A Multiplex Assay for the Detection and Mapping of Complex Glycerol Kinase Deficiency

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Background: Glycerol kinase deficiency (GKD) is an X-linked recessive disorder that presents in both isolated and complex forms. The contiguous deletion that leads to GKD also commonly affects NR0B1 (DAX1), the gene associated with adrenal hypoplasia congenita, and DMD, the Duchenne muscular dystrophy gene. Molecular testing to delineate this deletion is expensive and has only limited availability.

Methods: We designed a multiplex PCR assay for the detection and mapping of a contiguous deletion potentially affecting the IL1RAPL1, NR0B1, GK, and DMD genes in a 29-month-old male patient with GKD.

Results: Multiplex PCR detected a contiguous deletion that involved the IL1RAPL1, NR0B1, GK, and DMD genes. Although the patient had a creatine kinase concentration within the reference interval, further mapping with PCR revealed that exon 74 was the last intact exon at the 3′ end of the DMD gene.

Conclusions: Multiplex PCR is an effective and inexpensive way to detect and map the contiguous deletion in cases of complex GKD. The extension of a deletion to include DMD exon 75 in a patient with a creatine kinase concentration within the reference interval suggests that this region of the gene may not be essential for protein function.

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Glycerol kinase (GK)3 is a 553–amino acid protein (1) that catalyzes the phosphorylation of glycerol to glycerol 3-phosphate by ATP in a variety of tissues, most prominently liver and kidney. GK activity has also been identified in peripheral blood leukocytes, brain, cardiac and skeletal muscle, adipose tissue, adrenal and thyroid glands, intestinal mucosa, and lung. Glycerol 3-phosphate is a key intermediate in glucose and lipid metabolism. It serves as an important substrate in gluconeogenesis, particularly during prolonged starvation. Glycerol 3-phosphate is also a substrate for glycolysis and for the synthesis of triglycerides and glycerolipids. Because GK provides the major entryway of glycerol metabolism, loss of enzyme activity leads to hyperglycerolemia and hyperglyceroluria (2, 3).

The 21-exon GK gene,4 which spans ~50,000 bp, is highly conserved throughout evolution (1, 4, 5). Glycerol kinase deficiency (GKD) is an X-linked recessive disorder that presents in both isolated and complex forms. Isolated GKD can be expressed as juvenile or adult (benign) subtypes (4). A range of phenotypes can occur, even within kindreds. These vary from asymptomatic hyperglycerolemia to life-threatening metabolic crises, including vomiting, ketoacidosis, and central nervous system decompensation, presumably attributable to hypoglycemia (2, 3, 6–8). In general, genotype is not predictive of phenotype (4, 6–8). Deficiency of GK enzyme function can also cause pseudohypertriglyceridemia, an artifact of methods of triglyceride measurements based on serum or plasma glycerol concentrations (2, 3).

Complex GKD presents in infancy and is caused by a contiguous deletion in the Xp21 chromosomal region. The

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Received April 27, 2006; accepted July 6, 2006.
Previously published online at DOI: 10.1373/clinchem.2006.072397

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genetic deletions surrounding the GK gene, from telomere to centromere, are Xpter-interleukin 1 receptor accessory protein-like 1 (IL1RAPL1); nuclear receptor subfamily 0, group B, member 1 (NR0B1); GK, dystrophin (DMD), and ornithine carbamoyltransferase (OTC)-cent. Phenotypes in patients with complex GKD depend on the genes included within the deletions (2, 3, 9). Genes frequently involved in complex GKD include DMD, variations in which cause Duchenne muscular dystrophy (DMD) and Becker muscular dystrophy (BMD), and NR0B1 (DAX1), which is responsible for adrenal hypoplasia congenita (AHC) (2, 3). DAX1 is a 2-exon, 470-amino acid protein that plays an important role in development of the adrenal gland and hypothalamic-pituitary-gonadal axis. A C-terminal region of DAX1 is homologous to the ligand-binding domain in the nuclear hormone receptor superfamily but contains an atypical DNA-binding domain at its N-terminal region that lacks a characteristic zinc-finger DNA-binding domain (10). The most commonly reported phenotypes include AHC, GKD, and DMD (2, 3).

Patients with GKD and AHC usually present during the neonatal period with feeding problems, vomiting, hypoglycemia, failure to thrive, hyperpigmentation, and hyponatremic-hyperkalemic dehydration. These infants are usually developmentally delayed and have characteristic dysmorphic features. Hypogonadotropic hypogonadism manifests during adolescence, a consequence of luteinizing hormone and follicle-stimulating hormone deficiency secondary to both hypothalamic and pituitary dysfunction (11). Cryptorchidism is associated with AHC (11–15) and therefore may also be observed in complex GKD in patients in whom the deletion involves NR0B1. Patients with deletions extending into the DMD gene generally have signs and symptoms of classic DMD. Evaluation for adrenal insufficiency is important in patients with evidence of GKD because death may occur as a result of Addisonian crises in those who also have AHC (2, 3).

Complex GKD deletions that extend into the OTC gene, leading to OTC deficiency, have also been reported (2, 3). In addition, deletions have been identified that extend into the IL1RAPL1 gene, possibly producing or contributing to developmental delay in affected patients (9, 16, 17). Clinical testing for complex GKD by use of cytogenetic techniques such as fluorescence in situ hybridization or comparative genomic hybridization (array CGH) is available on a limited basis (18, 19). We describe a multiplex PCR assay for the detection and mapping of complex GKD in a 29-month-old male patient with presumptive GKD.

**Patient History and Hospital Course**

A 29-month-old male patient with global developmental delay and hypoglycemia was admitted to Yale–New Haven Hospital. The patient did not walk until 23 months of age, and spoke only 5 to 10 words. When the patient was 1 month old, he experienced feeding problems, and failure to thrive was diagnosed. However, his feeding difficulties appeared to resolve with a change in formula. The patient’s history was also important because of surgery to repair bilateral undescended testes, performed ~4 months before the most recent admission. Results of prior karyotyping by conventional cytogenetic methods had been unremarkable. Family history was notable only for a learning disability in the patient’s mother, who failed to walk until age 18 months.

Before the patient’s admission to Yale–New Haven Hospital, he had suffered a period of illness of ~6 weeks that began with gastroenteritis and decreased oral intake, accompanied by an episode of hypoglycemia and acidosis. The patient was taken to the emergency department at an outside hospital, where his glucose was found to be 1 nmol/L (18 mg/dL). The patient was admitted for 1 week for treatment of hypoglycemia and metabolic acidosis. Urine organic acids during this admission showed increased glyceral, leading to a presumptive diagnosis of GKD.

Shortly after the patient’s discharge, he developed flu-like symptoms with fever. His oral intake was poor, and he experienced weight loss, a cough with congestion, and intermittent irritability; he then suffered 2 generalized seizures at home and was again brought to the emergency department of the referring hospital, where another seizure occurred. Magnetic resonance imaging of the brain demonstrated evidence of acute and subacute infarctions in the right frontal and parietal lobes and right hippocampus. The patient continued to have poor oral intake, with a 1.5-kg weight loss, and he experienced several hospitalizations, until he was transferred to Yale–New Haven Hospital.

On admission to our hospital, the patient did not appear acutely ill. He was afebrile but tachypneic to 56 with a pulse of 144. Hyperpigmentation was not apparent. Physical examination findings were otherwise noncontributory. Laboratory values were remarkable for sodium of 124 mmol/L, chloride of 94 mmol/L, potassium of 4.7 mmol/L, and CO₂ of 17.7 mmol/L. Blood urea nitrogen, creatinine, and glucose were within reference intervals at 2.9 mmol/L (8 mg/dL), 44.2 μmol/L (0.5 mg/dL), and 5.1 mmol/L (91 mg/dL), respectively. The patient’s creatine kinase was 80 units/L (reference interval, 24–195 units/L). His urine glycerol concentration was 384.37 μmol/L (reference interval, 0–40 μmol/L), and his triglycerides were reported to be extremely increased at 8.2 mmol/L [726 mg/dL; reference interval, 0.3–1.7 mmol/L; (30–150 mg/dL)]. The remainder of the urine organic acid analysis demonstrated only mildly increased ethylmalonic acid. A follow-up urine acylglycine profile was within reference limits. Plasma amino acid and plasma acylcarnitine profiles were also unremarkable.

Morning cortisol was 110.4 nmol/L (4 μg/dL), which was below the lower reference limit (reference interval, 7–25 μg/dL). A corticotropin stimulation test yielded pre- and postadministration cortisol concentrations of 85.5...
nmol/L (3.1 μg/dL) and 171.1 nmol/L (6.2 μg/dL), consistent with adrenal insufficiency. The patient was treated with intravenous fluids, hydrocortisone, and fluconazole. Nocturnal hypoglycemia was not observed; nevertheless, the patient was treated prophylactically with 15 g of cornstarch in 1 oz of milk at bedtime to prevent the development of nocturnal hypoglycemia. Throughout the patient’s hospitalization at Yale, his creatine kinase remained within reference values. The patient’s lethargy and poor appetite resolved with steroid therapy, and he was discharged on hospital day 7.

Materials and Methods

Polymerase Chain Reaction
The 50-μL reaction contained 100 ng of DNA; 200 μmol/L dNTPs; 20 pmol of primers GKPRMF and GKPRMR; 12 pmol of primers DMD79F and DMD79R; and 8 pmol of primers DAX1PRF, DAX1PRR, IL1RAPL1F, IL1RAPL1R, PTCHEX7F, and PTCHEX7R (Table 1); 5 μL ×10 PCR buffer II (500 mmol/L KCl, 100 mmol/L Tris-HCl, pH 8.3; Roche); 0.25 mmol/L spermidine; 1.5 mmol/L MgCl2; 0.25 units of Taq DNA polymerase (Amplitaq®, Roche); and 34 μL of H2O. PCR was performed with an initial denaturation for 2 min at 97 °C; followed by 35 cycles of denaturation at 96 °C for 30 seconds, annealing at 55 °C for 30 seconds, and extension for 40 seconds at 72 °C; and a final extension at 72 °C for 5 min. PCR products were assayed on a 3% GPG/LE™ and 1% Suprasieve (Amersham) agarose/ethidium bromide gel and photographed under ultraviolet light.

Primers
The orientation of the included genes is: Xpter-IL1RAPL1 (5’ → 3’) NR0B1 (3’ ← 5’) GK (5’ → 3’) DMD (3’ ← 5’)-cent.

Primers were designed to flank the termination codon of the IL1RAPL1 coding sequence and junction, the start codon of the NR0B1 gene and junction, the GK promoter region, and the termination codon within DMD exon 79 and the accompanying 3’ junction with the untranslated region (Table 1, Fig. 1). Exon 7 of patched homolog (Drosophila) (PTCH) (20), which is located at chromosome 9q22.3, was used as a PCR amplification control gene. The primer sequences were as previously described (Table 1) (21).

Array CGH
We performed array CGH with Spectral Genomics Constitutional Chip™ 2.0, a commercially available 434 bacterial artificial chromosome (BAC) clone array targeted toward more than 40 specific chromosomally mediated syndromes. Patient genomic DNA was extracted from peripheral blood leukocytes by recognized methods (22, 23). Two Cy5/Cy3 signal intensity experiments were performed. For each experiment, 1 μg of patient DNA and 1 μg of control DNA obtained from a healthy female were differentially labeled with Cy3-dUTP and Cy5-dUTP by use of the Enzo BioArray CGH labeling system in accordance with the manufacturer’s instructions. The fluorochrome labels were reversed in each experiment. We purified the labeled DNA with the QiAquick PCR Purification Kit; otherwise, we followed the chip manufacturer’s instructions for the hybridization procedure and performed analyses with the manufacturer’s SpectralWare Web™ molecular karyotype analysis software.

Results
Multiplex PCR results showed a deletion involving the IL1RAPL1 gene at the telomeric end of the deletion and extending into the DMD gene at its centromeric end (Fig. 2). Further mapping of the deletion with PCR revealed exon 74 to be the last intact exon at the 3’ end of the DMD gene (Fig. 3). These findings were confirmed by array CGH. Array CGH demonstrated a deletion involving, at its ends, BAC RP11-487M22, which includes 19 919 nucleotides at the 5’ end of the IL1RAPL1 gene (20 019 270 total bp), and BAC RP11-46A23, which extends into intron 63 of the DMD gene (Figs. 1 and 4). These findings are consistent with the results obtained by PCR. Because these 19 919 nucleotides represent a small fraction of the total BAC (175 553 nucleotides), it appears that the deletion eliminated the entire IL1RAPL1 gene. No other known genes are within the additional chromosomal region covered by BAC RP11-487M22.

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<th>Table 1. Multiplex and mapping primers</th>
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<td>I. Multiplex primers (telomeric to centromeric)</td>
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<tr>
<td>IL1RAPL1F</td>
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<tr>
<td>DAX1PRF</td>
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<tr>
<td>GKPROMF</td>
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<td>DMD79F</td>
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<td>PTCH EX7F</td>
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<td>II. Primers for mapping the extent of the deletion in DMD</td>
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<td>DMDEX76F</td>
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<td>DMDEX74F</td>
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<td>DMDEX70F</td>
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Discussion

Complex GKD is a rare disorder but one that is important to recognize because of the potential for life-threatening Addisonian crises in patients whose deletions extend into the NR0B1 gene. GKD in both isolated and complex forms is indicated by an increased glycerol peak on urine organic acid testing. Another clue to GKD is the presence of pseudohypertriglyceridemia, an artifact of methods of triglyceride measurements that are based on serum or plasma glycerol concentrations (2, 3).

Molecular assays for complex GKD are not readily available. The 2 laboratories listed on http://genetests.org as providing testing for complex GKD use cytogenetic techniques such as fluorescence in situ hybridization, which requires the application of multiple expensive probes, or array CGH (18), a promising new technique that is expensive and offered by very few providers. Moreover, there is limited experience with array CGH for use in the diagnosis of complex GKD.

PCR-based assays are inexpensive and easy to perform. They potentially yield more information than do currently available cytogenetics-based assays. The PCR assay that we designed maps the gene deletion to the 4 loci most commonly associated with complex GKD (IL1RAPL1, NR0B1, GK, and DMD). This multiplex assay demonstrated extension of our patient’s deletion from IL1RAPL1 at its telomeric end to the 3’ end of exon 79 of DMD. Additional mapping revealed exon 74 to be the last intact exon at the 3’ end of the DMD gene. IL1RAPL1 has been implicated in mental retardation (9, 16, 17), suggesting that disruption of this gene may play a role in the developmental delay observed in the patient we describe.

DMD, the largest known human gene, contains 79 exons. Approximately 60% of cases of DMD are attributable to large deletions in the 5’ and central regions of DMD (24–27). Point variations have been found in ~30% of patients (24). Few central region deletions extending 3’ beyond exon 60 have been found in DMD or BMD patients. However, causative point variations, internal deletions, and contiguous gene deletions involving the most 3’ dystrophin exons or the 3’ untranslated region of the gene have been reported in DMD and BMD patients (17, 27–34).

Given creatine kinase concentrations within the reference interval, a dystrophinopathy is unlikely in our patient (35). Because the contiguous deletion extends to include all or part of exon 75, this patient must be contrasted with other reported patients who have DMD or BMD caused by variations in this region of the gene (17, 27–34). The finding calls into question the hypothesis that the 3’ exons of the DMD gene and the 3’ untranslated region of the gene are essential for protein function (27, 30). Moreover, our patient’s phenotype may be consistent with the reported phenotypic variability seen in affected patients, with variations in the most distal DMD exons (17, 27–29, 32).
In conclusion, we designed a simple, easy-to-perform, multiplex PCR assay for the detection and mapping of the responsible deletion in a patient with complex GKD. This assay can also be used for pre- or postnatal testing. Although in its present form the assay can be used only for detection of affected males, recently developed real-time approaches to assess gene dosage offer the potential to change the amplification format to allow carrier testing (36).

The finding of \(\text{IL1RAPL1}\) as part of a complex GKD deletion in a patient with global developmental delay is consistent with prior reports of the possible role of this gene in mental retardation. The finding of a deletion that

Fig. 3. PCR mapping of deletion into \(\text{DMD}\).

PCR amplification of coding region and intron–exon junctions of \(\text{DMD}\) exons 76 (A), 75 (B), and 74 (C). Absence of \(\text{DMD}\) exon 75 and 76 PCR products in patient specimens, with presence in healthy controls and presence of exon 74 PCR products in patient specimen and healthy controls can be seen. Note the larger, faint band in patient lane (B). This is the result of nonspecific priming attributable to the loss of target sequence in the specimen.
who appears not to have DMD or BMD suggests that the DMD leaves DMD exon 74 as the last intact exon in a patient who appears not to have DMD or BMD suggests that the 3'—most exons and untranslated region of the DMD gene may not be essential for protein function. Consequently, variations in these exons apparently do not inherently produce a dystrophinopathy phenotype.

R.D.K. was supported by National Institutes of Health Training Grant 5T32GM008753.

References