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MUTATION UPDATE

# Mutations in Muscle Phosphofructokinase Gene

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**Mutations in the muscle phosphofructokinase gene (PFK-M) result in a metabolic myopathy characterized by exercise intolerance and compensated hemolysis. PFK deficiency, glycogenosis type VII (Tarui disease) is a rare, autosomal, recessively inherited disorder. Multiple mutations, including splicing defects, frameshifts, and missense mutations, have recently been identified in patients from six different ethnic backgrounds establishing genetic heterogeneity of the disease. There is no obvious correlation between the genotype and phenotypic expression of the disease. PFK-M deficiency appears to be prevalent among people of Ashkenazi Jewish descent. Molecular diagnosis is now feasible for Ashkenazi patients who share two common mutations in the gene; the more frequent is an exon 5 splicing defect, which accounts for ~68% of mutant alleles in this population.**

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KEY WORDS: **Glycogenosis, Tarui disease, Exertional myopathy**

## BACKGROUND

Phosphofructokinase (PFK; E.C.2.7.1.11), an allosteric enzyme of glycolysis, catalyzes the irreversible conversion of fructose 6-phosphate to fructose 1,6-bisphosphate. Mammalian PFK is a complex isozyme pool of three subunits, muscle (M), liver (L) and platelet (P), derived from three structural loci on chromosomes 1, 21 and 10 respectively (Vora et al. 1982, 1983a; Van Keuren et al. 1986). The genes are differentially expressed in various tissues resulting in different amounts of the three subunits, which combine randomly to form homo- and heterotetramers with distinctive enzymatic and allosteric properties (Dunaway, 1983). A tetramer is apparently the smallest active form which further self-aggregates (Aaronson et al., 1972; Lad et al., 1973). The subunit composition of the PFK pools reflects the glycolytic and gluconeogenic activity of each tissue. PFK-L is the major form in liver and kidney. Only M type PFK isozyme is expressed in mature muscle, which therefore contains only homotetramers of four M subunits. Erythrocytes contain both L and M subunits, which randomly tetramerize to produce $M_4$, $L_4$, and three hybrid forms of the enzyme. PFK-M is also a major component in brain and heart; in these organs, M form constitutes about 50–90% of the PFK pool (Davidson et al., 1983; Dunaway et al., 1988).

A recessively inherited rare muscle disease associated with PFK-M deficiency was first recognized by Tarui et al. (1965) in a Japanese family. In the affected individuals, PFK activity was absent in muscle and was half-normal in erythrocytes; only $L_4$ isozyme remained in erythrocytes. Layzer et al. (1967) reported a similar case in the United States. In the Western hemisphere the disease seems to be prevalent among people of Ashkenazi Jewish descent, and six cases have been reported in Japan. This clustering, however, may reflect a referral bias because patients may not seek medical care for this relatively mild disorder.

The clinical symptoms of Tarui disease (glycogenosis type VII)—exertional myopathy and compensated hemolysis—reflect the deficiency of the enzyme in muscle and blood cells (Vora et al., 1983b; Rowland et al., 1986). Brain and heart are clinically spared. Muscle uses fatty acids as its primary source of energy; it relies on glucose when the demand for energy increases during exercise. Muscle symptoms develop at an early age; patients complain of exercise intolerance with associated nausea and vomiting. Short bursts of intense ac-

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tivity are particularly difficult. Severe muscle cramps and myoglobinuria develop after vigorous exercise. Most patients obtain a "second wind" when the onset of exercise is followed by a brief rest period. In time patients adjust their activity level and are well compensated. Laboratory data show elevated serum creatine kinase, increased bilirubin and reticulocytes with a mildly elevated MCV. Low levels of 2,3-diphosphoglycerate (2,3-DPG) in red blood cells cause increased hemoglobin–oxygen affinity (Vora et al., 1983b). The absence of anemia despite hemolysis has been explained by the stimulatory effect of the low 2,3-DPG on erythrocyte formation (Rowland et al., 1986). Hyperuricemia and clinical gout, probably secondary to increased purine catabolism, have been associated with this illness. Muscle biopsies show a mild increase in glycogen (~5%) and a significant decrease or absence of PFK activity. The presence of abnormal polysaccharide found in some patients has been explained by an increased ratio of glycogen synthetase to glycogen branching enzyme activities. While there are no proven therapeutic interventions, physiologic studies (Haller and Lewis, 1991) suggest that a low glucose and carbohydrate diet may increase exercise tolerance.

Two rare syndromes associated with PFK deficiency have been described: a rapidly progressive fatal infantile form (Guibaud et al., 1978; Danon et al., 1978; Danon et al., 1981; Servidei et al., 1986; Amit et al., 1992) and late-onset myopathy with progressive fixed muscle weakness (Vora et al. 1987; Danon et al., 1988). Atypical severe infantile form has been reported in six children from four families. None of these patients had evidence of hemolysis. One patient apparently had a phosphorylase b kinase deficiency in addition to the PFK deficiency (Danon et al., 1981). Cortical atrophy in two patients may have been associated with perinatal asphyxia unrelated to the enzyme deficiency. One of the two siblings described by Amit et al. (1992) had increased glycogen in muscle, heart, and liver, indicating the involvement of multiple PFK subunits. The genetic defects in this heterogeneous group have not been identified.

Late-onset PFK deficiency may represent a natural course of the disease rather than a separate nosologic entity because the patients give history of easy fatiguability and exercise intolerance since childhood (Argov et al., 1994).

## STRUCTURE OF THE PFK-M GENE

The human PFK-M gene is a single-copy gene that spans ~30 kb of genomic DNA and contains 24 exons (Sharma et al., 1990; Yamasaki et al., 1991; Vaisanen et al., 1992). The coding region encompasses 2,340 bp; the polypeptide encoded by the gene comprises 780 amino acids and has a predicted molecular mass of 85 kd (Nakajima et al., 1987). Exon 3 contains the initiator codon preceded by 8 nucleotides of untranslated sequence. The last exon contains the stop codon, the 3′ untranslated region (3′UT) and the polyadenylation signal. All the splice junctions in introns 2–23 agree with the consensus sequence for exon–intron boundaries of eukaryotic genes (Shapiro and Senapathy, 1987). The first and the last nucleotides of the introns are GT and AG for the 5′ and 3′ end, respectively.

The 5′ untranslated region (5′UT) of the gene contains two additional exons (exon 1 and exon 2), which are alternatively spliced to generate types A, B, and C mRNA (Nakajima et al., 1990). In type A mRNA, exon 2 is spliced to exon 3; the 89-bp intron 2 is removed from the transcript. In type B mRNA, the intron 2 sequence is retained. The 5′UT region in type C mRNA is different from that of types A and B; the region of intron 1, exon 2, and intron 2 is removed, and the exon 1 is spliced directly to exon 3. Alternative splicing of the transcripts is under the control of tissue-specific factors. Type C mRNA is expressed in various tissues, while types A and B are expressed almost exclusively in skeletal muscle (type B is the predominant message). Low levels of expression of the type B mRNA were also detected in kidney (Nakajima et al., 1990a).

Alternatively spliced mRNAs with different 5′UT regions differentially expressed in various tissues suggested the existence of two different promoters and tissue-specific transcription factors. Promoter 1, located upstream of exon 1, drives the transcription of the C type mRNA and contains Sp1-binding sites. Promoter 2, located within intron 1, initiates the transcription from exon 2 and facilitates the muscle-specific expression of types A/B mRNAs; it contains a muscle-specific enhancer (M-CAT-like sequence) and a TATA-like box. Two major transcription start sites (**tsp**) for types A and B mRNA are located in exon 2; type B mRNA has an additional **tsp** within intron 2 four nucleotides downstream from the exon 2–intron 2 junction (Nakajima et al., 1990a; Yamasaki et al., 1991). It has been recently shown that the promoter 2 is transcriptionally active in a variety of cells and contains four functional Sp1 binding sites (Le et al., 1994).

Similar structure of the 5′UT region was found

TABLE 1. Mutations and Polymorphisms in the PFK-M Gene

| No.[a] | E/I[b] | Nucleotide change | Amino acid change | Expected structure | Ethnic background | Reference |
|---|---|---|---|---|---|---|
| 1 | I 2[c] | $g \rightarrow t$ | | Polymorphism | Swiss | Raben et al., 1995 |
| 2 | E 4 | $G_{116} \rightarrow T$ | $Arg_{39} \rightarrow Leu$ | Missense | Ashkenazi[j] | Sherman et al., 1994 |
| 3 | E 4 | $G_{116} \rightarrow C$ | $Arg_{39} \rightarrow Pro$ | Missense | Italian | Tsujino et al., 1994 |
| 4 | I 5[d] | $g \rightarrow a$ | Splicing Defect | 26 aa deletion | Ashkenazi[j] | Raben et al., 1993 |
| 5 | E 6 | $G_{299} \rightarrow A$ | $Arg_{100} \rightarrow Gln$ | Missense | Swiss | Raben et al., 1995 |
| 6 | E 6 | $G_{246} \rightarrow A$ | $Thr_{82} \rightarrow Thr$ | Polymorphism | Swiss | Raben et al., 1995 |
| 7 | E 6 | $C_{306} \rightarrow T$ | $Ala_{102} \rightarrow Ala$ | Polymorphism | Ashkenazi | Sherman et al., 1994 |
| 8 | I 6[e] | $a \rightarrow c$ | Splicing Defect | 4 aa deletion Truncated | Italian | Tsujino et al., 1994 |
| 9 | E 7 | $C_{516} \rightarrow T$ | $Thr_{172} \rightarrow Thr$ | Polymorphism | Multiethnic | Sharma et al., 1989; Nakajima et al., 1990 |
| 10 | E 8 | $G_{626} \rightarrow A$ | $Gly_{209} \rightarrow Asp$ | Missense | Fr. Canadian | Raben et al., 1995 |
| 11 | E 13[f] | $G_{1127} \rightarrow A$ | $Arg_{376} \rightarrow Gln$ ? | Missense | Swedish | Raben, personal communication |
| 12 | I 15[g] | $g \rightarrow t$ | Splicing Defect | 25 aa deletion | Japanese | Nakajima et al., 1990 |
| 13 | E 18 | $A_{1628} \rightarrow C$ | $Asp_{543} \rightarrow Ala$ | Missense | Italian | Tsujino et al., 1994 |
| 14 | I 19[h] | $g \rightarrow a$ | Splicing Defect | 55 aa deletion | Japanese | Hamaguchi et al., 1994 |
| 15 | E 22[i] | $\Delta C_{2003}$ | Frameshift | Truncated | Ashkenazi[j] | Sherman et al., 1994 |
| 16 | E 22 | $G_{2058} \rightarrow T$ | $Trp_{686} \rightarrow Cys$ | Missense | Japanese | Nakajima, personal communication |
| 17 | E 22 | $G_{2087} \rightarrow A$ | $Arg_{696} \rightarrow His$ | Missense | Swiss | Raben et al., 1994b |
| 18 | E 24 | $T_{2334} \rightarrow G$ | $Ala_{778} \rightarrow Ala$ | Polymorphism | Ashkenazi | Sherman et al., 1994 |

[a]Number in order (5′ to 3′) along the PFK-M gene.
[b]Exon/intron.
[c]Base change at the transcription start site.
[d]First nt of intron 5; exon 5 skip in the transcript resulting in a 78-bp in-frame deletion.
[e]Penultimate nt of intron 6; two cryptic splice sites in exon 7 are used, resulting in transcripts with a 5- or 12-bp deletion.
[f]Base change at the last nt of exon 13; the effect of the mutation is unknown (a missense or a splicing defect).
[g]First nt of intron 15; a cryptic splice site in exon 15 is used resulting in a 75-bp in-frame deletion.
[h]First nt of intron 19; exon 19 skip in the transcript resulting in a 165-bp in-frame deletion.
[i]Δ, deletion of indicated nucleotide.
[j]Eleven families were screened for mutations; the other mutations were described in individual families.

in the rabbit PFK-M gene, suggesting that the two-promoter system is an evolutionarily conserved feature, contributing to the tissue-specific expression of the gene. Interestingly, the 89-bp intron 2 of the human gene exists as a part of the 1.7-kb intron of the rabbit gene, suggesting the importance of this region for transcription and alternative splicing (Li et al., 1990).

Another alternatively spliced PFK-M transcript with exon 11 skip (exon 9 based on the nomenclature for the rabbit PFK-M gene) was identified in normal human tissues (Sharma et al., 1990). The transcript codes for an 82-kd PFK-M related polypeptide lacking 31 amino acids from the N-terminal half of the protein. The significance of this transcript is not clear.

Mammalian PFKs are double the size of the prokaryotic enzyme. The sequence similarity among the two halves of mammalian PFK and the bacterial enzyme suggested that mammalian PFKs have evolved by a series of gene duplication and divergence (Poorman et al., 1984). The crystal structures of the bacterial enzymes (*Escherichia coli* and *Bacillus subtilis*) and their substrate binding and the allosteric effectors sites have been determined (Evans et al. 1981; Shirkihara and Evans, 1988). These data provided the basis for predictions concerning the structural organization of the human PFK and the effects of mutations on the protein function.

## MUTATIONS AND POLYMORPHISMS

Table 1 and Figure 1 summarize 13 mutations in the PFK-M gene in patients from six ethnic backgrounds. Table 1 also summarizes 5 polymorphisms identified in the PFK-M gene. The mutations represent three different classes: splicing defects, frameshifts, and missense mutations.

Three mutations are responsible for the disease in three unrelated Japanese families. A G-to-T transversion at the 5′ donor site of intron 15, resulting in a partial deletion of exon 15 (75 bp) from the transcript has been identified in the family described by Tarui et al. (1965); the second splicing defect is located at the 5′ donor site of intron 19 and completely removes exon 19 (165 bp) from the transcript (Nakajima et al., 1990b; Hamaguchi et al., 1994). The third case involves a G-to-T missense mutation at position 2058 in exon 22, substituting Trp for Cys-686 (H. Nakajima and T. Hamaguchi, personal communication).

Ashkenazi Jewish patients represent the largest group studied (nine unrelated families); they share two common mutations in the gene (Raben et al., 1993; Sherman et al., 1994). The predominant

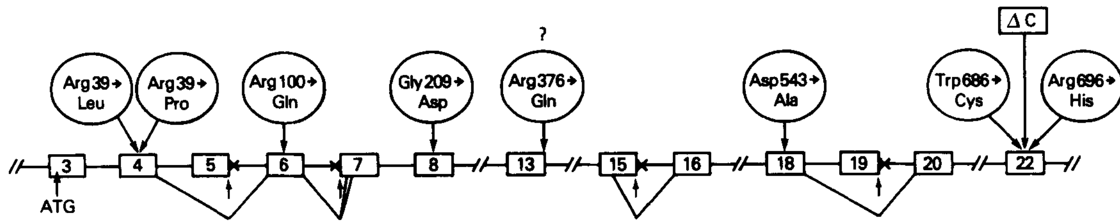


FIGURE 1. Distribution of mutations in muscle phosphofructokinase (PFK-M) gene. Numbered boxes represent exons; introns are shown as lines. The length of the boxes and lines does not reflect the map of the PFK-M gene. The start codon (ATG) is located in exon 3 as indicated. Circles indicate missense mutations; ΔC refers to a deletion of a C nucleotide (position 2003) resulting in a frameshift and a premature stop codon; X marks the position of splice site mutations.

mutation in this group (11 of 18 alleles) is a splicing defect at the 5′ donor site of intron 5, resulting in an in-frame deletion of exon 5 sequence in the transcript. The second, less frequent, mutation is a deletion of a C nucleotide at position 2003 in exon 22. The deletion results in a frameshift, introducing a stop codon 47 nucleotides downstream and would predict generation of a truncated protein with 16 amino acids of incorrect sequence at the C-terminus. Together these two mutations account for 17 of 18 alleles (94%). The remaining allele contains a G-to-T missense mutation at position 116 in exon 4, substituting Leu for Arg-39. The high frequency of the splicing defect in these families suggested the possibility of a founder effect. Most of these patients trace their ancestry to Russia and Poland. Interestingly, the patient with the missense mutation in the exon 4 on one allele was the only individual with partial non-Eastern-European lineage, indicating that this mutation may have arisen in a different part of the world. Screening of 250 unrelated Ashkenazi individuals revealed only one allele with the splicing defect and no evidence of the frameshift or the missense mutation (Sherman et al., 1994).

Recently two additional unrelated Ashkenazi patients (KP and MK), one with confirmed and a second with suspected PFK deficiency, were found to be homozygous for the exon 5 splicing defect (N. Raben, unpublished observations). The data bring the number of exon 5 mutated alleles to 15 of 22 (68%) and support the hypothesis of the founder effect in this population (Fig. 2).

Several mutations have been identified in non-Ashkenazi patients with the disease. A splicing defect at the acceptor site of intron 6 (an A-to-C base change at the penultimate nucleotide of the intron) is responsible for the PFK deficiency in an Italian family; two cryptic sites in the exon 7 are used generating two transcripts with a 5-bp or a 12-bp deletion (Tsujino et al., 1994). The more

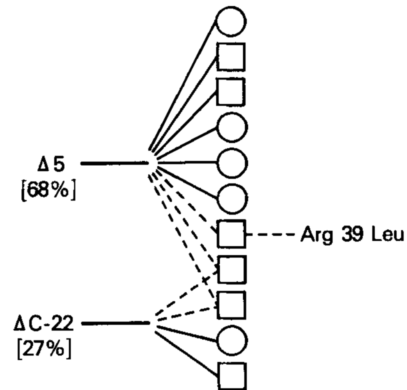


FIGURE 2. Mutations in muscle phosphofructokinase (PFK-M) gene in 11 unrelated Ashkenazi patients. In nine patients, a G-to-A transition at the 5′ splice donor site of intron 5 resulted in exon 5 removal from the transcript (Δ5); the mutation accounts for 15 of 22 alleles (68%). A C nucleotide deletion at position 2003 in exon 22 (ΔC-22) was identified in four patients; the mutation accounts for 6 of 22 alleles (27%). One allele has a G-to-T transversion at position 116 in exon 4, substituting Leu for Arg-39. The patient is a genetic compound who carries the exon 4 mutation and the exon 5 splicing defect.

abundant of these is a 12-bp deleted transcript that does not disrupt the reading frame. Interestingly, a low level of normally spliced mRNA was detected in this patient by PCR, but the amount was not enough to rescue the phenotype. Two other Italian patients as well as French Canadian and Swiss patients carry missense mutations involving exons 4, 6, 8, and 22 (Tsujino et al., 1994; Raben et al., 1995). Curiously, the same nucleotide in exon 4 (G at position 116) is mutated in two unrelated patients changing the encoded Arg-39 to Leu in an Ashkenazi patient or to Pro in an Italian patient. A compound heterozygous Swiss patient carries two point mutations (both are G-to-A transitions) at positions 299 and 2087 in exons 6 and 22. A homozygous French-Canadian patient harbors a G-to-A point mutation at position 626 in exon 8.

Finally, we are now investigating mutations in

the PFK-M gene in a Swedish family. The proband carries a base change at the last nucleotide of the exon 13 on one allele (N. Raben, unpublished observations); the consequences of the mutation (a splicing defect or a missense mutation) and the defect on the second allele remain to be identified.

Five polymorphic sites have been identified in the PFK-M gene. The most common of these, a silent C-to-T transition at position 516 in exon 7, was found in subjects with different ethnic backgrounds; other silent mutations include base changes in exon 6 (G-246 to A and C-306 to T) and exon 24 (T-2334 to G). A G-to-T transversion at the **tsp** in intron 2 was identified in a Swiss patients and four heterozygous and one homozygous unrelated control subjects of the same background.

The splicing defects and the frameshift mutations described in patients with the PFK deficiency would predict generation of proteins with profound structural abnormalities, and thus their "pathogenicity" seems obvious. The regions of the protein removed by the splicing defects at the introns 5, 15, and 19 may involve the putative ATP-inhibition site as well as ADP/AMP and fructose 1,6-bisphosphate-binding sites.

The missense mutations are spread throughout the gene; some of them are located in evolutionarily conserved regions, while others are not. Gly-209, the site of mutation in a homozygous French-Canadian patient, is located at a highly conserved domain present in all known PFK enzymes; both halves of the duplicated gene contain this amino acid, and the region is a part of the active site involved in substrate binding. Likewise, Arg-39, the site of the mutation in an Ashkenazi and an Italian patient, is located in the evolutionarily conserved region, and is considered to be a part of the ATP binding site.

In all cases of published or communicated missense mutations, the entire coding region of the gene was sequenced, and no other abnormalities were found. The effect of these mutations has not been tested in cultured mammalian cells, mainly because of the presence of endogenous PFK in these cells. The authors are currently using a yeast system devoid of endogenous PFK activity (Heinisch, 1993) to express the human mutant PFKs (Raben et al., 1995).

## DIAGNOSTIC RELEVANCE

Typical PFK-M deficiency is a rare, relatively mild disorder caused by multiple mutations in the gene. The molecular diagnosis seems practical in Ashkenazi patients who share two common pathogenic mutations. The predominant mutation in this population is a G-to-A base change at the first nucleotide of intron 5 resulting in exon 5 skipping phenotype. The mutation was identified in 9 of 11 families studied (6 homozygous and 3 heterozygous) and accounted for ~68% of mutant alleles. To avoid the need for muscle biopsy, we developed a simple PCR-based test to screen for this mutation. The following primers are used for PCR amplification: 5′-gggctgaacaggtataatgt (sense) and 5′-atatgggagagggagttctctggaa**g**ata (antisense). The antisense primer contains a mismatch (bold) located four nucleotides downstream from the mutation site and creates an *Eco*RV restriction site only in the PCR product from the mutated sequence. Using this method, we recently diagnosed PFK deficiency in a 49-year old woman (MK) who resisted muscle biopsy and gave a history of a lifelong exercise intolerance, fatigability, muscle cramps, and myoglobinuria. The patient is homozygous for the exon 5 splicing defect.

A simple diagnostic test could be also used to screen Ashkenazi patients for the second common mutation, which accounts for ~27% alleles in this population. The deletion of a C-2003 in exon 22 destroys a native *Ban*II restriction site in the wild-type sequence. Restriction digestion of the PCR product amplified from genomic region spanning exon 22 would result in two digested fragments in addition to an undigested fragment in heterozygous individuals; only undigested fragment could be expected in homozygous patients.

## NOTE ADDED IN PROOF

The Swedish patient has multiple intron retentions in the mRNA: part of intron 13 on one allele, and intron 10 and part of intron 16 on the second allele.

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