

A homozygous splicing mutation causing a depletion of skeletal muscle RYR1 is associated with multi-minicore disease congenital myopathy with ophthalmoplegia

Nicole Monnier¹, Ana Ferreiro², Isabelle Marty³, Annick Labarre-Vila⁴, Paulette Mezin⁵ and Joel Lunardi^{1,6,*}

¹Laboratoire de Biochimie de l'ADN-EA 2943, CHU Grenoble, France, ²INSERM U 582, Institut de Myologie, Paris, France, ³INSERM EMI 9931 and CIS-DRDC, CEA Grenoble, France, ⁴Laboratoire EFSN, CHU Grenoble, France, ⁵Laboratoire de Pathologie Cellulaire, CHU Grenoble, France and ⁶BECF-EA 2943/DRDC; CEA Grenoble, France

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The ryanodine receptor (RYR1) is an essential component of the calcium homeostasis of the skeletal muscle in mammals. Inactivation of the *RYR1* gene in mice is lethal at birth. In humans only missense and in-frame mutations in the *RYR1* gene have been associated so far with various muscle disorders including malignant hyperthermia, central core disease and the moderate form of multi-minicore disease (MmD). We identified a cryptic splicing mutation in the *RYR1* gene that resulted in a 90% decrease of the normal RYR1 transcript in skeletal muscle. The 14646+2.99 kb A→G mutation was associated with the classical form of MmD with ophthalmoplegia, whose genetic basis was previously unknown. The mutation present at a homozygous level was responsible for a massive depletion of the RYR1 protein in skeletal muscle. The mutation was not expressed in lymphoblastoid cells, pointing toward a tissue specific splicing mechanism. This first report of an out-of-frame mutation that affects the amount of RYR1 raised the question of the amount of RYR1 needed for skeletal muscle function in humans.

INTRODUCTION

Excitation–contraction (E–C) coupling in skeletal muscle involves mechanical and functional interactions between Ca²⁺ release channels (dihydropyridine receptor and ryanodine receptor), and peripheral modulating proteins (triadin, calsequestrin, calmodulin, FKBP12). Ryanodine receptor (RYR1) present in the terminal cisternae of the sarcoplasmic reticulum (SR) controls the release of luminal calcium. RYR1 is essential to skeletal muscle development as demonstrated by the lethal birth defects with gross abnormalities of the skeletal muscle observed after inactivation of the *RYR1* gene in mice (1).

In the SR membrane, E–C coupling units comprise RYR1 subunits that organize to form homotetrameric structures facing tetrad assemblies of dihydropyridine receptors (DHPR). Dihydropyridine receptor is a voltage-dependant L-type Ca²⁺ channel located in the T-tubule plasma membrane that acts as a voltage sensor in E–C coupling. In humans, the 106 exons of

the *RYR1* gene that maps to chromosome 19q13.2 code for a protein of 5038 amino acids (2) that contains two functional domains: a large N-terminal cytoplasmic domain with regulatory sites that interacts with the DHPR and a C-terminal transmembrane domain that forms the channel pore.

The *RYR1* gene has been involved in several congenital myopathies including central core disease (CCD) and multi-minicore disease (MmD) families (3–7). Furthermore *RYR1* gene has been associated with malignant hyperthermia, a sub-clinical myopathy occasionally associated with histological core pictures (8–10). CCD and MmD are congenital myopathies. Both are characterized by neonatal hypotonia, delayed motor development, generalized non- or slowly progressive muscle weakness and presence of 'core' lesions in muscle biopsies. Both entities are usually distinguished on the basis of their inheritance patterns, the different length of the histological lesions in skeletal muscle and their clinical expression. Although considered as genetically heterogeneous,

*To whom correspondence should be addressed at: Laboratoire Biochimie de l'ADN-EA 2943, CHU Grenoble 217X, 38043 Grenoble Cedex, France. Tel: +33 476765573; Fax: +33 476765837; Email: jlunardi@chu-grenoble.fr

autosomal-dominant CCD was linked to the *RYR1* gene in a majority of investigated families. The mutations identified so far in *RYR1* were missense mutations and in-frame micro-deletions that mostly mapped to the C-terminal domain of the protein. Four clinical subgroups have been distinguished in MmD (11). The most prevalent classical form is characterized by the predominance of axial muscle weakness leading in most cases to respiratory insufficiency and scoliosis. Ophthalmoplegia associated with this form of MmD represents a specific subgroup. The moderate form consists of generalized muscle weakness predominant in the pelvic girdle, joint hyperlaxity, hand weakness and amyotrophy. The last subgroup includes antenatal onset with arthrogyriposis. MmD is clearly genetically heterogeneous. Mutations in the *selenoprotein N* gene (*SEPN1*) have been identified in 50% of classical severe phenotypes of MmD (12). A homozygous mutation in the *RYR1* gene has recently been identified in the moderate form of MmD with hand involvement whose evolution was compatible with a recessive form of CCD (13). The genetic basis of the two remaining MmD forms remains unknown.

We reported here the identification, in a consanguineous family, of a homozygous mutation in the *RYR1* gene in a patient presenting with a severe classical form of MmD with scoliosis, respiratory insufficiency and ophthalmological paresis. The cryptic splicing site generated by the mutation inserted an out-of-frame intronic fragment in the cDNA. This led to a dramatic decrease in the amount of *RYR1* present in the patient's skeletal muscle. This is the first report of an out-of-frame mutation in the *RYR1* gene. The loss of *RYR1*, a protein usually considered as essential in skeletal muscle, will be discussed in term of disease phenotype.

RESULTS

Clinical and histological data

As shown in Figure 1, we report here a consanguineous family in which the proband presented with clinical features suggestive of a particularly severe form of classical MmD associated with a mild limitation of external eye movements. The proband (II3) is a 17-year-old Tunisian boy. His parents are first cousins. He has one brother (II1) and one sister (II2), none of whom present with clinical symptoms.

The clinical picture was highly consistent with the classical form of MmD (11): neonatal hypotonia, delayed motor development (autonomous gait acquired at 3 years of age), generalized muscle weakness and amyotrophy with walk lost at 12 years of age, respiratory insufficiency (36% vital capacity requiring transitory ventilatory assistance) and a severe scoliosis (up to 40°). Facial dysmorphism, thorax deformity and ocular paresis were also present. No cardiac involvement was reported. The EMG performed at 15 years of age disclosed a severe myopathic pattern while CPK level was normal. Furthermore, the patient presented with a factor XI deficiency.

As shown in Figure 2, eccentric cores associated with mini cores were evidenced in the proband's skeletal muscle biopsy. Serial sections showed an abnormal variability in the fiber size diameter. The predominant type 1 fibers were either hypotrophic or hypertrophic, while all the type 2 fibers had large

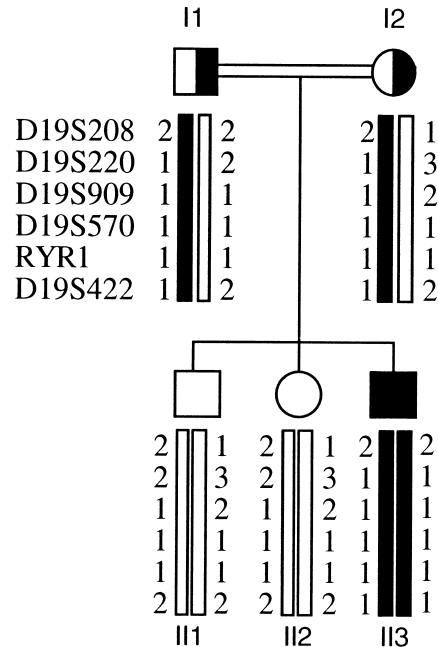


Figure 1. Pedigree and haplotyping analysis of the MmD consanguineous family. Solid symbols indicate the affected individuals. The double line indicates consanguinity. Genotyping data and schematic segregating haplotypes bars for chromosomes 19q13.1–13.2 are shown below the symbol for each individual. Black bars indicate the haplotype segregating with the gene underlying MmD; gray bars show the wild-type haplotypes.

diameters. Centrally located nuclei, often multiple, were present in almost 50% of the small fibers (Fig. 2A). Core lesions, localized areas of sarcomere disorganization and mitochondria depletion, were visible as clear areas devoid of oxidative activity (Fig. 2B–D). Since longitudinal sections of this biopsy could not be performed, we established the length of cores through analysis of serial transversal sections. The core lesions were short and did not span more than two successive sections. Poorly circumscribed core lesions (Fig. 2B and C, black arrows) coexisted with well-defined cores (Fig. 2D, white arrow). Most of the cores were unique and eccentric in transversal sections. This myopathological pattern is almost identical to that previously reported in a patient with moderate MmD linked to chromosome 19q13.1–13.2 (13).

Haplotyping studies

The pathological and clinical phenotypes of the proband suggested that the *RYR1* gene might be involved. Genotyping studies allowed to define a common *RYR1* haplotype shared by both parents on chromosome 19q13.1–13.2. It was present in a homozygous state in the proband and absent in the two unaffected siblings (Fig. 1, black bars). While not fully significant *per se*, the LOD score value of 1.19 at $\Theta = 0.0$ was nevertheless compatible with an involvement of the *RYR1* gene. However, as the *SEPN1* gene had been recently associated with MmD (12), we performed haplotyping studies on chromosome 1p35. The *SEPN1* locus on chromosome 1p35 was clearly excluded as a candidate gene (LOS score value of -2.65 at $\Theta = 0.0$).

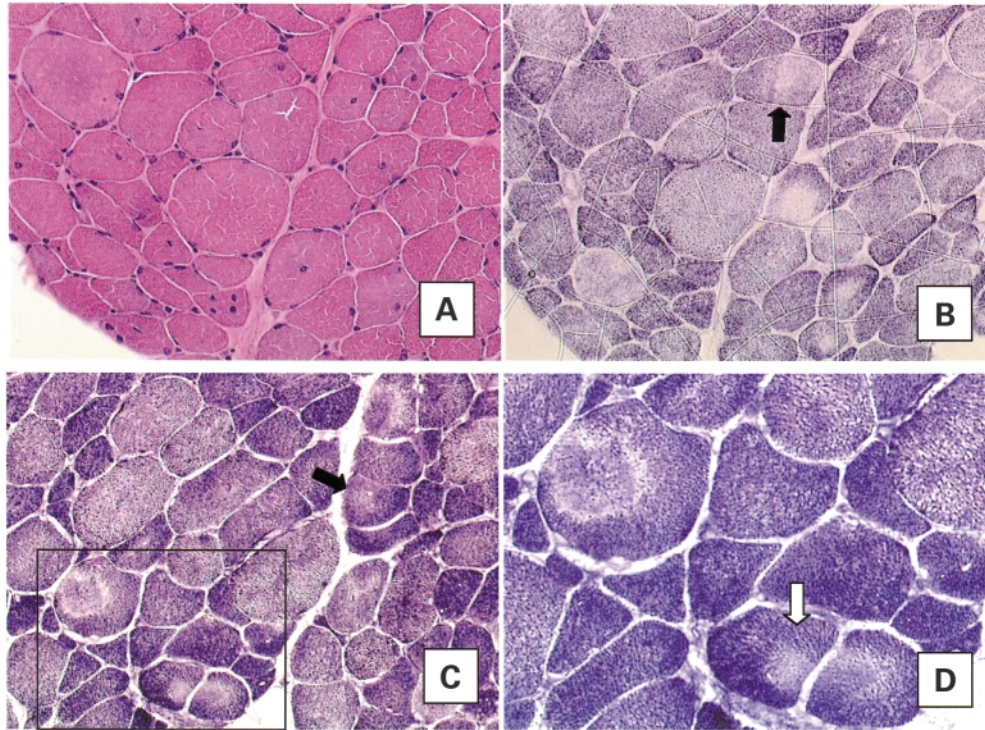


Figure 2. Morphological findings in a deltoid muscle biopsy of the proband. Biopsy of individual II3 was performed at 14 years of age. Serial transverse cryostat sections: hematoxylin–eosin 20 \times (A); succinate dehydrogenase (SDH) 20 \times (B); NADH-tetrazolium reductase 20 \times (C) and 40 \times (D). The area framed in (C) is enlarged in (D).

Mutation screening and detection of an abnormal RYR1 transcript in skeletal muscle

Direct sequencing of all the overlapping fragments spanning the entire cDNA obtained from the proband's muscle biopsy only revealed the presence of eleven silent polymorphisms: L198L (590G \rightarrow A), D993D (2979C \rightarrow T), I1152I (3456C \rightarrow T), H2621H (7863C \rightarrow T), T2659T (7977G \rightarrow A), I2706I (8118T \rightarrow C), D2730D (8190T \rightarrow C), S2863S (8580T \rightarrow C), P3062P (9186G \rightarrow A), A4439A (13317C \rightarrow T) and T4748T (14244A \rightarrow C). More interestingly, amplification of the coding cDNA fragment spanning from nucleotide 14 388 to nucleotide 14 941 that included exons 101–103 generated two products, the normal 553 bp fragment and an abnormal fragment of larger size (Fig. 3). Sequencing of the abnormal transcript identified a 119 bp out-of-frame insertion at the junction of exons 101 and 102. The presence of two different transcript products was in apparent contradiction to the homozygosity evidenced by genomic haplotyping. Although a double recombination event between the two D19S570 and D19S422 flanking markers in one of the two alleles could not be ruled out, a more likely explanation for the presence of two transcripts was either an unusual mutation with incomplete penetrance or/and a neomutation.

Identification of a 14646+2.99 kb A \rightarrow G cryptic splice-site mutation in the RYR1 gene

When aligned with the sequence of the 3' end of the RYR1 gene (clone F15472, GeneBank entry AC005933) the 119 bp

insertion fragment present in the cDNA almost perfectly matched with an intronic sequence located at +2990 bp from exon 101. The only difference was in the number of adenine residues present in a polyA tract located at the 3' end of the fragment. Fifteen adenine residues were reported on the clone F15472 sequence while direct sequencing of the insertion fragment demonstrated the presence of only 14 adenine (Fig. 4A). Computer analysis of the sequence of intron 101 showed that a putative AG acceptor splice site was present at the 5' end of the insertion fragment while a potential GT donor splice site could be present providing that a A \rightarrow G transition occurred at nucleotide +1 from the 3' end of the fragment (Fig. 3A). Specific primers were designed and sequencing of the genomic region encompassing the 119 bp insert clearly identified a A \rightarrow G mutation at nucleotide +1 from the insertion fragment (Fig. 4A). According to the recommendations from the Nomenclature Working Group (Medical School of the University of Geneva, Geneva, Switzerland), the mutation was named 14646+2.99 kb A \rightarrow G. The mutation led to a frameshift additional exon that would introduce a STOP codon 94 amino acids downstream the insertion site. If expressed, the truncated RYR1 protein would thus contain 4976 amino acid residues with a modified C terminal region devoid of the last transmembrane domain coded by exon 102 (2,14).

The intronic 14646+2.99 kb A \rightarrow G mutation that abolished a *Nde*I restriction site was present at a heterozygous state in both parents and absent in the two unaffected siblings (Fig. 4B). This genomic mutation that generated a cryptic splicing site in intron 101 was not present in a panel of 100 independent

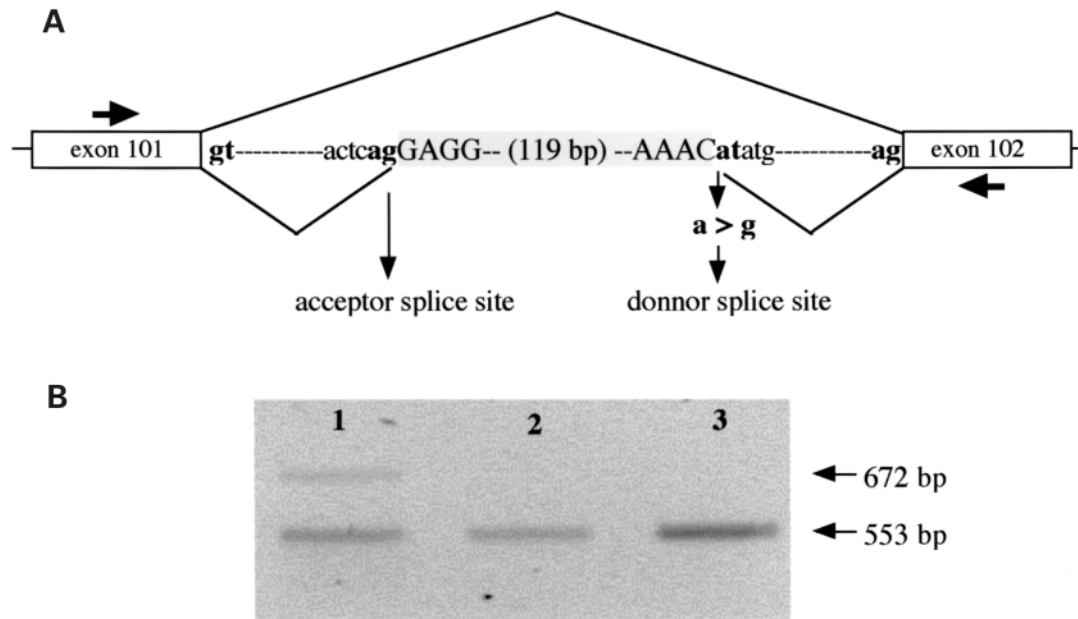


Figure 3. RT-PCR evidence of the mutation. (A) Schematic representation of the intron 101 region containing the additional exon that has been abnormally spliced in the cDNA of the affected proband. Horizontal arrows indicate the position of the primers used for amplification of the normally and abnormally spliced cDNA. (B) Agarose gel analysis of the PCR products resulting from amplification of the cDNA spanning nucleotides 14388–14941. cDNAs were obtained using total RNA isolated from the patient's (lane 1) or control's (lanes 2 and 3) muscle biopsies. Arrows on the right indicate the size of the normal (553 bp) and abnormal (672 bp) RT-PCR products.

chromosomes from a general population originating from North Africa.

Expression studies in skeletal muscle and lymphoblastoid cell lines

Consequences of the 14646+2.99 kb A→G mutation for RYR1 expression were investigated using total RNA and proteins homogenates prepared from a muscle biopsy of the proband that were respectively analyzed by RT-PCR and immuno-blotting. As presented in the upper lane of Figure 5A, semi-quantitative PCR analysis showed that the amount of normal RYR1 transcript was in a 10-times lower range in the patient's skeletal muscle than in the muscle of control cases. Likewise, the amount of abnormally spliced transcript present in the patient's muscle was also in the same low range as compared to the normal RYR1 transcript present in control muscle (middle lane of Fig. 5A). The low amount of normal transcript in the patient muscle suggested that the cryptic site generated by the 14646+2.99 kb A→G mutation was preferentially used. On the other hand, the low amount of abnormally spliced RYR1 transcript was likely to be the consequence of the instability of the mutant transcript as it has been reported for other pathological situations (15). These results were confirmed by western blot analysis that showed a massive decrease of the amount of RYR1 present in the patient's muscle as compared with the control muscle while the α 1-DHPR and γ -sarcoglycan content remained in the normal range (Fig. 5B). Although the immunoblotting analysis did not allow a precise quantification of the proteins, the amount of RYR1 present in the proband's muscle was estimated at 10% of the control muscle RYR1 content using a Molecular Analyst software

(BioRad, USA). Furthermore, no truncated form of RYR1 corresponding to the potential 62 amino acid shorter mutated RYR1 could be evidenced because the protein was either absent or present in a non-detectable amount. The western blot was done using polyclonal antibodies that reacted with the first N-terminal half of RYR1 and in SPAGE conditions that allowed detection of the 65 amino acid difference between the RYR1 and RYR2 isoforms (16).

Recent studies have shown that the RYR1 gene was significantly expressed in lymphoblastoid cell lines (17,18). Since no muscle specimen from the parents could be obtained, we took advantage of this situation to try to investigate the expression of the 14646+2.99 kb A→G mutation either at a homozygous or at a heterozygous level using the lymphoblastoid cell lines established from the proband and the parent's blood samples. Results presented in Figure 6 showed that the abnormal transcript observed in the proband's skeletal muscle (lane 4) was not present in the lymphoblastoid cell lines originating from the proband (lane 1) or his parents (lanes 2 and 3). This unexpected result was further confirmed by RT-PCR amplification performed with specific primers of exons 101–102 and of exon 101–intron 101 insert junction fragments designed to allow the specific amplification of cryptically spliced RNA in absence of the wild-type background (not shown).

DISCUSSION

In the present report, we described the first identification of a frameshift mutation in the RYR1 gene. The intronic 14646+2.99 kb A→G mutation identified in a consanguineous family led to the creation of a cryptic donor splice site and to

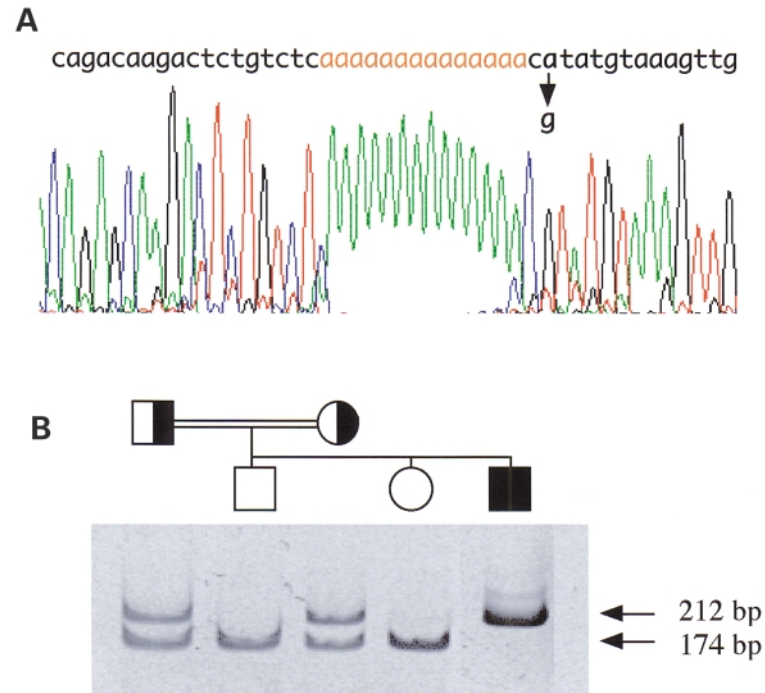


Figure 4. Sequence analysis and segregation of the MmD mutation. **(A)** Mutation analysis of genomic *RYR1* was performed as described in the Methods section. Mutated nucleotide is pointed by an arrow. Only 14 adenine residues indicated in red were identified in the poly A stretch. **(B)** Segregation analysis of the 14646+2.99 kb A→G mutation in the family was performed by restriction analysis of intron 101 as described in Methods. The affected proband is indicated with a solid symbol while heterozygous carriers are indicated with half-solid symbols. As the mutation abolishes a restriction site for *NdeI*, fragment sizes obtained after enzymatic digestion were 174 and 38 bp for the normal allele and 212 bp for the mutated allele. To help the presentation only the two largest fragments are shown.

the insertion of an additional exon at the 101–102 exonic junction in the *RYR1* cDNA. This out-of-frame insertion would change the 94 last amino acids downstream the insertion site and introduced a premature stop codon. Surprisingly, while the proband was homozygous for the genomic mutation, both mutated and normal transcripts were characterized in the patient's muscle biopsy. This apparent paradox can be explained by the respective information content of the mutated cryptic and natural splice sites. When analyzed using a neural network software (BDGP), the newly created cryptic donor splice site showed a 0.94 prediction score and the acceptor splice site failed to be recognized, while intron 101 donor and acceptor splice sites, respectively, showed 0.97 and 0.83 prediction scores. However, when analyzed using an 'in context' splice site prediction software to improve the prediction accuracy (SpliceProximalCheck, EBI, UK), both cryptic sites were recognized as potential splicing sites. It is likely that intron 101 splicing used stronger splice sites than cryptic splicing did, allowing competitive production of both normal and pathological transcripts as observed in the patient's muscle. On the other hand, a homozygous mutation leading to the complete suppression of the *RYR1* protein would probably have been lethal. This has already been observed in dyspedic mice that contain two disrupted *RYR1* alleles (1).

Taken together, the data obtained through RNA and protein analyses favored the hypothesis of a quantitative defect in *RYR1* expression due to an alteration of the transcript level. The preferential use of the cryptic splicing site associated with

the instability of the abnormally spliced mRNA would be the more straightforward explanation for the low amount of normal transcript in the patient's skeletal muscle. The residual normal splicing would be then responsible for the low amount of *RYR1* still present in the proband's skeletal muscle. Other possible explanations can also be put forward. The translation of the normal transcript can be inhibited by the mutated transcript or protein (19). Alternatively, one cannot exclude the truncated protein being expressed and integrated into ryanodine receptor tetramers. Incorporation of aberrant monomeric units would lead to a premature degradation of the *RYR1* tetramers, resulting in the observed decrease of the *RYR1* content. In all cases, the homozygous 14646+2.99 kb A→G mutation would result in a quantitative defect of *RYR1* in the skeletal muscle triad of the proband. The small amount of *RYR1* detected in the proband's skeletal muscle would be responsible for the unusually severe MmD phenotype observed in this patient. He lost gait at 12 years of age and presented with predominantly axial weakness, respiratory insufficiency and severe scoliosis, and had been initially considered to have classical MmD until a partial ophthalmoplegia was detected. The 14646+2.99 kb A→G mutation of the *RYR1* gene constitutes the first genetic defect associated with a quantitative loss of *RYR1* causing a congenital myopathy. The particular phenotype described in this family suggests a possible overlap between MmD with ophthalmoplegia and CCD.

The loss of expression associated with the mutated allele should normally lead to a significant decrease of the expression

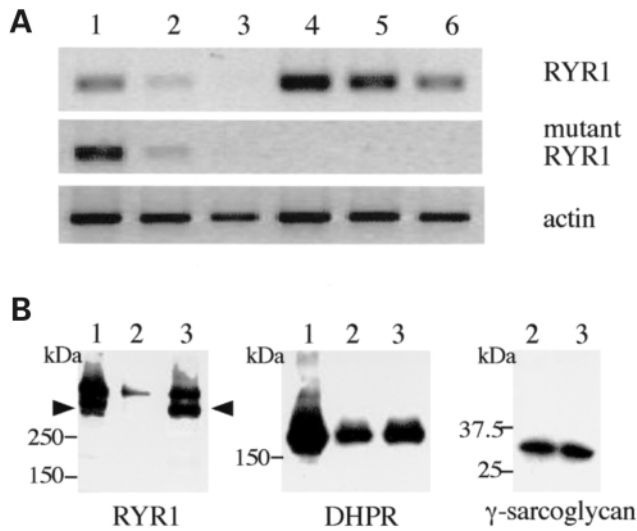


Figure 5. Expression of the mutated allele in skeletal muscle. (A) Total RNAs were isolated from skeletal muscle biopsies obtained from the patient (lanes 1–3) and a control subject (lanes 4–6). RT-PCR were carried out as described in Methods using specific primers to amplify either the normal RYR1 cDNA or the abnormally spliced RYR1 cDNA. Actin was used as standard to normalize the amount of skeletal muscle RNAs. Dilutions of RNA were used as follows: lanes 1 and 4—pure RNA; lanes 2 and 5—1/10 RNA dilution, lanes 2 and 6—1/100 RNA dilution. (B) Protein extracts obtained from the skeletal muscle of the patient (lane 2) and of a control (lane 3) were submitted to SDS-PAGE and then immunodecorated using antibodies directed against RYR1, α 1-DHPR subunit and γ -sarcoglycan. The lower band (arrow) visualized in lanes 1 and 3 corresponds to a proteolytic fragment of RYR1. A skeletal muscle extract obtained from rat (lane 1) was used as a control to test the reactivity of the DHPR and RYR1 antibodies. Values indicated on the left correspond to the position of molecular weight markers.

of the *RYR1* gene in both heterozygous parents since one allele harbored the cryptic splicing mutation. Since skeletal muscle samples for the parents could not be obtained, we investigated the pathogenic effect of the mutation on the expression of the *RYR1* gene in lymphoblastoid cell lines. Recent studies have shown that the *RYR1* gene was expressed in these cells (17) thus providing a valuable tool for functional studies (7,18). Unexpectedly, no abnormally spliced transcript was detected in lymphoblastoid cells originating either from the homozygously mutated proband or his heterozygous parents (Fig. 6). The absence of the mutated transcription product in lymphoblastoid cells strongly suggested a tissue-specific expression of the 14646+2.99 kb A→G cryptic splicing mutation. A tissue-specific regulated splicing has been documented in mice skeletal muscle *RYR1* mRNA (20). This pointed toward tissue-specific splicing apparatus and one can speculate that, depending on the nature of the specific splicing machinery present in a given cell type, the cryptic splicing site could be preferentially used in skeletal muscle and ignored in lymphoblastoid cells. A major problem in studying the mutation is linked to the nature of the mutation since its expression would actually require use of the *RYR1* gene and not only the *RYR1* cDNA for functional studies.

Two distinct mechanisms, the leaky channel and the E–C uncoupling mechanisms, were proposed to explain how altered calcium release channel function caused by different mutations

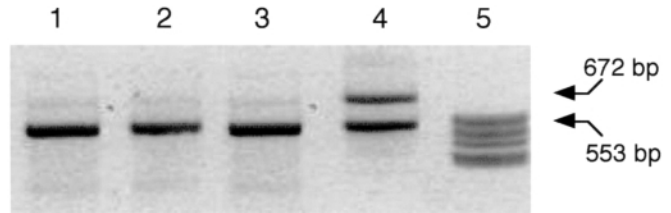


Figure 6. Expression of RYR1 in lymphoblastoid cells. RT-PCR were performed using specific primers described in the Methods section and total RNAs isolated from lymphoblastoid cell lines established from the proband (lane 1), his father (lane 2), his mother (lane 3) and from the proband's skeletal muscle (lane 4) were analysed. Lane 5 corresponds to molecular weight markers.

in RYR1 could result in muscle weakness in congenital myopathies (21). Recent functional studies performed using dyspedic myotubes provided evidence that the Y522S mutation in RYR1 was associated with leaky channel, store depletion and elevation in resting Ca^{2+} while the I4898T mutation resulted in E–C uncoupling (21,22). Noticeably, the Y522S mutation was identified in a core-associated MHS French family that presented without congenital myopathy (23), while the I4898T mutation was responsible for a severe form of CCD (3). The leaky channel hypothesis was first proposed to explain core development and muscle weakness in congenital myopathies (24) and was later supported by functional analysis based on expression of mutant proteins in HEK-293 cells (4,25) or using lymphoblastoid cells from affected patients (7). Although these cellular models did not take in account physical interactions between RYR1 and additional triad components (DHPR, triadin, calsequestrin, etc.), the leaky-channel hypothesis can explain core development associated with (in CCD) or without (in MHS) muscle weakness depending on the extent of the SR Ca^{2+} leak induced. The alternative E–C uncoupling mechanism was made evident so far only for the I4898T mutation associated with CCD. The 14646+2.99 kb A→G mutation described in this paper led to a 90% decrease in the RYR1 protein content in the skeletal muscle triad. The quantitative loss of E–C coupling units could therefore represent a new cause to consider when referring to muscle weakness.

Both parents were heterozygous for the cryptic splicing mutation. The loss of expression associated with the mutated allele could be predicted to induce a significant decrease in the RYR1 protein content. Although minor muscular features cannot be formally excluded in absence of a muscle biopsy, the two parents were reported to be clinically unaffected. The absence of a congenital myopathy in both of them implies that such decrease in RYR1 expression was not sufficient to significantly alter the E–C coupling in skeletal muscle. This suggested a threshold effect. A major point raised by the 14646+2.99 kb A→G mutation was the question of the necessary amount of RYR1 for a normal skeletal muscle development and function in humans. It has been reported that in mice complete inactivation of the *RYR1* gene results in lethal birth defects with gross abnormalities of the skeletal muscle including loss of E–C coupling (1). However, many ultrastructural details of the triadic junction as well as expression of other critical of the Ca^{2+} release complex such as α 1-DHPR, FKBP12 or calsequestrin are preserved in dyspedic mice (26). The observation that the level of the α 1-DHPR was not

significantly affected by the reduced amount of RYR1 was in agreement with data obtained in dyspedic mice, which carry a targeted mutation of the *RYR1* gene (26). In our case, however, while the proband is clinically severely affected in response to a massive depletion of RYR1, the degree of abnormality observed in his skeletal muscle cannot be compared with the pathological picture described in dyspedic mice. Although we cannot exclude the RYR1 function being, to some extent, different in mice and humans, other explanations can also be discussed. One involves the requirement for only a minimal amount of RYR1 to support triad structuration and skeletal muscle development at birth, provided that other proteins of the triad are present. A compensatory mechanism resulting from the presence of other RYR isoforms during embryonic development in humans can also be considered. Alternatively, the existence of specific *RYR1* splicing machineries in different skeletal muscles and/or developmental stages can be speculated. Although we substantiated the massive depletion of RYR1 only in a deltoid muscle biopsy, the clinical picture of the proband pointed toward a more generalized depletion involving many other muscles. Further investigations, both at a genomic and protein levels of RYR1 mutations associated with different clinical, histological and biochemical phenotypes, are clearly needed to gain new insight regarding the essential point of how much RYR1 is needed to support normal skeletal muscle development and function.

MATERIALS AND METHODS

Morphological studies

A deltoid muscle biopsy was performed in the proband. Specimens for histological and ultrastructural studies were immediately frozen in liquid nitrogen and stored at -80°C or fixed and processed as previously described (27).

Acid nucleic isolation

Blood samples from each members of the family were collected after informed consent was obtained. Lymphoblastoid cell lines were established with EBV from the proband and his parents. Genomic DNA was extracted using a guanidine method (28). Total RNA was extracted from a proband's frozen muscle specimen and from lymphoblastoid cell lines of the patient and his parents, using a guanidine thiocyanate-phenol-chloroform method (29).

Haplotyping analysis

An haplotyping study was performed as described previously (5,12) in the two candidate regions known to be involved in MmD: chromosome 1p36, including the *SEPN1* gene, and chromosome 19q13.1–13.2, including the *RYR1* gene. The following markers were used: D1S3766, D1S3767, D1S2885, D1S3768, D1S3769 for the *SEPN1* locus and D19S208, D19S220, D19S909, D19S570, RYR1, D19S897, D19S422 for the *RYR1* locus. Linkage analysis were performed using FASTLINK (30) assuming an autosomal recessive inheritance, a disease-gene frequency of 0.0001 and a penetrance of 0.95.

RYR1 mutation screening

First-strand cDNA was synthesized from total RNA using specific primer mixes and long expand reverse transcriptase (Roche, Switzerland) and was then amplified in 31 overlapping fragments using *Taq* Plus Precision Polymerase (Stratagene, USA). The amplified products spanning the entire RYR cDNA were subsequently purified and directly sequenced. Fragments were mapped according to the numbering of the normal cDNA (31).

The 14388–14941 cDNA abnormal fragment was extracted from low-melting agarose gel using a QIAEX II purification kit (QIAGEN, FRG) and subsequently sequenced. Specific primers encompassing the junction point between exon 101 and intron 101 (5'-agtgtgatgacatgatgacggagg) and the junction point between exon 101 and exon 102 (5'-gtgtgatgacatgatgacgtgttacc) were synthesized and used on cDNA with a common reverse (5'-gatcagacctggatgatgac) primer located in exon 102 to confirm the presence of the mutated transcript. To identify the mutation on genomic DNA, part of intron 101 containing the insert was amplified using the following forward (5'-gtacaaaaattagctgggtgggg) and reverse (5'-agcatggtctacc-tggcatttg) primers. The 212 bp amplified product was then cloned in a Bluescript SK+ plasmide (Stratagene, USA) and sequenced using universal sequencing primers.

Mutation analysis

NdeI restriction analysis of intron 101 was used to screen for the 14646+2.99 kb A→G mutation in the proband's family and in 100 independent chromosomes from a population originating from North Africa. The mutation abolished a restriction site in the 212 bp intronic amplified fragment. Fragment sizes obtained after enzymatic digestion were respectively 174 and 38 bp for the normal allele and 212 bp for the mutated allele.

Western blot

Thin slices of frozen muscle were solubilized in Tris-HCl 10 mM (pH 7.4), EDTA 5 mM, NaCl 150 mM, Triton X100 2%, protease inhibitor cocktail (Roche, Germany). After centrifugation at 10 000g for 8 min, the solubilized proteins were collected and stored at -80°C until use. The amount of protein was estimated using a bicinchoninic assay (Sigma, USA). Proteins were analysed by SDS-PAGE and immuno-blotting using anti-RYR1 (31), anti-DHPR $\alpha 1$ subunit (Upstate Biotechnology Inc, USA) and anti- γ sarcoglycan (Novo Castra, UK) antibodies as previously described (32).

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