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ORIGINAL PAPER

Samaritan myopathy, an ultimately benign congenital myopathy, is caused by a RYR1 mutation

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Abstract Congenital myopathies describe a group of inherited muscle disorders with neonatal or infantile onset typically associated with muscle weakness, respiratory involvement and delayed motor milestones. We previously reported a novel congenital myopathy in an inbred Samaritan family. All patients displayed severe neonatal hypotonia and respiratory distress, and unlike other congenital myopathies, a constantly improving health status. As clinical and pathological data did not point to preferential candidate genes, we performed exome sequencing complemented by linkage analysis to identify the mutation causing the benign Samaritan congenital myopathy. We identified the homozygous p.Tyr1088Cys mutation in *RYR1*, encoding the skeletal muscle ryanodine receptor. This sarcoplasmic reticulum calcium channel is a key regulator of excitation–contraction coupling (ECC). Western blot and immunohistofluorescence revealed a significant decrease of the RYR1 protein level and an abnormal organization of skeletal muscle triad markers as caveolin-3, dysferlin and amphiphysin 2. *RYR1* mutations are associated with different myopathies and malignant hyperthermia susceptibility. The index patient had mild hyperthermia following anesthesia, indicating that the inbred Samaritan population might be a risk group for this disorder. Our results suggest an aberrant ECC as the primary cause of this disease, and broaden the clinical consequences of RYR1 defects.

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Introduction

Congenital myopathies are clinically and genetically heterogeneous and vary in symptoms, complications and treatment options [11, 18]. They are usually associated with neonatal or childhood onset, progressive or non-progressive muscle weakness, breathing difficulties and delayed motor milestones. Congenital myopathies are characterized by the predominance of particular histological anomalies on muscle biopsy as central nuclei, nemaline rods, vacuoles or central cores. However, clinical and morphological features sometimes overlap and can involve genetic or phenotypic heterogeneity.

We previously reported a novel myopathy found in the Samaritan population and named it benign Samaritan congenital myopathy [6]. In contrast to other congenital myopathies, where muscle weakness is progressive or stable, the benign Samaritan congenital myopathy is characterized by an "inverse" course of disease with patients severely affected at birth, progressively improving and minimally affected at adult stage. The Samaritans are an ethno-religious group of 700 people living in Israel. Ancestrally, they consider themselves as descendants of the 10 lost tribes that formed the Kingdom of Israel. The Samaritan population has the highest recorded inbreeding coefficient in human populations due to almost exclusive intra-lineage marriages. As a result of the small allele pool, recessive disorders as Usher syndrome and hereditary spastic paraplegia appeared in several generations [2]. There is no ancestral history for the muscle phenotype, which segregates as a pseudo-dominant trait.

In the present study, we specify the clinical and histological phenotypes of four patients with initially severe, but ultimately benign congenital myopathy and we characterize the molecular cause of the benign Samaritan congenital myopathy. By combining linkage analysis and exome sequencing, we identified a novel ryanodine receptor mutation involving a significant decrease of the protein level, and revealed an abnormal skeletal muscle triad organization on muscle biopsies. Our data strongly suggest an aberrant excitation–contraction coupling (ECC) as the primary cause of this disease.

Patients and methods

Patients

Patients were from the Samaritan population living close to Nablus and Tel-Aviv in Israel. Informed consent was provided according to the declaration of Helsinki. Genomic DNA was prepared from peripheral blood and quality-controlled for high-throughput sequencing.

Homozygosity-by-descent

The genomic DNA of patients LR53 and LR54 was hybridized on Affymetrix 250K SNP arrays according to the manufacturer's instructions. Loss of heterozygosity was analyzed with GeneChip DNA Analysis and Chromosome Copy Number Analysis software (Affymetrix).

Targeted massively parallel sequencing and bioinformatic analysis

The exomes of patients LR53 and LR54 were targeted for enrichment using the Agilent SureSelect Array v1. Oligonucleotides covered all coding exons and intron–exon boundaries corresponding to the NCBI Consensus CDS database (CCDS) and represented 38 Mb. Genomic DNA was sheared using the Covaris E210 (KBioscience, Herts, UK) followed by automatic library preparation with the SPRI-TE machine. Capture was ensured by the Agilent SureSelect custom target enrichment kit (Agilent Technologies, Santa Clara, CA). Enriched DNA fragments were sequenced on an Illumina Genome Analyzer IIX to generate 72 nt paired-end reads. Sequence data were analyzed using Illumina Pipeline RTA (Real-Time Analysis) version 1.7 and aligned to the reference genome GRCh37/hg19 using BWA [7]. Variant calling and filtering of reads sharing the same start position and strand was done with SAMtools [8]. For SNP/indel annotation and filtering SVA, Ensembl60 and dbSNP131 and 1,000 genomes were used. The identified *RYR1* mutation was numbered according to GenBank NM_000540.2 and NP_000531.2. Nucleotide position reflects cDNA numbering with +1 corresponding to the A of the ATG translation initiation codon.

Western blot, immunohistofluorescence and histology

Whole protein extraction from the deltoid biopsy, Western blot, and protein quantification was performed using routine methods. For immunohistofluorescence, primary antibodies for RYR1 (homemade; 1/10,000 for WB, 1/1,000 for IF), dihydropyridine receptor (DHPR, Chemicon, Billerica, USA; 1/600), caveolin-3 (Santa Cruz Biotechnology, Santa Cruz, USA; 1/200), Trisk51 (homemade; 1/10,000 for WB, 1/1,000 for IF), amphiphysin 2 (BIN1, homemade; 1/400) and dysferlin (Novocastra, Newcastle, UK; 1/25) and secondary antibodies (Invitrogen, Carlsbad, USA; 1/1,000) were used following the manufacturer's instructions. For histochemical analyses, transverse sections (10 μm) were stained with hematoxilin-eosin, Gomori trichrome, NADH-TR and ATPase and

assessed for nuclei position, fiber morphology, fiber type distribution, cores and accumulation/infiltrations.

RNA

RNA was extracted from muscle biopsies by routine procedures and reverse transcribed using the SuperScript® III kit (Invitrogen, Carlsbad, USA).

Results

Case reports

The clinical presentations of the index patient LR52 and the two children LR53 and LR54 have been described previously [6] and are specified in supplemental Table 1. All were severely hypotonic at birth requiring ventilation. Hypotonia and weakness gradually improved and the oldest patient is now only minimally affected. Since, a third child (LR55, Fig. 1a) was born and presented as the other affected family members with a low Apgar score, severe neonatal hypotonia, a thick and doughy skin and lax hands. For all patients, motor milestones were delayed, but hypotonia improved after a few weeks. LR56 and LR57 are healthy children of the index patient.

Histological characterization

A muscle biopsy was available for the index patient at the age of 23 years and revealed abnormal nuclei positioning in 25.8 % of the fibers ($n > 300$), comprising internalized (16.4 %) and central (9.4 %) nuclei, central areas devoid of oxidative enzyme activity in 9.5 % of the fibers, and moth-eaten appearance (Fig. 1b). Fiber size variability, atrophy, fiber type predominance or radial arrangements of sarcoplasmic strands were not noted.

Homozygosity-by-descent and exome sequencing

The low percentage of central compared to internal nuclei and the lack of fiber size alteration and predominance of type 1 fibers were not in favor of centronuclear myopathy [5]. In addition, the clinical presentation of the patients differed from classical congenital myopathies and did therefore not point to obvious candidate genes. Assuming recessive inheritance of the disorder due to the high consanguinity in the Samaritan population, we hybridized the genomic DNA of patients LR53 and LR54 on Affymetrix 250K SNP arrays and we identified 16 homozygous regions larger than 1 Mb, encompassing a total of 1,553 genes (supplemental table 2). We then sequenced the exomes of LR53 and LR54. By comparing both patients, we found 40 common SNVs within the regions determined by linkage analysis (supplemental table 3). We lastly ranked the remaining variants based on the type of mutation and the known implication of the genes in muscle functions.

A novel pathogenic *RYR1* mutation as the genetic cause of the benign Samaritan congenital myopathy

We identified a homozygous c.3263A>G (p.Tyr1088Cys) missense mutation in the *RYR1* gene, encoding the ryanodine receptor calcium channel. The presence of the mutation was verified by sequencing of the cDNA obtained by RNA extraction from the biopsy. In total, DNA samples from 11 members of the Samaritan family were available. The *RYR1* c.3263A>G mutation segregates with the disease and was tracked over four generations. All affected family members were homozygous and all obligate carriers with available DNA were healthy and heterozygous for the mutation (Fig. 1a). The mutation is not noted in the SNP databases listed in the web resources.

Decreased RYR1 protein level and defects of the skeletal muscle triad

RYR1 codes for the skeletal muscle ryanodine receptor implicated in ECC at the skeletal muscle triad. To assess the impact of the p.Tyr1088Cys mutation, whole protein extracts of the LR52 biopsy were analyzed by Western blot and revealed a significant RYR1 decrease relative to GAPDH and Trisk51 (Fig. 2a), a triadin isoform associated to RYR1 in the sarcoplasmic reticulum. Decreased RYR1 protein levels as seen in our patient were also reported for central core disease (CCD) patients [23].

We next analyzed markers of the skeletal muscle triad, harboring the excitation–contraction machinery (ECC), by immunohistofluorescence. Trisk51 is a marker of the sarcoplasmic reticulum and dihydropyridine receptor (DHPR), amphiphysin 2 (BIN1), caveolin-3 (CAV3) and dysferlin (DYSF) are markers of the mature or regenerating T-tubule (Fig. 2b and supplemental figure). In contrast to the homogeneous RYR1 distribution in the age-matched control, the Samaritan biopsy revealed locally restricted abnormal accumulations or weak staining in some fibers. In addition, accumulations and co-localization of RYR1, DHPR, CAV3, BIN1 and DYSF were noted within the myofibers and often around internalized nuclei. This was not seen in the control biopsy, demonstrating a defective organization of the skeletal muscle triad in the Samaritan patient.

Discussion

By combining homozygosity-by-descent and exome sequencing, we identified the pathogenic mutation causing

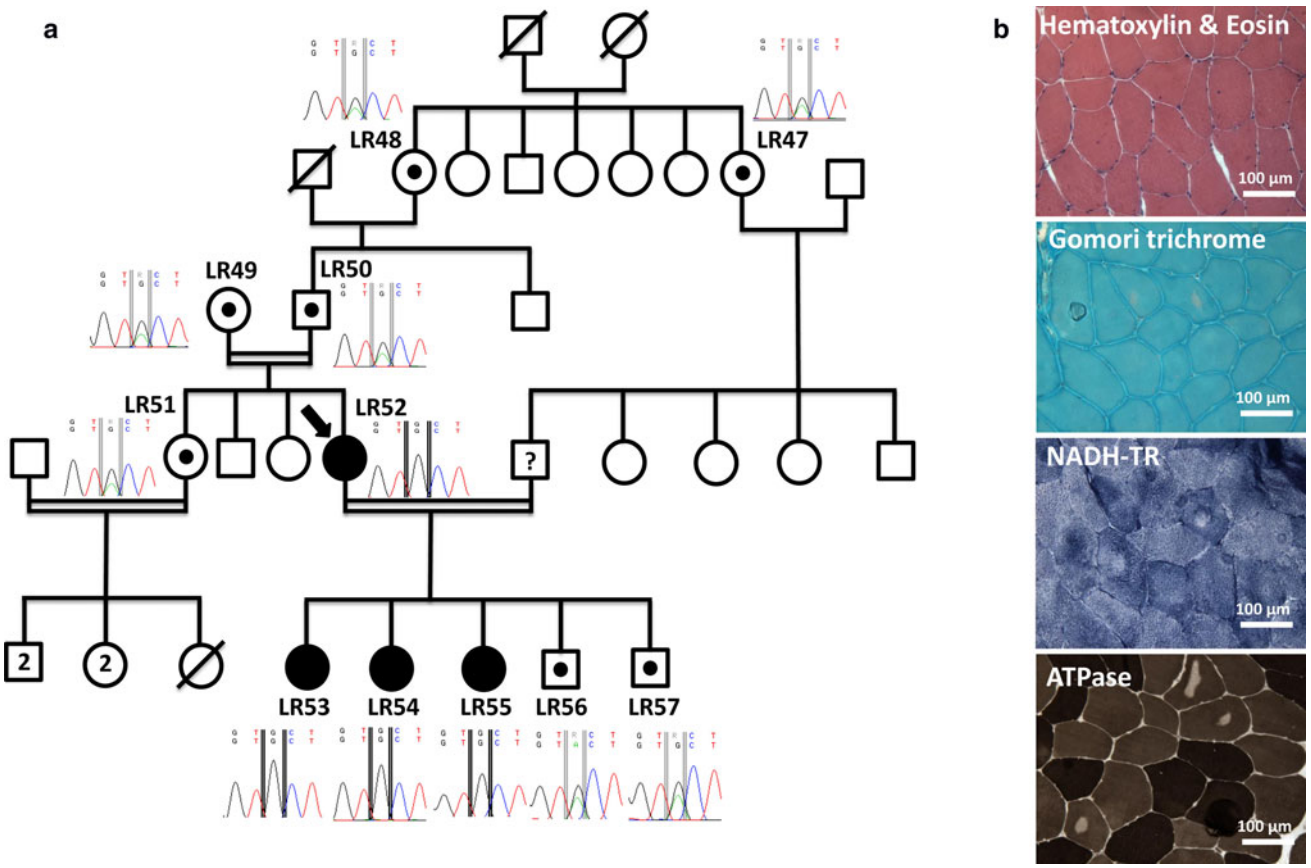


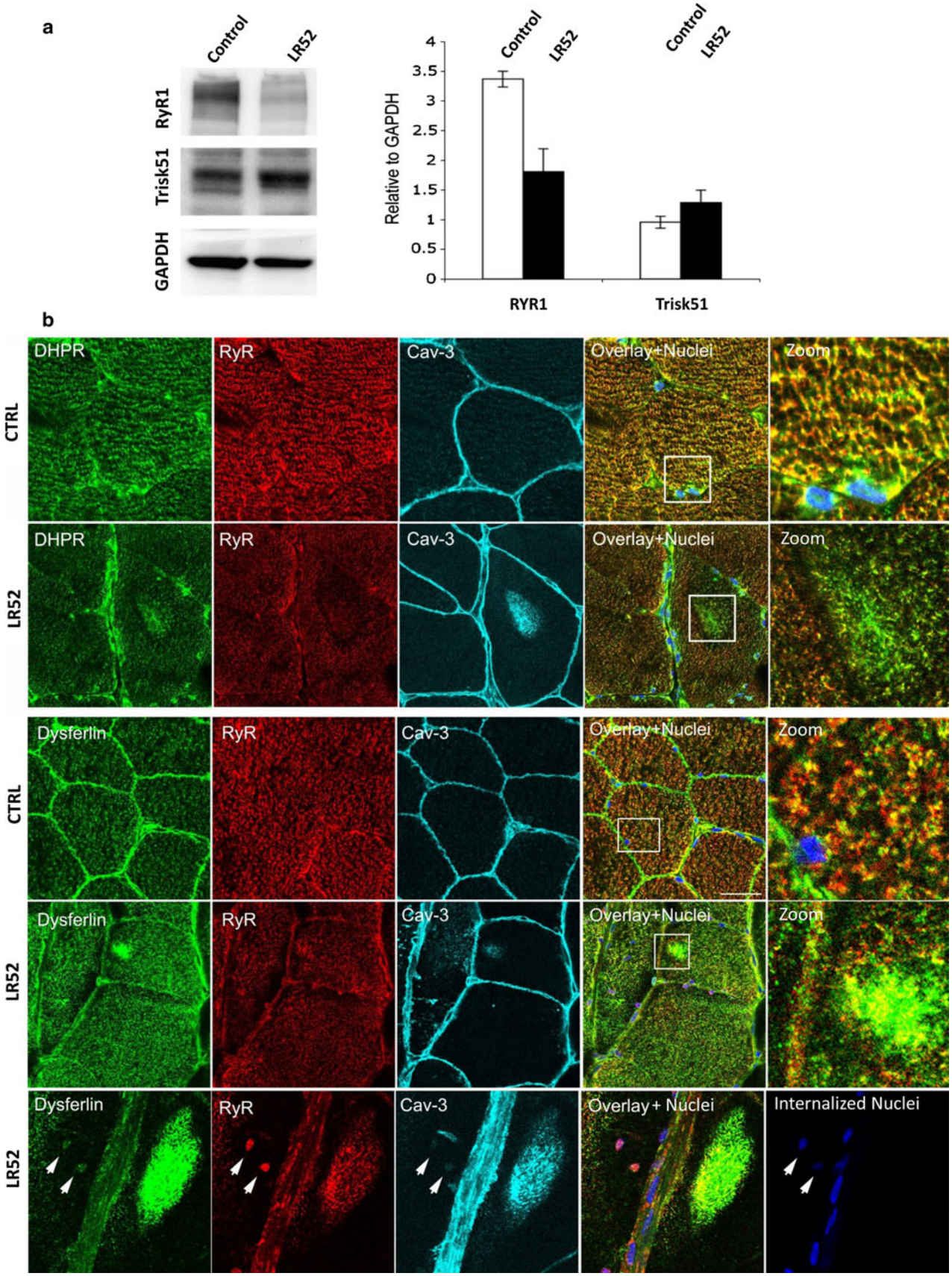
Fig. 1 Pedigree, segregation of the *RYR1* c.3263A>G mutation and histological analysis of a deltoid muscle biopsy of the index patient. **a** The muscle disorder segregates as a pseudo-dominant trait. All four affected family members (LR52, LR53, LR54 and LR55) were homozygous for the *RYR1* exon 25 mutation and all obligate carriers with available DNA were heterozygous. **b** Histological analysis revealed moderate abnormal nuclei positioning by haematoxylin and eosin staining and core-like structures uniquely in type I fibers on Gomori trichrome, NADH-TR and ATPase staining

the benign Samaritan congenital myopathy. The homozygous *RYR1* p.Tyr1088Cys mutation is predicted to alter the physicochemical properties of a conserved residue and is not listed in the SNP databases. Abnormal accumulations of excitation–contraction machinery proteins within the myofibers strongly suggest that the Samaritan *RYR1* mutation leads to a defective organization of the skeletal muscle triad.

RYR1 codes for the skeletal muscle ryanodine receptor, which forms a homotetrameric structure on the sarcoplasmic reticulum and acts as a calcium release channel. RYR1 is regulated by the T-tubule channel DHPR and plays a central role in excitation–contraction coupling [12, 19, 24]. *RYR1* mutations have been implicated in different neuromuscular phenotypes as CCD [13, 22], multiminicore disease (MmD) [10], centronuclear myopathy-like [1, 20], congenital fiber-type disproportion (CFTD) [3], fetal akinesia [15] and malignant hyperthermia susceptibility (MH) [4]. The vast majority of the *RYR1* mutations are associated with CCD and MH, their functional impact is however only partially understood. It is supposed that MH-related *RYR1* mutations result in a hypersensitive calcium channel, while CCD-related *RYR1* mutations rather involve channel hyposensitivity [16].

The benign Samaritan congenital myopathy is considered as a distinct muscle disorder due to its "inverse" course with patients severely affected at birth with neonatal muscle weakness and respiratory distress, progressively improving and minimally affected at adult stage [6, 9]. Temporal improvement of the phenotype has been reported for CNM and CFTD, but to our knowledge, the Samaritan myopathy is the only ultimately benign congenital myopathy to date. The histopathology was not indicative of a specific congenital myopathy, there was however a partial

Fig. 2 RYR1 quantification and immunofluorescence of skeletal muscle triad markers. **a** Quantification relative to GAPDH demonstrated a strong RYR1 reduction in the deltoid muscle of the index patient, while the RYR1-associated Trisk51 protein was similar to the control. **b** Immunohistofluorescence analysis of transverse muscle sections revealed irregular RYR1 staining compared to the age-matched control. Accumulation of RYR1 with the T-tubule markers caveolin-3 and dysferlin were often seen around internalized nuclei ▶


a
Control
LR52
RyR1
Trisk51
GAPDH
Relative to GAPDH
4
3.5
3
2.5
2
1.5
1
0.5
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Control
LR52
Control
LR52
RYR1
Trisk51
b
CTRL
LR52
CTRL
LR52
DHPR
RyR
Cav-3
Overlay+Nuclei
Zoom
Dysferlin
Overlay+ Nuclei
Internalized Nuclei

overlap with centronuclear myopathy concerning abnormal localization of nuclei, and with CCD concerning core-like structures. A recent study reported a RYR1-related congenital myopathy with prominent nuclear internalization and large areas of myofibrillar disorganization [1] and another study reported *RYR1* mutations with a low frequency of central nuclei [20]. However, atrophy and type 1 fiber predominance were major histological aspects in both studies. In contrast, the Samaritan biopsy displayed invariable fiber size and regular fiber type distribution (supplemental Table 1). Similarly, ophthalmoparesis is a common feature of CNM and RYR1-related congenital myopathies with prominent nuclear internalization [1, 20] whereas it is not observed in our patients. Taken together, the course of disease, the clinical features and the histopathology suggest that the benign Samaritan congenital myopathy is a distinct and unique muscle disorder.

To address the question, why the Samaritan myopathy is the only known myopathy with benign course of disease, we have extracted the *RYR1* RNA from the muscle biopsy of the index patient to investigate alternative splicing of the exon harboring the mutation. There was no indication that exon 25 was alternatively spliced in the adult (data not shown). The ultimately benign course of disease of the Samaritan myopathy is possibly due to the specificity of the mutation. This is sustained by the fact that mutations in exon 25 have never been reported in RYR1-related myopathies that do not follow a benign course. It is possible that the region encoded by this exon has an important role during development and that it is not essential for muscle maintenance after birth. Future investigations aiming to understand the partial recovery of the disease in the benign Samaritan congenital myopathy may reveal possibilities to attenuate or reverse the progression of other RYR1-related myopathies.

It is known that specific heterozygous *RYR1* mutations can involve both a muscle phenotype and malignant hyperthermia. The p.Tyr1088Cys mutation is the first *RYR1* exon 25 mutation and no missense mutation in proximity has been associated with a muscle phenotype yet. The closest reported mutations are p.Arg1043Cys in exon 24 and p.Arg1140Cys in exon 26, both associated with malignant hyperthermia [14], indicating that the Samaritan mutation potentially involves ryanodine receptor hypersensitivity like the MH-related *RYR1* mutations, even though the Samaritan mutation is homozygous. This has however to be conciliated with the reduced RYR1 protein level found in the Samaritan biopsy. The pharmacogenetic disorder MH results from anesthetics-induced disruption of the skeletal muscle calcium homeostasis. The massive calcium release from the sarcoplasmic reticulum causes muscle contracture followed by a hypermetabolic state and is a potential death cause. Importantly, upon interrogation based on our molecular findings, the index patient LR52 reported to have suffered from a mild transient hyperthermia following anesthesia. As MH is inherited as a dominant trait, and as the Samaritan population is highly inbred, it would be of medical interest for all Samaritans to be sequenced for *RYR1* exon 25 to potentially adapt anesthesia.

The identification of the causative mutation of the benign Samaritan congenital myopathy can have major consequences on patient care and disease management. Treatment with salbutamol (albuterol) has been shown to significantly reduce muscle weakness in CCD patients [17]. Salbutamol increases the level of the sarcoplasmic reticulum Ca^{2+}-pump SERCA1a [21], which is supposed to amplify Ca^{2+} uptake in the sarcoplasmic reticulum, and thereby to partially compensate for the RYR1 dysfunction. It is therefore possible that salbutamol might have a beneficial effect on the Samaritan patients.

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Conflict of interest None of the authors reports conflicts of interest.

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Web resources

1,000 genomes, http://www.1000genomes.org/
dbSNP, http://www.ncbi.nlm.nih.gov/projects/SNP/
Exome Variant Server, NHLBI Exome Sequencing Project (ESP), Seattle, WA (URL: http://evs.gs.washington.edu/EVS/)
Online Mendelian Inheritance in Man (OMIM), http://www.omim.org/
Polyphen, http://genetics.bwh.harvard.edu/pph/
SIFT, http://sift.jcvi.org/