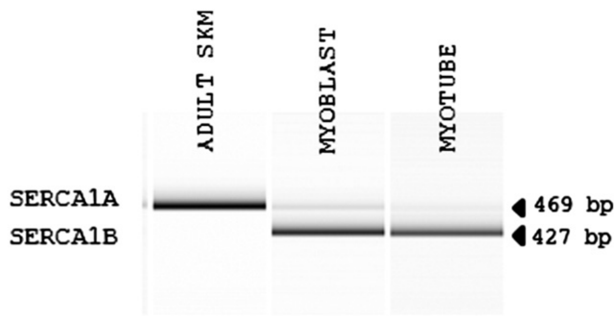

Fig. 5. *ATP2A1* RNA isoforms analysis on primary human muscle cultures. Adult skeletal muscle (lane 1) shows a 469 bp band corresponding to SERCA1a isoform. Myoblasts and myotubes (lane 2 and lane 3) present an almost exclusive expression of SERCA1b (427 bp).

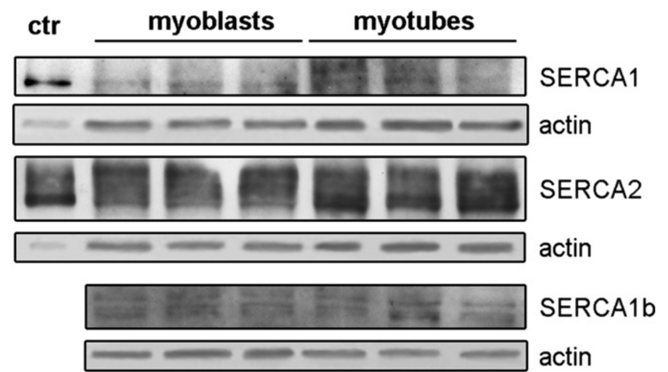

Fig. 7. Immunoblot of SERCA1, SERCA1b and SERCA2 on primary human muscle cultures. SERCA1, SERCA1b and SERCA2 display a band of about 110 kDa in both myoblasts and myotubes. SERCA2 protein expression is slightly increased at the stage of myotubes. Normal skeletal muscle is loaded as control (ctr).

compared to myoblasts (Fig. 7). A faint band for SERCA1 was observed in human cultured muscle fibers (Fig. 7).

Immunoblot with increased loading of SR fraction from infant vastus lateralis muscle showed a linear increase of band intensity corresponding to SERCA1b and the total amount of SERCA1. Regression analysis revealed no difference ($p < 0.01$) between the linearity of changes in band intensities detected by SERCA1b or pan SERCA1 antibody suggesting that SERCA1b is the exclusive SERCA1 isoform at that age in this type of muscle (Fig. 8).

4. Discussion

We report the clinical, morphological and molecular features of a male patient affected by BS (BS1). In our patient the age of disease onset was in childhood and the clinical picture consisted of exercise-induced delay in muscle relaxation and of muscle cramping. Light microscopic changes included only fiber size variation and the compound SERCA activity, measured on whole muscle homogenates, resulted significantly decreased with the residual activity of 60% in patient's muscle. The sequence analysis of *ATP2A1* from genomic DNA of patient did not detect any mutation in the 23 exons and in the intronic sequence flanking each exon. Intronic mutations and deletion/duplication events in the *ATP2A1* gene have also been excluded. Our extensive molecular analysis excluded that *ATP2A1* is the causative gene for the disease in this patient which therefore belongs, according to the classification proposed by MacLennan, to the class of BS. Voermans and collaborators suggested that patients with BD present clinical features different from patients affected by BS; the former usually have the age of onset in childhood, delayed muscle relaxation after repetitive contraction and a generalized muscle involvement while the latter more often complain of myalgia and experience a considerable impact on daily life [7]. However clinical findings in our patient were similar to those described for the phenotype of BD.

We also conducted a search for mutations in the *ATP2A1* gene from a patient (BD1) which has been clinically described previously [14]. The patient resulted compound heterozygote for two previously unreported mutations within exon 3; one was a missense variation which leads to the substitution of a leucine with an arginine at position 67 of the protein and the other a deletion in codon 56 leading to the premature

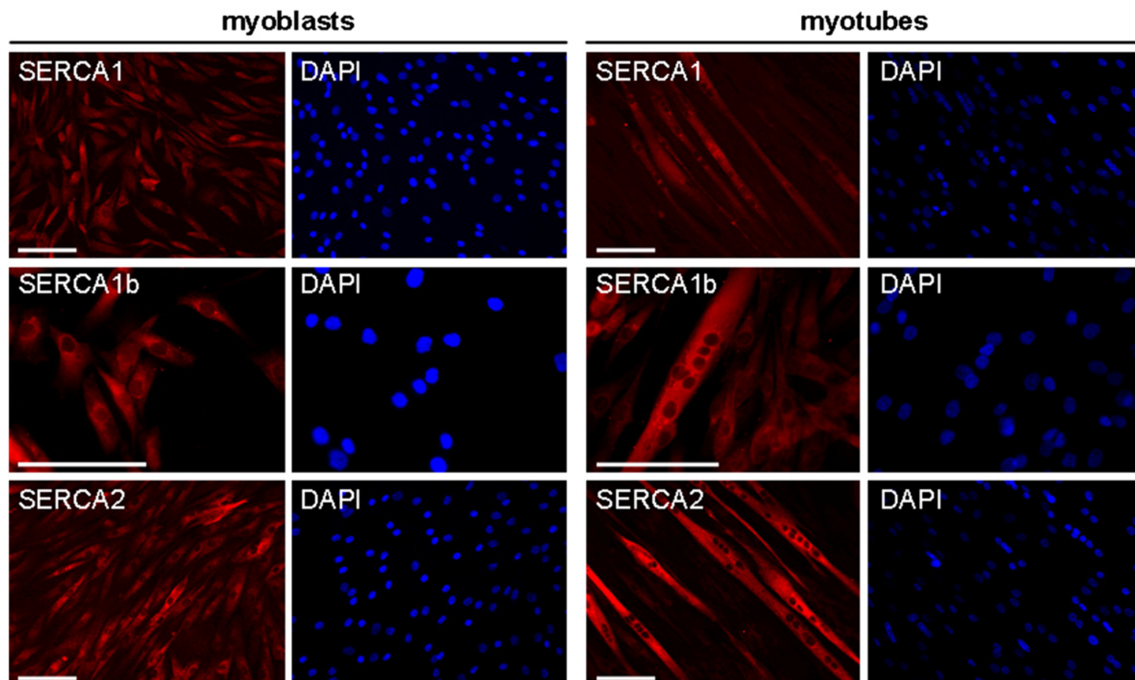

Fig. 6. Immunohistochemistry for SERCA1, SERCA1b and SERCA2 on primary human muscle cultures. SERCA1, SERCA1b and SERCA2 staining is detected in myoblasts and myotubes grown in vitro. Nuclei contrastained with DAPI. Bars, 100 μm.

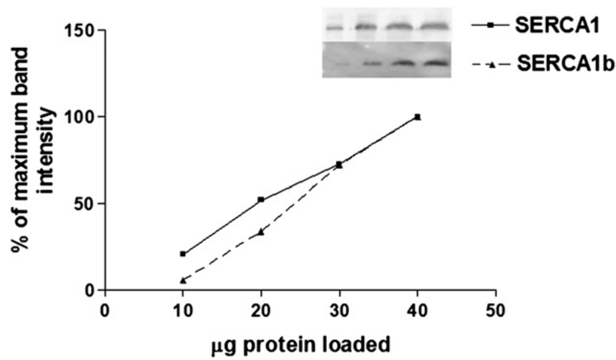

Fig. 8. Linearity of SERCA1 and SERCA1b on the same immunoblot of infant vastus lateralis muscle. Note the largely similar increase of band intensities showing that SERCA1b practically accounts for the total of SERCA1 in this infant muscle.

formation of a stop codon. The transcriptional analysis predicted that the protein is produced only from the allele with the missense mutation. The Leu67 is located in transmembrane helix M1 which has a fundamental role in the formation of the occluded state of the enzyme [17,18]. Indeed, M1 helix assumes different positions in all of the four states of the enzyme catalytic cycle and its conformational changes participate in the regulation of the access to the cytoplasmic or to the luminal gates of the ion pathway [19]. The alteration of M1 hydrophobicity, as a consequence of the substitution at position 67 of the aliphatic amino acid leucine with the hydrophilic residue arginine, could have a negative influence on the structural stability of M1, since the highly hydrophobic environment in which the helix resides, and could interfere with the ability of M1 to undergo conformational changes during the catalytic cycle.

In this study, we performed a detailed analysis of SERCA1 protein expression in muscle biopsy specimens of seven patients by using immunohistochemical and immunoblotting analysis. We observed SERCA1 immunoreactivity in type 2 muscle fibers of patients with and without *ATP2A1* mutations. The staining pattern and intensity of SERCA1 were similar in muscle of controls and of patients with and without *ATP2A1* mutation. However, immunoblot analysis after SDS-PAGE and 2D-PAGE showed a significant reduction of SERCA1 protein in muscle of patients with *ATP2A1* mutation as compared to muscle of control subjects and of patients without gene mutation.

We assumed that the discrepancy between the immunohistochemical data and the immunoblotting findings could be due to the lower recovery of mutated SERCA1 during total proteins extraction. Differences in SERCA1 recovery from muscle tissue could be a consequence of the different solubility features of *wild type* protein compared to the mutated SERCA1. Therefore we evaluated the solubility of the protein using three different solubilizing solutions including the RIPA buffer and the 2D buffers with a different detergent composition. First of all we observed that SERCA1 migrated at faster rate in RIPA buffer rather than in 2D buffers during electrophoretic separation; the difference in protein migration could be due to the presence of the reducing agent DTT in 2D buffers [20]. In addition, we found that the extraction yield of SERCA1 protein was variable when different lysis buffers were used for the muscle homogenization. We documented that SERCA1 recovery from muscle of controls and of patients regardless from their association with *ATP2A1* mutation was strongly higher with RIPA buffer as compared to 2D buffers and among these, the one with the zwitterionic detergent ASB-14 turned out to be better than the other containing Triton X-100 and CHAPS, a non-ionic and a zwitterionic detergent, respectively. Despite RIPA buffer was better than 2D buffers for solubilization of both *wild type* and mutated SERCA1, the behavior of the *wild type* and mutated protein was similar within RIPA and 2D buffers. In particular, when loading of total proteins in patients with *ATP2A1* mutation was five times greater than that in controls and patients without *ATP2A1* mutation, the intensity of SERCA1 band was similar in patients and controls, regardless of the lysis buffer used for muscle homogenization. These results make unlikely that the mutated and *wild type* SERCA1 exhibit different solubility properties at least with the three solubilizing solutions.

Despite differences in protein expression are more readily observed by immunoblotting, the striking difference between our immunohistochemical data and immunoblot findings can be only partially explained by the lower sensitivity of immunohistochemistry.

The reduction of mutated SERCA1 highlighted by our immunoblotting data may be useful for the diagnosis of patients with reduced SERCA activity and *ATP2A1* mutation. BD is considered a rare disease with an incidence of 1 in 10,000,000 new births; nevertheless the frequency could be underestimated [1]. Indeed the clinical diagnosis is quite difficult because symptoms are not specific and may be ascribed to other muscle disorders. Furthermore, the heterogeneity of clinical findings between patients makes the disease difficult to be recognized. A decreased sarcoplasmic reticulum Ca^{2+} ATPase activity has been reported in all patients regardless of the association with *ATP2A1* mutation. However, reduced SERCA activity has been observed in muscle of patients with myotonic dystrophy type 1 and can be related to other non-muscle pathological conditions including hypothyroidism [21,22]. Therefore, up to now, only mutation analysis of *ATP2A1* allows a definite diagnosis of BD. Our study demonstrated that immunohistochemistry is not a reliable technique for the diagnosis of the disease while provided evidence that immunoblot analysis could be a valuable tool. Indeed SERCA1 immunoblotting allows us to identify patients with mutation of *ATP2A1* gene using a small amount of total proteins from muscle tissue lysates. Therefore we propose an additional criterion, namely the reduced expression of SERCA1 protein in patients with *ATP2A1* mutation, for the biochemical distinction of patients with Brody disease from patients with Brody syndrome.

In line with the considerably reduction of SERCA activity, patients are able to relax their fast-twitch skeletal muscles only at a reduced rate. An unsolved question is which mechanisms could participate to the lowering of cytoplasmic Ca^{2+} concentration in these patients. The detection of SERCA2 immunoreactivity in type 1 muscle fibers of all patients regardless of the association with *ATP2A1* mutations and the absence of hybrid fibers co-expressing both SERCAs exclude the ectopic compensatory expression of SERCA2 which accordingly does not take part to relaxation of the fast-twitch skeletal muscle fibers in these patients. Moreover immunoblotting for SERCA2 revealed no remarkable differences between muscle of controls and of patients, further confirming the immunohistochemical data.

Finally, we investigated whether SERCA1splicing variants are present in primary cultures of normal human muscle and in infant muscle. In mammals, SERCA1 is alternative spliced in two major variants, SERCA1a which represents the adult form and SERCA1b which is the neonatal form. The difference between these two isoforms results from the alternative splicing of exon 22 and consequently affects the C-terminal region of the proteins. SERCA1a transcript contains exon 22 and encodes 994 amino acids, while SERCA1b encodes 1001 amino acids because of exon 22 skipping that results in a frameshift and use of a downstream termination codon [23]. The SERCA1a is expressed in fast-twitch fibers while the SERCA1b is found in neonatal and developing skeletal muscle [16,23]. Using a specific antiserum raised against the C-terminal high charged octapetide (DPEDERRK) of SERCA1b, we observed a cytoplasmic staining for SERCA1b isoform in cultured human muscle fibers at both non confluent myoblast and full differentiated myotube stages. Immunoblot analysis confirmed the presence of the neonatal form in both myoblast and myotube protein extracts. We also studied muscle biopsy of newborn infant for the dominant SERCA1 isoform. Linear regression of the band intensities revealed that the amount of SERCA1 is practically identical with SERCA1b in the SR fraction. Therefore these findings clearly documented that SERCA1b is the main SERCA1 isoform expressed in cultured human muscle fibers and infant muscle. However it will be important for future studies to clarify functional differences between

SERCA1a and 1b and mechanisms involved in the regulation of SERCA1 isoforms expression in view of using cultured human muscle fibers to study the disease.

In conclusion, the principal new findings from the present study are: (a) the demonstration that SERCA1 expression is reduced in patients with BD but not in BS patients; (b) the proof that immunohistochemistry is not a reliable technique for the diagnosis of BD while immunoblot analysis could be a valuable tool allowing the identification of patients with *ATP2A1* gene mutation; (c) the evidence that SERCA1b is the main SERCA1 isoform expressed in primary cultures of normal human muscle at both non confluent myoblast and myotube stage; (d) the identification of two novel mutations in the *ATP2A1* gene from a previously reported patient with BD; and (e) the clinical, morphological and biochemical data of a new patient with BS.

Conflict of interest

There is no conflict of interest.

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