Clinical and Molecular Variability in Patients with *PHKA2* Variants and Liver Phosphorylase b Kinase Deficiency

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Abstract Glycogen storage disease (GSD) type IX is a rare disease of variable clinical severity affecting primarily the liver tissue. Individuals with liver phosphorylase b kinase (PhK) deficiency (GSD IX) can present with hepatomegaly with elevated serum transaminases, ketotic hypoglycemia, hyperlipidemia, and poor growth with considerable variation in clinical severity. PhK is a cAMP-dependent protein kinase that phosphorylates the inactive form of glycogen phosphorylase, phosphorylase b, to produce the active form, phosphorylase a. PhK is a heterotetramer; the alpha 2 subunit in the liver is encoded by the X-linked *PHKA2* gene. About 75% of individuals with liver PhK deficiency have mutations in the *PHKA2* gene; this condition is also known as X-linked glycogenosis (XLG). Here we report the variability in clinical severity and laboratory findings in 12 male patients from 10 different families with X-linked liver PhK deficiency caused by mutations in *PHKA2*. We found that there is variability in the severity of clinical features, including hypoglycemia and growth. We also report

additional *PHKA2* variants that were identified in 24 patients suspected to have liver PhK deficiency. The basis of the clinical variation in GSDIX due to X-linked *PHKA2* gene mutations is currently not well understood. Creating systematic registries, and collecting longitudinal data may help in better understanding of this rare, but common, glycogen storage disorder.

Synopsis: Liver phosphorylase b kinase (PhK) deficiency caused due to mutations in X-linked *PHKA2* is highly variable.

Introduction

Glycogen storage diseases (GSDs) are a heterogeneous group of inherited disorders characterized by the storage of glycogen in various tissues, particularly the liver and/or muscle. GSDs are caused by deficiencies of enzymes and transport proteins that are important in the metabolism of glycogen. Deficiency of liver phosphorylase b kinase (PhK), also known as glycogen storage disease type IX (GSD IX), accounts for about 25% of individuals with GSD. PhK is one of the key regulatory enzymes in glycogen metabolism. In response to physiological conditions, PhK activates phosphorylase enzyme in the liver and muscle, which in turn catalyzes glycogen breakdown in these organs (Newgard et al. 1989; Kishnani et al. 2009). It is estimated to affect about 1 in 100,000 people (Maichele et al. 1996) but may be underdiagnosed due to the variable presentation in some individuals. Children with liver PhK deficiency typically present in the first 2 years of life with hepatomegaly,

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growth delay, elevated liver transaminases, and ketotic hypoglycemia of variable severity. Mild elevations in serum cholesterol and triglyceride are common, and postprandial lactic acid elevations may also be present (Wolfsdorf et al. 1999; Davit-Spraul et al. 2011). Based on available reports in the literature, symptoms generally improve with age, and most adults are virtually asymptomatic (Willems et al. 1990).

PhK is a complex enzyme composed of four copies of four different subunits (alpha, beta, gamma, and delta/calmodulin). The activity of the catalytic gamma subunit is regulated by the phosphorylation state of the alpha and beta subunits and by calmodulin via calcium levels (Brushia and Walsh 1999). Tissue-specific expression and alternative splicing of different genes for the various PhK subunits gives rise to differential expression of different isoforms of PhK in different tissues. In the liver, *PHKA2*, *PHKB*, and *PHKG2* encode the alpha, beta, and gamma subunits of PhK, respectively. While mutations in each of these genes can cause liver PhK deficiency, mutations in the X-linked *PHKA2* gene (Xp22.13; OMIM# 300798) are by far the most common cause of liver PhK deficiency, accounting for about 75% of cases (Beauchamp et al. 2007a, b; Davit-Spraul et al. 2011; Roscher et al. 2014). Mutations in *PHKB* (16q12.1; OMIM# 172490) and *PHKG2* (16p11.2; OMIM# 172471) genes cause autosomal recessive forms of liver PhK deficiency and are responsible for most, if not all, of the remainder of PhK deficiency cases.

Traditionally, analysis of glycogen content and PhK enzyme activity in affected tissue samples, such as liver biopsy specimens and erythrocytes, has been important in the diagnosis of liver PhK deficiency. In liver biopsy specimens, glycogen content is highly elevated, with normal structure. PhK activity is usually reduced or absent. However, in a subgroup of patients (those with X-linked glycogenosis type 2, XLG 2), PhK activity can be normal or equivocal in blood cells and variable in liver specimens (Hendrickx et al. 1996; Burwinkel et al. 1997a, b, 1998a, b; Hendrickx et al. 1999). Therefore, a normal in vitro PhK activity does not rule out the diagnosis of liver PhK deficiency. DNA analysis, by sequencing individual genes, gene panels, or whole exome sequencing (WES), can confirm the diagnosis and circumvent the need for liver biopsy if causative pathogenic mutations are detected.

Although there is no clear gene/phenotype correlation seen in PhK deficiency, patients with mutations in *PHKG2* have been reported to have more severe symptoms and increased risk of developing liver cirrhosis in childhood (Maichele et al. 1996; van Beurden et al. 1997; Burwinkel et al. 1998a, b, 2000, 2003; Beauchamp et al. 2007a, b; Davit-Spraul et al. 2011; Fahiminiya et al. 2013; Albash et al. 2014; Bali et al. 2014; Roscher et al. 2014), while those with mutations in *PHKB* tend to have milder

involvement (Burwinkel et al. 1997a, b; van den Berg et al. 1997; Beauchamp et al. 2007a, b; Davit-Spraul et al. 2011; Roscher et al. 2014). A wide variability in clinical presentation and severity has been recognized among patients with the most common subtype, X-linked PhK deficiency, ranging from mild involvement to severe, recurrent hypoglycemia and liver cirrhosis in some reported cases (Burwinkel et al. 1998a, b; Morava et al. 2005; Beauchamp et al. 2007a, b; Davit-Spraul et al. 2011; Tsilianidis et al. 2013; Roscher et al. 2014). In this manuscript we further report the variability in clinical severity and laboratory findings in 12 male patients from 10 different families with X-linked liver PhK deficiency caused by mutations in *PHKA2*. We also describe *PHKA2* variants identified in another 24 patients who were suspected to have liver PhK deficiency but for whom limited clinical information was available and/or the variants were not clearly pathogenic.

Materials and Methods

Patients

This study includes 12 male patients from 10 unrelated families. All subjects have a “pathogenic” or “likely pathogenic” variant in *PHKA2*, based on the American College of Medical Genetics and Genomics guidelines for the interpretation of sequence variants (Richards et al. 2015). Patients 1 and 2 are maternal first cousins, and Patients 6 and 7 are maternal second cousins. All of the patients were followed at medical centers in the United States.

Medical records were reviewed to obtain relevant clinical and laboratory data. Hypoglycemia was defined as blood glucose level <70 mg/dL. Height and weight percentiles were calculated according to the CDC stature-for-age and weight-for-age data (http://www.cdc.gov/growthcharts/cdc_charts.htm). Short stature was defined as height that is two standard deviations or more below the mean for children of that gender and chronologic age. This study was performed in accordance with the Institutional Review Board requirements of Duke University Health System.

PHKA2 Gene Sequencing

PHKA2 gene variants were detected through full gene Sanger sequencing of all 33 coding exons of the gene (NM_000292.2). Sequencing was performed either in a clinical diagnostic laboratory or by a research laboratory at Duke followed by confirmation in the Duke Molecular Diagnostics Laboratory. Sequence variants were named according to the recommendations of the Human Genome Variation Society (www.hgvs.org). The pathogenicity of

PHKA2 variants was investigated using the following in silico tools: the Berkeley Drosophila Genome Project (BDGP) Splice Site Prediction Tool (http://www.fruitfly.org/seq_tools/splice.html) for alterations to the splice site consensus sequences, PolyPhen-2 (<http://genetics.bwh.harvard.edu/pph2/>) (Ramensky et al. 2002), Sorting Intolerant from Tolerant (<http://sift.jcvi.org/>) (Kumar et al. 2009) for amino acid substitutions, and Mutation Taster (<http://www.mutationtaster.org/>) for splice site consensus sequence changes, amino acid substitutions, and small in-frame deletions. To determine if any novel variants identified in this study are common variants, we searched the NCBI dbSNP (<http://www.ncbi.nlm.nih.gov/sites/entrez?db=Snps>) and ExAC databases (<http://exac.broadinstitute.org/>). Variants were classified as pathogenic, likely pathogenic, uncertain significance, likely benign, or benign based on the American College of Medical Genetics and Genomics guidelines for the interpretation of sequence variants (Richards et al. 2015).

Results

Subjects were aged <1 year–4 years at the time of diagnosis (median 2 years 6 months) and 5–17 years at the last evaluation (median 8 years 6 months). Subjects were followed for 2–14 years (median 5 years). A detailed summary of the clinical features and other findings in the 12 patients included in this study is given in Table 1.

Diagnosis

Six patients were initially diagnosed by PhK enzyme analysis in red blood cells ($n = 5$) or liver ($n = 1$) with enzyme activity levels of <10% of normal means. The diagnosis was further confirmed for these patients by the finding of a pathogenic/likely pathogenic mutation in *PHKA2*. One subject (Patient 8) had normal PhK activity in the liver but was later found to have a likely pathogenic variant in *PHKA2* that was previously reported to be associated with X-linked glycogenosis type 2 (XLG 2). Five patients were diagnosed by DNA testing alone.

Initial Clinical Presentation

For all of the patients, symptoms were first noticed in early childhood (3 years and under). Abdominal distension/hepatomegaly was the first symptom to be noticed for the majority of cases ($n = 8$). For another two patients (Patients 9 and 11), the initial concern was growth failure. Upon further evaluation, both of these patients had hepatomegaly and elevated serum transaminases. Another patient (Patient 7) first came to medical attention due to hypoglycemic seizures, but

had additional features of GSD on further evaluation including hepatomegaly, elevated serum transaminases, elevated serum triglycerides and cholesterol, and a “doll-like face.” Although Patient 2 had a known family history of X-linked PhK deficiency (affected first cousin; Patient 1), his family sought an evaluation after noticing that he fed frequently and had a cherubic face.

Hepatomegaly and Abdominal Imaging

Hepatomegaly, based on palpation, varied from 3 to 10 cm below the right costal margin, at the time of first evaluation. Abdominal imaging results (ultrasound $n = 10$; ultrasound and CT scan $n = 1$) were available for 11 patients. Typical findings on abdominal ultrasound were heterogeneously/diffusely echogenic and enlarged liver. One (Patient 1) had hepatosplenomegaly identified on ultrasound. Results of serial ultrasounds, performed approximately every 2 years, were available for eight patients. No signs of liver cirrhosis, adenoma, or focal abnormality were found on abdominal imaging for any of the patients at any time in the study.

Liver Histology

Liver histology reports were available for eight patients (Table 1), all of whom were less than 5 years old at the time of the biopsy. Liver histology universally showed distended hepatocytes with cytoplasmic glycogen accumulation shown by PAS staining. Mild to moderate steatosis was described for two patients (Patients 7 and 9). On electron microscopy, glycogen accumulation in hepatocytes appeared granular or particulate. Four patients had evidence of fibrosis (two with portal fibrosis, two with bridging fibrosis), but none had any signs of liver cirrhosis (Table 1). Muscle histology was performed for one patient (Patient 6) and was normal.

Glucose and Ketone Levels

Glucose levels were not measured routinely when patients were asymptomatic; therefore, the degree and frequency of hypoglycemia is not known. Blood glucose levels were available for seven patients who were treated with cornstarch. Pretreatment glucose levels for these patients varied from fasting lows of 60's mg/dL ($n = 1$) to 50's mg/dL ($n = 3$) to 40's mg/dL ($n = 3$). One of these patients (Patient 7) had hypoglycemic seizures prior to diagnosis, with glucose levels in the 30's mg/dL. After initiation of cornstarch treatment, occasional low blood glucose levels were still seen during times of illness or poor food intake. Four patients were not on regular cornstarch treatment; of these two patients had no documented low blood glucose levels, one had very occasional levels in the 60's, and one had some documented

Table 1 Key clinical features in the male patient cohort with *PHKA2* variants

Patient	Age at diagnosis (years)	Age at last evaluation (years)	Family history ^a	Hepatomegaly (liver cm below RCM ^b by palpation at time of diagnosis)	Liver pathology ^c (age at evaluation)	Fasting hypoglycemia ^d	Growth ^e	Musculoskeletal	Others
1	2	8	Yes	Hepatomegaly (10 cm); splenomegaly	ND	Hypoglycemia	Normal	Mild hypotonia in early childhood	Cherubic face, loose stools
2	0	5	Yes	Hepatomegaly (2 cm)	ND	Hypoglycemia	Normal	Normal	Cherubic face, occasional constipation
3	2	9	No	Hepatomegaly (10 cm)	No fibrosis, glycogen accumulation, no fatty change (2 years)	Hypoglycemia	Normal	Bilateral external rotation of the feet, mild spinal asymmetry ^f	Asthma, constipation, speech delay, learning difficulties
4	1	10	Yes	Hepatomegaly (7 cm)	No fibrosis, glycogen accumulation (1 year)	Mild hypoglycemia	Normal	Bilateral pronation of the feet, scoliosis, pectus excavatum	ECG change of incomplete right bundle branch block
5	1	9	Yes	Hepatomegaly (4 cm)	ND	Hypoglycemia	Normal	Bilateral pronation of the feet and mild scoliosis, resolved	
6	3	5	Yes	Hepatomegaly (10 cm)	Portal fibrosis, glycogen accumulation (3 years)	Hypoglycemia	Short stature	Medial pronation of feet and broad gait ^f	Premature birth with bowel obstruction caused by necrotizing enterocolitis
7	3	7	Yes	Hepatomegaly (9 cm)	Minimal portal fibrosis with areas of bridging fibrosis, moderate steatosis, glycogen accumulation (3 years)	Hypoglycemia	Short stature	Mild postural deviations, genu valgus ^f	Cherubic face, loose stools, exercise intolerance
8	2	17	No	Hepatomegaly (ND)	Mild portal fibrosis with areas of bridging fibrosis, glycogen accumulation (1 year)	Mild hypoglycemia	Normal	Mild genu recurvatum and pronation and eversion at feet and ankles ^f	Hyperflexibility
9	4	8	No	Hepatomegaly (mild)	No fibrosis, glycogen accumulation, mild steatosis (4 years)	No documented hypoglycemia	Short stature	Normal	Mild right hydronephrosis, meatal stenosis, flat top of the head, learning difficulties, ADD

(continued)

Table 1 (continued)

Patient	Age at diagnosis (years)	Age at last evaluation (years)	Family history ^a	Hepatomegaly (liver cm below RCM ^b by palpation at time of diagnosis)	Liver pathology ^c (age at evaluation)	Fasting hypoglycemia ^d	Growth ^e	Musculoskeletal	Others
10	4	11	No	Hepatomegaly (10 cm)	Bridging fibrosis, glycogen storage (4 years)	Hypoglycemia	Normal	Normal	
11	3	8	No	Hepatomegaly (ND)	No fibrosis, glycogen storage, no steatosis (3 years)	No documented hypoglycemia	Short stature	Medial pronation of the feet	Cherubic face
12	1	3	No	Hepatomegaly (ND)	ND	Mild hypoglycemia	Normal	Lumbar lordosis, bilateral pronation of the feet, mild muscle weakness ^f	Food allergies, hypothyroidism, global developmental delay

^a Patients 1 and 2 are maternal first cousins; Patients 6 and 7 are maternal second cousins. Patients 4 and 5 each have a maternal male relative with a reported diagnosis of liver PhK deficiency

^b RCM right costal margin, ND not done or not defined

^c Based on liver histology

^d Severity of fasting hypoglycemia varied. Hypoglycemia = episodes of fasting hypoglycemia <60 mg/dL; mild hypoglycemia = episodes of fasting hypoglycemia <70 mg/dL

^e Growth delay is defined as <2 standard deviations below the mean

^f Identified on physical therapy evaluation

Fig. 1 Height (a) and weight (b) at different ages for our patient cohort. CDC percentiles were plotted using height and weight data for the 3rd, 50th, and 97th percentiles for 1–36 months and 3–20 years

using data tables from the CDC (http://www.cdc.gov/growthcharts/percentile_data_files.htm)

lows in the 40–50's after overnight fast. Of note, two patients had blood glucose levels in the 50's mg/dL range without overt clinical symptoms.

Less data is available on ketone levels ($n = 4$). However, where data is available, blood ketone levels began to rise as glucose levels fell, and ketones were elevated in the presence of hypoglycemia, as expected (Brown et al. 2015).

Growth

Both growth and weight were variable in this group of patients (Fig. 1). Four of the 12 patients had short stature at some point during childhood (height <2 standard deviations below the mean). For all subjects, including those with normal stature, our data show a general trend of

reduction in growth velocity in early childhood and later improvement in growth over time. Due to variations in treatment strategy, it was not possible to separate the effects of age and treatment on growth. One patient was treated with growth hormone from age 3 years; his height increased from the 1st to the 16th percentile over the next 14 months, and by age 8 years his height was at the 90th percentile.

Cardiology Evaluations

Seven patients had an echocardiogram (ages 2–4 years for six patients and age 17 years for one patient); three of them also had an EKG. Cardiac findings were present in two patients; one had incomplete right bundle branch block that did not affect cardiac function, and the other had a very small patent foramen ovale that did not require intervention. Otherwise, no cardiac problems were identified.

General Laboratory Testing and Evaluations

A summary of serum transaminase results is given in Table 2. At the time of diagnosis, serum transaminases (AST, ALT) were elevated by 2–36 times the upper limit of normal in all patients. Serum transaminase levels decreased with increasing age in all patients, regardless of treatment. Elevations of serum cholesterol and triglyceride were common at diagnosis and tended to decrease with increasing age. Plasma lactate levels were available for seven patients and were usually normal with occasional mild elevations. However, one patient (Patient 8) had several unexplained elevated lactate levels (around 2× upper limit), with increased anion gap, which later resolved. Plasma uric acid level, where measured ($n = 6$), was normal. Creatine

kinase level was normal in all patients in which it was analyzed ($n = 7$). Urine organic acid analysis was performed for four patients. Results were normal for two patients (Patients 7 and 10); one patient had marked elevation of lactate and prominent ketosis in one sample (Patient 6); another patient (Patient 9) had slight elevation of 3-methylglutaconic acid which subsequently normalized. Serum total protein and albumin levels at diagnosis and prior to initiating dietary treatment were normal in patients in whom this data was available ($n = 6$).

Treatment

Treatment strategies varied considerably between patients; most (8 out of 12 patients) took oral uncooked cornstarch and some of those patients ($n = 4$) also had protein supplementation; the amount and frequency of cornstarch and protein supplementation varied between patients according to age and current needs. General treatment guideline is to recommend small frequent meals to avoid hypoglycemia.

Cornstarch dose was individualized based on the risk of hypoglycemia and varied between 0.4 g and 2 g/kg throughout the day or at bedtime, protein to provide ~20% calories (3–4 g protein/kg/day) and distributed throughout the day, and fat to meet the general criteria of a heart healthy diet.

Current need for CS and protein was assessed by measuring blood glucose and ketone levels over a 2–3-day period, upon waking in the morning, before meals, and after activity. Due to the clinical variability and differences in individualized treatment regimens, it was not possible to compare the effects of treatment on parameters such as growth and laboratory values (AST, ALT).

Table 2 Key laboratory findings in the male patient cohort with *PHKA2* variants

Patient	AST – multiples of ULN at the time of initial diagnosis	AST – multiples of ULN at the time of last evaluation	ALT – multiples of ULN at the time of initial diagnosis	ALT – multiples of ULN at the time of last evaluation
1	16.2	0.7	36.4	1.0
2	7.5	3.8	8.9	4.4
3	4.7	1.8	3.1	1.6
4	2.7	0.6	3.8	0.4
5	23.9	0.8	16.8	0.9
6	13.5	1.4	6.9	1.6
7	30.7	3.0	20.8	3.6
8	23.4	0.6	16	1.2
9	2.0	1.7	1.8	1.5
10	2.7	1.3	2.9	0.8
11	3.6	0.7	2.9	0.3
12	11.1	0.9	11.6	1.1

ULN upper limit of normal, AST aspartate transaminase, ALT alanine transaminase

PHKA2 Gene Sequencing

The *PHKA2* mutations in these patients are listed in Table 3 and in the Supplementary Tables 1, 2, and 3. The Supplementary Tables 1, 2, and 3 also includes *PHKA2* variants that were identified in another 24 families for whom limited clinical information was available. Overall, we identified 17 different pathogenic/likely pathogenic mutations (7 missense, 5 frameshift, 2 splice site, 2 non-sense mutations, and 1 multi-exon deletion), 9 variants of unknown significance, and 6 benign/likely benign variants. Ten of the pathogenic/likely pathogenic variants are novel (Supplementary Tables 1, 2, and 3).

Discussion

Here, we present the clinical and laboratory features of 12 patients with PhK deficiency caused by mutations in the *PHKA2* gene. Similar to previous case series, symptoms of hepatomegaly, elevated serum transaminases, ketotic hypoglycemia, and growth delay were common among our cohort, and there was variability in severity (Beauchamp et al. 2007a, b; Achouitar et al. 2011; Davit-Spraul et al. 2011; Kido et al. 2013; Roscher et al. 2014). Historically, liver PhK deficiency was reported to be “benign,” but more recently, patients have been described with symptoms ranging from growth retardation alone (Hirono et al. 1998) to asymptomatic hepatomegaly (Willems et al. 1990; Kim et al. 2015), to severe recurrent hypoglycemia

and growth delay (Hidaka et al. 2005; Achouitar et al. 2011) and progression to liver cirrhosis (Johnson et al. 2012; Tsilianidis et al. 2013). There are considerable differences in severity among patients with the same *PHKA2* mutation (Hirono et al. 1998; Achouitar et al. 2011) suggesting that other genetic and environmental factors may influence disease severity. In this regard, it is interesting that two of the more severely affected patients in our cohort (Patients 6 and 7) are also heterozygotes for a pathogenic mutation, c.79delC, in *GSDI* gene, *G6PC*. This gene encodes glycogen-6-phosphatase, which catalyzes the hydrolysis of glucose-6-phosphate (G6P) to glucose and phosphate in the terminal step of gluconeogenesis and glycogenolysis. It is possible that there is a synergistic effect between carrier status for GSD Ia and liver PhK deficiency in Patients 6 and 7 which results in a more severe phenotype for these patients.

Interestingly for two patients in our cohort, low blood glucose levels (in the 50’s mg/dL) were not associated with any obvious clinical symptoms but were found by chance during a clinic visit. Another patient was ketotic in the absence of symptoms and with normal glucose levels. The presence of ketosis with minimal hypoglycemia has been reported in patients with liver PhK deficiency (Tsilianidis et al. 2013). These findings, both of asymptomatic hypoglycemia and ketosis, indicate the importance of monitoring glucose and ketone levels routinely, even in the absence of symptoms, because undetected hypoglycemia and ketosis can affect growth and development, and dietary intervention may be needed.

Table 3 *PHKA2* variants, predicted to be pathogenic or likely pathogenic, identified in our patient cohort

Patient	Location	cDNA change	Amino acid change	Reference
1	Exon 2	c.133C>T	p.Arg45Trp	Davit-Spraul et al. (2011), Tsilianidis et al. (2013), Wang et al. (2013), and Brown et al. (2015)
2	Exon 2	c.133C>T	p.Arg45Trp	Davit-Spraul et al. (2011), Tsilianidis et al. (2013), Wang et al. (2013), and Brown et al. (2015)
3	Exon 2	c.134G>A	p.Arg45Gln	This study
4	Intron 5	c.537+2T>C		This study (c.537+5G>A) reported (Davit-Spraul et al. 2011; Choi et al. 2016)
5	Exon 8	c.811delG	p.Glu271Lysfs*3	This study
6	Exon 9	c.883C>T	p.Arg295Cys	Ban et al. (2003)
7	Exon 9	c.883C>T	p.Arg295Cys	Ban et al. (2003)
8	Exon 9	c.884G>A	p.Arg295His	Hendrickx et al. (1999) and Choi et al. (2016)
9	Intron 16	c.1715-2A>G		This study
10	Exon 21	c.2238-2239delTG insGAACAGGCC	p.Ser746Argfs*11	This study
11	Exon 31	c.3334G>T	p.Glu1112*	This study
12	Exon 33	c.3614C>T	p.Pro1205Leu	van den Berg et al. (1995), Hirono et al. (1998), Achouitar et al. (2011), Davit-Spraul et al. (2011), and Roscher et al. (2014)

Patient 3 also has a variant of unknown significance, p.Phe71Val (c.211T>G), in *PHKB*

Of the four patients for whom urine organic acid analysis was performed, one (Patient 9) had mild elevations of 3-methylglutaconic acid. Elevation of urine 3-methylglutaconic acid has previously been reported in some patients with liver PhK deficiency caused by *PHKA2* (Achouitar et al. 2011) and *PHKG2* mutations (Bali et al. 2014) and also in patients with GSD I (Law et al. 2003). The cause of urinary 3-methylglutaconic acid elevations in some patients with glycogen storage disease is unknown but may reflect abnormal cholesterol metabolism or general mitochondrial dysfunction (Law et al. 2003).

Three patients in our cohort had developmental delays including speech delay with learning difficulties (Patient 3); motor delays, dyslexia, ADD, language-based learning disability, and behavior problems (Patient 9); and global developmental delay (Patient 12). While most individuals with liver PhK deficiency do not have these problems, cognitive impairment or speech delay has been reported (Burwinkel et al. 1998a, b; Beauchamp et al. 2007a, b; Roscher et al. 2014). It is not clear if PhK deficiency contributes to neurocognitive problems in any way. It is possible that undetected hypoglycemia, particularly early in life, could be a factor. It is known that neonatal hypoglycemia can cause brain injury leading to developmental delay/cognitive impairment, particularly if it is severe and prolonged (Montassir et al. 2009). We do not have evidence for severe, prolonged hypoglycemia in any of our subjects with developmental delays/learning difficulties, but this cannot be ruled out. It is also possible that PhK deficiency may have a more direct effect on brain function. ESTs from PhK subunit genes, including *PHKA2*, are found in the brain (Winchester et al. 2007) suggesting that PhK activity may have a role in brain function. Further studies are needed to determine whether developmental delays are any more common in children with liver PhK deficiency when compared to the general population.

In this study, we identified ten novel pathogenic/likely pathogenic variants in *PHKA2*, further showing the genetic heterogeneity involved in X-linked liver PhK deficiency as indicated in previous studies (Hendrickx et al. 1999; Beauchamp et al. 2007a, b; Davit-Spraul et al. 2011; Roscher et al. 2014). One of these novel variants, p. Arg45Gln, was found in three families; it is not known if these families are related or whether this variant is recurrent.

Molecular testing for patients suspected to have GSD type IX (PhK deficiency) is very helpful in confirmation of the diagnosis and identifies which PhK subunit gene is altered and provides information on the inheritance pattern (Davit-Spraul et al. 2011). Finding of rare pathogenic mutations in families with this rare but variable GSD disorder could provide important education about genotype and phenotype correlations. Gene panels or whole exome

sequencing (WES) can be helpful because similar symptoms can present in individuals with various types of glycogen storage disease including GSD III, VI, and IX. However, due to the limitations of WES, including the possibility of missing a mutation if coverage is poor or if a large deletion or duplication is present, single gene or panel sequencing should be considered if the clinical suspicion for liver PhK deficiency is high.

In conclusion, liver PhK deficiency caused by mutations in the *PhKA2* gene is a highly variable condition in terms of clinical presentation and severity. Although our study highlights the variability among our patient cohort, it has the inherent limitations of a retrospective review of medical records. Since some subjects had limited data, while certain trends were noted, we do not know if they apply to the population as a whole. Secondly, early data availability was very limited; therefore, analysis of trends early in life was not possible except for growth parameters. In addition, there may be bias toward more severely affected patients because very mildly affected patients may not come to medical attention (Willems et al. 1990). It would be ideal to follow the early development data and parameters in a population of children with *PHKA2* mutations, from birth, and look for environmental and genetic factors that alter the presentation of the condition. In order to achieve this, creating a detailed registry for individuals diagnosed with liver PhK deficiency would be very helpful and educational for GSD treating community.

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