A Proposed Molecular Diagnostic Flowchart for Myophosphorylase Deficiency (McArdle Disease) in Blood Samples from Spanish Patients

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Grant sponsor: J.C. Rubio was supported by a contract from Fondo de Investigación Sanitaria (FIS) CA05/0039 Ministerio de Sanidad y Consumo, Madrid, and M.A. Martin is supported by Programa de Intensificación de la Actividad Investigadora, ISCIII (MSC) and Agencia Laín Entralgo (Consejería de Sanidad, Comunidad de Madrid). This work was supported by grants from FIS numbers PI040487, PI041157, PI040362, PI020907 and PI050579.

Communicated by Sergio Ottolenghi

McArdle disease is a metabolic myopathy due to molecular defects in the myophosphorylase gene (PYGM), usually diagnosed in muscle biopsy. The aims of this study were to characterize genetically a large series of patients and to establish a protocol of molecular diagnosis on blood samples. We studied 55 Spanish unrelated patients with McArdle disease. Screening for the three more frequent mutations in the PYGM gene in the Spanish population (c.148C>T, p.R50X; c.613G>A, p.G205S; and c.2392T>C, p.W798R) were performed with polymerase chain-reaction and restriction fragment length polymorphism (PCR-RFLP) methods. To identify other mutant alleles, the coding region of PYGM gene was sequenced. The p.R50X mutation was observed in 38 patients, the p.G205S substitution in eight, and the p.W798R change in nine. Nine novel mutations, five missense (c.247A>T, p.I83F; c.521G>A, p.G174D; c.1.094C>T, p.A365V; c.1468C>T, p.R490W; and c.1730A>G, p.Q577R), one nonsense mutation (c.2352C>A, p.C784X), three frameshift (c.402del, p.N134KfsX161; c.1470dup, p.R491AfsX7), and nine previously reported mutations were found. In addition, we also updated the molecular data of 95 unrelated patients with McArdle disease studied thus far in our center. Of these patients, 56 were either homozygous or compound heterozygous for the p.R50X, p.G205S, or p.W798R mutation. By including in the molecular diagnosis protocol sequencing of the exons 1, 14, 17 and 18 of the PYGM gene, 16 further patients were characterized, and therefore we were able to detect the molecular defect in 72 out of 95 patients. A proposed molecular diagnosis protocol of the disease based on blood DNA would avoid muscle biopsy in 75.8% [95% confidence interval (95%CI): 62.1% - 78.6%] of patients with McArdle disease.

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KEY WORDS: molecular diagnostics; McArdle disease; PYGM; mutation

INTRODUCTION

Genetic defects of the PYGM gene (MIM# 608455) cause a typical metabolic myopathy, McArdle disease or Glycogen storage disease type V (MIM# 232600), characterized by onset in the second or third decade of life, exercise intolerance, premature fatigue, myalgia, cramps in exercising muscles, and sometimes recurrent

Received 21 April 2006; accepted revised manuscript 16 August 2006.

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DOI: 10.1002/humu.9474
myoglobinuria (Arenas et al. 2006). Brief efforts involving isometric contraction and less intense but sustained dynamic exercise are the activities more prone to cause symptoms (Dimaruo and Tsujino, 1994). Most patients present a “second wind” phenomenon on exercise testing, even those not being able to report it to the clinician. It has been suggested that second wind is pathognomonic for McArdle disease (Vising et al. 2003). Aerobic and anaerobic exercise forearm tests display no increase in lactate values (lactate flat response) (Kazemi-Stephani et al. 2002). Molecular heterogeneity has been demonstrated by the identification of various different mutations in the coding regions or splice sites of the gene (Andreu et al. 1999, Bartram et al. 1993, Bruno et al. 1999a, Bruno et al. 1999b, Bruno et al. 2006, Deschauer et al. 2003, Fernandez-Cadenas et al. 2003, Gamez et al. 1999, Kubisch et al. 1998, Martin et al. 2001a, Martin et al. 2000a, Martin et al. 2000b, Martin et al. 2001b, Quintans et al. 2004, Rubin et al. 2000a, Rubin et al. 2000b, Tsujino et al. 1994a, Tsujino et al. 1993, Tsujino et al. 1994b, Vorgerd et al. 1998). The most common among European and American patients is a nonsense mutation at codon 50 in exon 1 (p.R50X) (Andreu et al. 1998, Andreu et al. 1999, Bartram et al. 1993, Bruno et al. 2006, el-Schahawi et al. 1996, Martin et al. 2001a, Martinuzzi et al. 1996, Tsujino et al. 1993, Tsujino et al. 1995, Vorgerd et al. 1998). In this article, we present molecular studies in 55 Spanish patients with McArdle disease, and describe nine novel mutations in the PYGM gene. In addition, we update the molecular data of 95 patients with McArdle disease studied so far in our center and propose a molecular diagnosis protocol based on blood DNA that avoids muscle biopsy in nearly 76 % of patients.

**MATERIAL AND METHODS**

**Patients and Controls**

We studied 55 Spanish unrelated probands with McArdle disease (28 male and 27 female), ranging in age at onset from 8 to 59 years (age at diagnosis ranging from 10 to 70 years). We also studied nine McArdle’s patients who were relatives of probands. All patients presented with the typical manifestations of the disease: exercise intolerance, muscle cramps, myalgia and myoglobinuria. Eighty percent of these 64 patients experienced the “second wind phenomenon” during moderate to intense physical activities in the past. Thus, most of them have remained totally sedentary in the last years to abolish the occurrence of such unpleasant event. In all patients, resting serum creatine kinase levels were increased (range: 500 U/L to 2,800 U/L, normal < 170 U/L), and forearm ischemic exercise testing revealed no increase in venous lactate. The fifty-five index patients showed negative myophosphorylase histochemical staining in muscle biopsy, confirming the lack of the enzyme activity. In 27 out of these 55 patients, biochemical measurement of the enzyme in the biopsy material showed undetectable activity and further confirmed the diagnosis. In the remaining 28 index patients we were not able to measure myophosphorylase activity. We also updated the molecular data of 95 unrelated patients with McArdle disease [55 patients of the present study plus 40 patients documented before (Martin et al. 2001a)] studied in our center so far.

Fifty control muscle samples consisted of biopsies obtained for diagnostic purposes from individuals ultimately deemed to be free of neuromuscular diseases. We analyzed control genomic DNA from muscle or blood of 100 normal individuals.

Written consent was obtained from all individuals. The study was approved by the institutional ethics committee (Hospital Universitario 12 de Octubre, Madrid, Spain) and was in accordance with the Declaration of Helsinki for Human Research.

**Genomic DNA Extraction and Screening for Three Known Mutations**

Mutations were numbered based on protein (GenBank NP_005600.1) or c.DNA sequence (GenBank NM_005609.1). The nucleotide A of the ATG translation initiation codon is the number +1, and this codon is numbered as 1.

Genomic DNA was extracted from muscle using standard methods based on Proteinase K and phenol/chloroform isolation, or from whole blood (Nucleon BACC-2, GE Healthcare Europe GMBH, Chalfont St. Giles, UK, www.gehealthcare.com).


To avoid false positive PCR-RFLP results, we verified the nucleotide change in each mutation by direct sequencing of a second amplified PCR product. Both strands were sequenced in both directions using the conditions described below with the ABI-DyeDeoxy Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA, U.S.A., www.appliedbiosystems.com).
Molecular Diagnosis in McArdle Disease

PCR Amplification of Genomic DNA and Sequencing

The coding sequence of the entire PYGM gene (20 exons) was amplified by PCR from genomic DNA in 14 fragments with the primers described by Kubisch et al. (Kubisch et al. 1998). In this method, intron primers are chosen so that the entire coding region, including its splice junctions, can be analyzed. For PCR analysis, 100 ng of genomic DNA was amplified with a DNA Thermocycler System (Applied Biosystems, Foster City, CA, U.S.A., www.appliedbiosystems.com) for 35 cycles consisting of denaturation at 94°C for 1 min, annealing for 1 min [temperatures as given by Kubisch et al. (Kubisch et al. 1998)], and extension at 72°C for 1 min. Initial denaturation at 94°C was performed for 4 min, and a final extension step at 72°C for 10 min stopped the program. Each 50 μl reaction contained 1.0 U Taq polymerase (Bioline, Randolph, MA, www.bioline.com), 20 pmol each primer, 200 μM each dNTP, and 1.5 mM MgCl₂ in the buffer supplied. The PCR products were purified by electrophoresis in 1 to 2% low-melting-point agarose gel and extracted from the slices by the GFX Gel Band Purification Kit (GE Healthcare Europe GMBH, Chalfont St. Giles, UK, www.gehealthcare.com) according to the manufacturer’s protocol and eluted in 50 μl H₂O. Approximately 200 ng of the PCR products were directly sequenced with 5 pmol of each primer with the ABIDyeDeoxy Terminator Cycle sequencing kit (Applied Biosystems, Foster City, CA, U.S.A., www.appliedbiosystems.com) on an ABI Prism System 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, U.S.A., www.appliedbiosystems.com) according to the manufacturer’s specifications. Sequences were compared with the revised genomic structure of PYGM (Kubisch et al. 1998).

Statistical Analysis

Categorical variables are reported as percentages and 95% confidence intervals (95%CI) were calculated using SPSS for Windows, ver. 11.5

RESULTS

Screening by PCR-RFLP for the three commonest mutations p.R50X, p.G205S and p.W798R in 55 McArdle’s index patients showed that: i) nineteen patients were homozygous for the p.R50X mutation, two patients for the p.G205S mutation, and one patient for the p.W798R mutation, ii) three patients were compound heterozygotes for the p.R50X and p.G205S mutations, and five were compound heterozygotes for the p.R50X and p.W798R mutations, and iii) eighteen patients had one of these three mutations in one allele and an unidentified mutant allele. The alleles of the seven remaining patients did not harbor any of these three mutations.

To detect mutations other than those stated above in the unidentified alleles we amplified and sequenced genomic DNA fragments encompassing the entire coding region and intron/exon boundaries of the PYGM gene. Eighteen additional mutations were identified. Of them, nine were reported elsewhere: c.13_14del, p.L5VsX22; (Rubio et al. 2006, in press); c.280C>T, p.R94W (Deschauer et al. 2001); c.1726C>T, p.R576X (Vorgerd et al. 1998); c.1768 +1G>A (Tsujino et al. 1994b); c.1804C>T, p.R602W (Martin et al. 2001a); c.2262del, p.K754NfsX49 (Kubisch et al. 1998, Martin et al. 2001b).

Nine novel mutations were found: five missense mutations, p.I83F, p.G174D, p.A365V, p.R490W and p.Q73HfsX17; and one nonsense mutation, p.C784X; one single base pair deletion, p.N134KfsX161; one seven base pair duplication, p.Q73HfsX7; and one single base pair insertion, p.R491AfsX7. It is noticeable that mutation p.A365V is derived from a c.1094C>T. In this regard, Bruno et al. (Bruno et al. 2006) have reported this nucleotide change at c.DNA level, but they documented this mutation as p.A365E, that would be predicted by a c.1094C>A change. DNA from 150 control individuals did not have any of these nine mutations. PCR-RFLP analysis confirmed the existence of these novel mutations.

One mutant allele was solely identified in five patients, and their muscle biopsies showed both absence of histochemical staining and undetectable activity for myophosphorylase.

The results of the molecular analysis and distribution of mutant alleles in the 55 patients with McArdle disease are shown in Table 1.

Of the 95 patients overall studied so far by us, 56 were either homozygous or compound heterozygous for the p.R50X, p.W798R, or p.G205S mutations. By including in the molecular diagnosis protocol sequencing of the exons 1, 14, 17 and 18 of the PYGM gene, in 16 further patients the two alleles of PYGM gene were characterized, and therefore we were able to detect the molecular defect in 72 out of 95 patients (Fig. 1).
<table>
<thead>
<tr>
<th>No. Patients</th>
<th>Gender (M/F)</th>
<th>Allele 1</th>
<th>Allele 2</th>
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<tr>
<td>3</td>
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<td>c.148C&gt;T p.R50X</td>
<td>c.13_14del p.L5VfsX22</td>
</tr>
<tr>
<td>2</td>
<td>0/2</td>
<td>c.2392T&gt;C p.W798R</td>
<td>c.2392T&gt;C p.W798R</td>
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<tr>
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<td>1/0</td>
<td>c.2392T&gt;C p.W798R</td>
<td>c.1768+1G&gt;A -------</td>
</tr>
<tr>
<td>1</td>
<td>0/1</td>
<td>c.148C&gt;T p.R50X</td>
<td>c.280C&gt;T p.R94W</td>
</tr>
<tr>
<td>1</td>
<td>0/1</td>
<td>c.148C&gt;T p.R50X</td>
<td>c.2012_218dup p.Q73HfsX7</td>
</tr>
<tr>
<td>1</td>
<td>1/0</td>
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<td>c.1804C&gt;T p.R602W</td>
</tr>
<tr>
<td>1</td>
<td>1/0</td>
<td>c.148C&gt;T p.R50X</td>
<td>c.1726C&gt;T p.R576X</td>
</tr>
<tr>
<td>1</td>
<td>1/0</td>
<td>c.148C&gt;T p.R50X</td>
<td>c.1094C&gt;T p.A365V</td>
</tr>
<tr>
<td>1</td>
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<td>c.2111C&gt;T p.A704V</td>
</tr>
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<td>1</td>
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<td>c.1827G&gt;A -------</td>
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<td>0/1</td>
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<tr>
<td>1</td>
<td>0/1</td>
<td>c.402del p.N134KfsX161</td>
<td>c.1470dup p.R491AfsX7</td>
</tr>
<tr>
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<td>1/0</td>
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<td>c.280C&gt;T p.R94W</td>
</tr>
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<td>c.? p.?</td>
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<td>0/1</td>
<td>c.13_14del p.L5VfsX22</td>
<td>c.? p.?</td>
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</tbody>
</table>

Novel mutations described in this study are shown in bold.

M, male; F, female; Reference GenBank sequences used were NP_005600.1 for protein and NM_005609.1 for cDNA. The nucleotide A of the ATG translation initiation codon is the number +1, and this codon is numbered as 1.
Clinical findings
- Exercise intolerance
- Myoglobinuria (+/-)
- Moderate elevation of resting serum CK (mean 1,500 U/L)
- Flat response of lactate in aerobic or anaerobic forearm test.
- Positive cycle test to address second wind phenomenon

Blood DNA
Molecular diagnosis

- p.R50X
  - 34.7%
  - (25.1% - 44.3%)
- p.R50X + p.W798R
  - 48.4%
  - (38.3% - 58.4%)
  - 58.9%
  - (49.0% - 68.8%)
  - 75.8%
  - (67.2 % - 84.4%)

Muscle Biopsy
Histochemical and/or biochemical diagnosis.
Definitive molecular characterization is based on complete PYGM gene sequencing.

Figure 1. Proposed diagnostic flow chart for diagnosis of McArdle disease. (+/-) indicates presence or absence of the particular clinical feature. In the Blood DNA molecular diagnosis square, left boxes indicate the mutations and exons screened, left flow chart (thick arrow-lines) shows each of the consecutive steps of molecular screening, and right boxes represent the percentage (95% confidence interval) of patients in whom the two mutant alleles were identified by using the corresponding consecutive step of molecular screening (horizontal thin arrow-lines). E: exon.
DISCUSSION

We have identified nine novel molecular genetic defects in Spanish patients with McArdle disease: five missense mutations, one nonsense mutation, and three frameshift mutations. The p.A365V missense mutation disrupts a highly conserved buried site localized near various residues involved in glycogen storage (Hudson et al. 1993). The p.G174D missense mutation modifies a buried conserved site that lies close to two clusters of amino acid residues that are important in dimerization (Hudson et al. 1993). The p.R490W missense mutation alters a buried conserved site that lies next to a pyridoxal-5'-phosphate (PLP) binding site (Hudson et al. 1993). The p.I83F mutation disrupts a conserved buried site that resides in a fragment of the protein important for dimerization and binding to PLP and AMP (Hudson et al. 1993). The p.Q577R missense mutation modifies a strictly conserved buried site that is located in clusters of amino acid residues that are related to active site, glucose-binding, PLP-binding, and purine nucleoside-inhibitor sites (Hudson et al. 1993). Therefore, these missense mutations would presumably affect protein sites that are important for the enzyme activity. In addition, they are likely to be the cause of myophosphorylase deficiency, because (a) they were the only nucleotide alteration in the coding region and adjacent exon/intron boundaries of the PYGM gene; (b) they lead to the replacement of amino acid residues that are identical not only in the glycogen phosphorylase of various species but also in the three human isoforms of this enzyme (Hudson et al. 1993) which is consistent with a crucial role of these amino acids in the normal function of myophosphorylase; and 100 normal controls and 50 disease controls did not have any of these mutations in their alleles.

The p.C1768 +1G>A mutation is likely to cause premature termination of translation and to generate a truncated 783-amino acid peptide instead of the normal 842-residue myophosphorylase protein. The p.N134KfsX161 mutation predicts a frameshift with premature termination of the protein 161 amino acids downstream from the mutation; the p.Q73HfsX7 mutation predicts a frameshift with premature termination of the protein seven aminoacids downstream from the mutation; and the p.R491AfsX7 mutation also predicts a frameshift and premature termination of translation seven codons downstream from the mutation. In these four mutations, the difference in length of the resulting peptide is expected to be crucial for myophosphorylase function. The abnormal enzyme proteins may be more prone to degradation and the resulting enzymes are missing substantial and relevant parts of the total protein. The fact that these four mutations were absent in 150 controls further supports their pathogenicity.

We also identified nine mutations already described. The mutation c.1768 +1G>A has been reported in patients from different ethnic backgrounds (Martin et al. 2001b, Tsujino et al. 1994b). In this regard, the mutations p.R94W and p.R576X identified in two patients, were previously described in McArdle disease patients of German origin (Deschauer et al. 2003, Vorgerd et al. 1998) suggesting that these mutations are not private, and might be found in individuals from other ethnic backgrounds. The p.K754NfsX49, p.A660D, p.R602W, p.A704V, c.1827G>A and p.L5VfsX22 mutations have already been reported in Spanish patients with McArdle disease (Fernandez-Cadenas et al. 2003, Gamez et al. 1999, Martin et al. 2001a, Martin et al. 2001b, Rubio et al. 2006, in press).

In this series of 55 patients, the p.R50X mutation was observed in 39 patients and 58 alleles, the G205S mutation in 8 patients and 10 alleles, and the p.W798R substitution in 9 patients and 10 alleles.

In five patients only one mutant allele was identified. These patients are presumably manifesting heterozygotes, (Dimauro and Tsujino, 1994). Although residual activity of the enzyme in muscle was expected to be found, we failed to detect it. In this regard, the presence of mutations in non-sequenced intronic or regulatory regions of the PYGM gene cannot be excluded. Molecular studies based on RNA rather than DNA analysis could shed light to this issue (Fernandez-Cadenas, et al. 2003), but we must keep in mind that human muscle specimen is mandatory, and this sample is not always available.

Taken together these molecular data on 55 patients with those reported by us before on 40 unrelated patients (Martin et al. 2001a), we have studied thus far 95 Spanish patients with McArdle disease. The p.R50X mutation is the commonest in Spanish patients, accounting for 68.4% (95%CI: 59.1%–77.7%) of patients (65 out of 95) and 51.6% (95%CI: 44.5%-58.7%) of alleles (98 out of 190). The p.W798R is second most frequent underlying cause of myophosphorylase deficiency representing 16.8% (95%CI: 9.2%-24.3%) of patients (16 out of 95) and 11.6% (95%CI: 7.0%-16.2%) of alleles (22 out of 190). Our data overall indicate that blood DNA analysis based on PCR-RFLP methods in patients with clinical suspicion of McArdle disease (Figure 1) could detect as much as 56 of patients (58.9%; 95%CI: 49.0%-68.8%) and 142 of mutant alleles (74.7%; 95%CI: 68.5%-80.9%). Therefore, we propose to screen in blood for: first the p.R50X, second the p.W798R, and third, the p.G205S mutations (Figure 1). To increase the sensitivity of the molecular diagnosis in blood, and as a
second line of screening, we propose to sequence the exons 1, 14, 17 and 18, because by doing so we were able to identify molecularly 16 additional patients in this cohort (Figs. 1 and 2, Table 1). This second line of sequencing analysis is an additional effort that yields a moderate but significant diagnostic improvement. Every center should evaluate whether this strategy is adequate and if it is really worth implementing it. This way, we could avoid an invasive muscle biopsy in as much as 72 patients with McArdle disease [(75.8% of patients; 95%CI: 67.2%-84.4%) and (85.3%; 95%CI: 80.3%-90.3% of alleles)]. In the remaining percentage of patients who were not characterized using blood DNA, a muscle biopsy should be obtained to establish either the diagnosis of McArdle disease or other muscle glycogenoses also giving a flat lactate response in ischemic forearm testing (Figure 1). Given that the W798R mutation is only present in Spaniards, this flowchart is not useful for other populations (Arenas et al. 2006).

Moreover, our data further confirm the genetic heterogeneity of Spanish patients with McArdle disease and expand the crowded map of mutations (Fig. 2) within PYGM gene.

Figure 2. Map of the mutations in the PYGM gene in patients with McArdle disease. Open boxes represent the 20 exons of the PYGM gene. Novel mutations identified in this study are above the gene map.
REFERENCES


