Molecular analysis of LPIN1 in Jordanian patients with rhabdomyolysis

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ABSTRACT
Recessive mutations in LPIN1, which encodes a phosphatidate phosphatase enzyme, are a frequent cause of severe rhabdomyolysis in childhood. Hence, we sequenced the 19 coding exons of the gene in eight patients with recurrent hereditary myoglobinuria from four unrelated families in Jordan. The long-term goal is to facilitate molecular genetic diagnosis without the need for invasive procedures such as muscle biopsies. Three different mutations were detected, including the novel missense mutation c.2935G>C ( Gly799Arg), which was found in two families. The other two mutations, c.2174G>A (Arg725His) and c.1828C>T (Arg368X), have been previously identified, and were found to cosegregate with the disease phenotype in the other two families. Intriguingly, patients homozygous for Arg725His were also homozygous for the c.1828C>T (Arg615Ser) polymorphism, and were exercise-intolerant between myoglobinuria episodes. Notably, patients homozygous for Arg368X were also homozygous for the c.2250G>C silent variant (Gly750Gly). Taken together, the data provide family-based evidence linking hereditary myoglobinuria to pathogenic variations in the C-terminal lipin domain of the enzyme. This finding highlights the functional significance of this domain in the absence of structural information. This is the first analysis of LPIN1 in myoglobinuria patients of Jordanian origin, and the fourth such analysis worldwide.

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1. Introduction
Rhabdomyolysis results from acute breakdown of skeletal muscle sarcolemma due to acquired factors, which constitute over 75% of the cases, or due to familial factors (Zutt et al., 2014; Hamel et al., 2015). In these patients, sustained activation of several Ca²⁺-dependent signaling cascades causes the leakage of electrolytes, myoglobin, and the sarcoplasmic enzymes creatine kinase, aspartate aminotransferase, and alanine transaminase into the blood stream (Zutt et al., 2014). Notably, Ca²⁺ in skeletal muscle fibers is increased 11 times in patients with acute rhabdomyolysis (López et al., 1995). Acute renal failure and cardiac arrhythmia are the most critical clinical complications, and are usually associated with high mortality rate (Melli et al., 2005; Michot et al., 2010).

In lipid metabolic diseases of which rhabdomyolysis is a component, defects in mitochondrial fatty acid oxidation are a frequent cause. However, where such defects have been excluded by biochemical analysis, mutations in LPIN1 were identified in a significant percentage of the cases. In 2008, homozygosity mapping and DNA sequencing identified a Glu215X mutation in LPIN1, along with five other mutations, in seven patients with severe rhabdomyolysis and myoglobinuria (Zeharia et al., 2008). Subsequently, Michot and colleagues found other mutations in a larger cohort of patients (Michot et al., 2010; Bergounioux et al., 2012; Michot et al., 2012). Notably, loss-of-function mutants of Lpin1 were originally associated with fatty liver dystrophy in mice, which also exhibit lipodystrophy and insulin resistance (Péterfy et al., 2001). In contrast, children with LPIN1 mutations do not present lipodystrophy, nor are LPIN1 mutations present in lipodystrophy patients (Cao and Hegele, 2002; Zeharia et al., 2008; Michot et al., 2010). Nevertheless, single nucleotide polymorphisms in LPIN1, or specific haplotypes composed of several such polymorphisms, are significantly associated with body mass index, insulin sensitivity, obesity, and other metabolic traits in several ethnic groups (Suvio-lahti et al., 2006; Loos et al., 2007; Fawcett et al., 2008; Wiedmann et al., 2008; Chang et al., 2010; Zhang et al., 2013). Moreover, a correlation between metabolic phenotypes and LPIN1 mRNA abundance in adipose tissue has been consistently observed (Suvio-lahti et al., 2006; Chang et al., 2010).

The mechanism that drives rhabdomyolysis in LPIN1-deficient mice was recently established to be impaired autophagy of damaged mitochondria (Zhang et al., 2014). On the other hand, pro-inflammatory cytokines have been found to notably increase the volume of lipid droplets and upregulate ACACB in LPIN1-deficient myoblasts (Michot et al., 2013).

LPIN1 encodes lipin-1, an enzyme that dephosphorylates phosphatidic acid to generate diacylglycerol, a precursor to triglycerides (Takeuchi and
Multiple isoforms are expressed by alternative splicing of the \( \text{LPIN1} \) transcript. Three isoforms, lipin-1\( \alpha \), \( \beta \), and \( \gamma \), have been characterized in humans and mice (Péterfy et al., 2005; Han and Carman, 2010). The isoforms are enzymatically active, but have different substrate affinities. The human \( \text{LPIN} \) homologs \( \text{LPIN1}, \text{LPIN2}, \) and \( \text{LPIN3} \), share the same structural organization, in which all introns are located at homologous positions. These homologs are also differentially expressed in tissues (Péterfy et al., 2001). Lipin-1 is predominantly expressed in skeletal muscle and adipose tissue (Péterfy et al., 2001; Donkor et al., 2007; Donkor et al., 2009), and accounts for most, if not all, of phosphatidate phosphatase activity in skeletal muscle (Donkor et al., 2007).

The N-terminal lipin (N-LIP) and C-terminal lipin (C-LIP) domains are highly conserved among lipin proteins and across species (Péterfy et al., 2001). The C-LIP domain contains the \( \text{Mg}^{2+} \)-dependent catalytic Asp-X-Asp-X-Thr motif, which is shared among halocid dehalogenase-like enzymes, along with three other signature motifs (Burroughs et al., 2006; Han et al., 2006; Seifried et al., 2013). These four motifs have been mapped to corresponding regions in human lipin-1 (Donkor et al., 2009). An additional motif, Leu-X-X-Ile-Leu, facilitates complex formation with transcriptional coactivators and nuclear factors to induce expression of several enzymes in mitochondrial fatty acid \( \beta \)-oxidation and in the tricarboxylic acid cycle in hepatocytes (Finck et al., 2006). Similar transcriptional coactivation activity has been described for lipin-2 (Donkor et al., 2009).

In this study, we sequenced \( \text{LPIN1} \) in eight Jordanian patients with severe recurrent myoglobinuria, a condition associated with high mortality and significant morbidity. The goal was to facilitate molecular genetic diagnosis without the need for invasive procedures such as muscle biopsies.

2. Materials and Methods

2.1. Families

Eight patients (5 males and 3 females) from four different consanguineous families (A, B, C, and D) that reside in different locations in Jordan were referred to the Metabolic Genetics Clinic at King Hussein Medical Centre, Amman, Jordan with rhabdomyolysis and severe myoglobinuria. In family A, four children were affected (A-1, A-2, A-3, and A-4), and two were healthy. Two of three children in family B were affected (B-1 and B-2), as was one individual in family C (C-1). A child in family D was affected (D-1), although a sibling died at the age of 1 year with severe weakness and dark urine. This patient was not characterized at time of death. Each patient experienced 1–4 episodes of myoglobinuria, the first of which occurred between the ages of 3 months and 6 years (Table 1). Patients underwent extensive examinations including full blood count, kidney and liver function tests, and assays for creatine kinase, alanine transaminase, aspartate amino transferase, lactate and lactate dehydrogenase, acylcarnitine, urine organic acids, and plasma amino acids. Patient B-1 underwent a muscle biopsy. DNA samples were collected from 50 control individuals, and DNA samples are available from all patients, parents, and unaffected siblings of families A and B for screening of mutations in \( \text{LPIN1} \). Blood samples were provided to the Princess Haya Biotechnology Center Laboratory with informed consent from parents. This study was approved by the institutional review board committees at Jordan University of Science and Technology and King Abdullah University Hospital.

2.2. Sequence analysis

The 19 coding exons of \( \text{LPIN1} \) were amplified from genomic DNA by polymerase chain reaction (PCR). Forward and reverse primers were designed in Primer3 (http://frodo.wi.mit.edu/primer3/) to encompass exon-intron junctions in the NCBI reference sequences for the \( \text{LPIN1} \) gene (NG_012843.2) and cDNA (NM_145693.2). Primer sequences and PCR parameters are available upon request.

PCR products were purified, and sequenced in both directions using Big-Dye Terminator v3.1 Cycle Sequencing Kit on a 3130xl Genetic Analyzer (Applied Biosystems). Sequencing data were compared to reference sequences using ChromasPro 1.34 (Technelysium Pty. Ltd., Australia). Mutations and variants were designated according to guidelines of the Human Genome Variation Society (http://www.hgvs.org/mutnomen).

2.3. Phylogenetic and pathogenicity analyses

Multiple sequence alignments of lipin proteins were generated using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/). The potential pathogenicity of mutations in Arg725 and Gly799 was analyzed using PolyPhen2 (http://genetics.bwh.harvard.edu/pph2/).

3. Results

3.1. Clinical and biochemical features

All patients voided dark urine and experienced muscle weakness in at least one episode in early life (Table 1). In all patients except A-3, the first of these episodes presented along with acute febrile illnesses. At age 3 months, patient A-3 was not breast-fed for a week, during which the first episode manifested. During episodes, patients had significant weakness and decreased deep-tendon reflexes, especially in the lower limbs. Muscles presented mild to moderate tenderness, but not significant swelling. Patients did not present symptoms of complications in the central nervous system, and had normal electrocardiograms. Patients were healthy between episodes, except A-1, A-2, and A-4, who were exercise-intolerant. In addition, these three children have impaired renal function during episodes, but recover without sequelae.

Patient B-1, who had mild, residual weakness of the limb girdle, underwent muscle biopsy immediately after the third episode at the age of 4 years. Results indicated myositis and atrophy of type II fibers, but normal histochemistry staining for all enzymes including cytochrome

| Patient | Sex | Age | Age at onset | Number of episodes | Creatine kinase UI/L | Mutation | Amino Acid Change |
|---------|-----|-----|-------------|--------------------|---------------------|----------|-------------------|
| A-1     | F   | 16 years | 6 years | 2                  | 37,787             | c.2174G>A | Arg725His         |
| A-2     | M   | 15 years | 3 years  | 2                  | 15,000             | c.2174G>A | Arg725His         |
| A-3     | M   | 9 years  | 3 months | 1                  | 296,000            | c.2174G>A | Arg725His         |
| A-4     | M   | 19 years | 3 years  | 3                  | 36,000             | c.2174G>A | Arg725His         |
| B-1\(1\) | F   | 5 years\(4\) | 18 months | 4                  | 142,000           | c.2395G>C | Gly799Arg         |
| B-2     | M   | 2 years  | 16 months | 1                  | 32,668             | c.2395G>C | Gly799Arg         |
| C-1     | F   | 8 years  | 2 years  | 3                  | 70,000             | c.2395G>C | Gly799Arg         |
| D-1\(1\) | M   | 6 years  | 8 months | 3                  | 200,000           | c.1162C>T | Arg388X           |

\( ^{1} \) Normal range: 45–195 UI/L

\(^{4}\) Died at this age.

\(^{4}\) Had a sibling who died of myoglobinuria.
oxidase, Gomori trichrome, and NADH. She died at the age of 5 years in a regional hospital after a very short bout of febrile illness and diarrhea secondary to cardiac complications. Her parents presented myalgia, whereas all other parents were unaffected.

Creatine kinase (normal range: 45–195 UI/L) was elevated in all patients (Table 1), while acylcarnitine, urine organic acids, and plasma amino acids were normal. In patients A-1, A-2, A-3, B-1, B-2, and D-1, aspartate aminotransferase (normal range: 0–37 UI/L) and alanine transaminase (normal range: 0–41 UI/L) ranged from 217 to 2390 UI/L and from 203 to 2141 UI/L during episodes.

3.2. LPIN1 mutations and polymorphisms

All four affected children in family A were homozygous for the transition point mutation c.2174G>A (Fig. 1A), which is in exon 17 and results in the substitution of Arg725 with His. The parents were carriers, but the unaffected children were not. This mutation was not found in 100 control chromosomes. Haplotypes for this mutation were phased using four variants identified in family A (Fig. 1B). The disease-causing mutation, c.2174G>A (Arg725His), appears to be carried on a chromosome with a haplotype that includes alleles C, T, T, and T at the four polymorphic loci: c.192+17C>T (rs10209969, intron 2); c.193-47C>T (rs45509591, intron 2); c.552C>T, p.Ile184Ile (rs11538448, exon 4); and c.1828C>T, p.Pro610Ser (rs4669781, exon 14).

In families B and C, a novel c.2395G>C transversion (Fig. 1C) was identified in exon 18. This mutation replaces Gly799 with Arg. Patients B-1, B-2, and C-1 were homozygous, while parents were heterozygous, and the unaffected child in family B was homozygous wild type. The mutated allele was not detected in control individuals. Notably, all three patients were also homozygous for the ‘T’ allele, a c.1698+41G>T (rs7561070) polymorphism in intron 12.

The c.1162C>T transition (Fig. 1D) in exon 8, which converts Arg388 to a stop codon and truncates the enzyme by 502 residues, was found in both alleles in patient D-1. Both parents were carriers. The patient was also homozygous for the ‘C’ allele, a c.2250G>C silent variant (Gly750Gly, rs61732581), for which one of the 50 control individuals was heterozygous.

3.3. Pathogenicity potential of lipin-1 missense mutations

Arg725 and Gly799 are conserved among orthologous lipin-1 proteins (Fig. 2). Additionally, mutations of both residues were predicted by PolyPhen2 to be probably damaging, with score 1.000. In PolyPhen2, scores represent the probability that missense substitutions are damaging, and values approaching 1.000 suggest deleterious consequences (Adzhubei et al., 2010).

4. Discussion

In this study, we identified a novel LPIN1 missense mutation in Jordanian patients with autosomal recessive rhabdomyolysis syndrome. We also detected missense and nonsense mutations that have been
previously associated with the disorder. All patients were homozygous for the underlying mutation in LPIN1, and Mendelian inheritance was confirmed in all four families.

In one family, cosegregation of Arg725His with the disease phenotype confirms the pathogenicity of this mutation, which was originally described as a compound heterozygous genotype in a Caucasian baby who presented myoglobinuria at 18 months (Michot et al., 2012). In contrast, this mutation was found homozygous in all four affected children in the family. In addition, the patients were homozygous for the Pro610Ser variant, while unaffected siblings were wild type at both positions. Pro610Ser was originally characterized in an adult Ashkenazi patient who was treated with statins for hypercholesterolemia, and then developed myopathy and high levels of plasma creatine kinase (Zeharia et al., 2008). Notably, this variant reverses the mutant phenotype in yeast from which the LPIN1 homolog has been deleted, indicating that the enzyme is functional. However, the homozygosity of all patients in one family for both Arg725His and Pro610Ser is intriguing. Whether this haplotype reflects linkage disequilibrium or not is a possibility, and could be resolved by additional genotyping and analysis of allele and haplotype frequency in Jordan.

In conclusion, we have detected three LPIN1 mutations in four Jordanian families. Two of these mutations have been previously identified, and were confirmed in two different families to segregate with the disease. The third mutation is reported for the first time. These results will facilitate molecular genetic diagnosis of severe rhabdomyolysis with myoglobinuria, and potentially eliminate the need for painful and invasive muscle biopsies.

Conflict of interest

The authors declare no conflict of interest.

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Fig. 2. Multiple sequence alignment of lipin proteins from various species. Arg725 and Gly799 in the C-LIP domain, which were found mutated in different families, are highlighted in green. Asp678, Asp680, Ser723, and Asp804, which are strictly conserved in haloacid dehalogenase-like proteins including lipins, were used to anchor alignments, and are highlighted in red. Alignments were generated in CLUSTAL Omega.
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