Clinical/Scientific Notes

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MYOGLOBINURIA AND MUSCLE PAIN ARE COMMON IN PATIENTS WITH LIMB-GIRDLE MUSCULAR DYSTROPHY 2I

Fukutin-related protein (*FKRP;* OMIM #606596) is critical for the appropriate glycosylation of α-dystroglycan, a component of the dystrophin-glycoprotein complex. The 12-kb *FKRP* gene is composed of 3 noncoding exons and 1 exon encompassing the entire open reading frame.[1] Mutations in *FKRP* cause autosomal recessive muscular dystrophy with a wide range of clinical severity. A common missense mutation, c.826C>A (p.L276I), has been identified. Generally, individuals homozygous for this mutation have a mild form of limb-girdle muscular dystrophy (LGMD) 2I, compound heterozygotes with 1 c.826C>A allele have a more severe form of LGMD 2I, and those with 2 unique alleles are most severely affected, including some with congenital muscular dystrophy.[1,2]

We report a high incidence of myoglobinuria and muscle pain in a retrospective study of patients with LGMD 2I.

Methods. ***Standard protocol approvals, registrations, and patient consents.*** Institutional Review Board approval was obtained for all recruitment and data collection. Written informed consent was obtained from all participants or their legal guardians. This study was posted on clinicaltrials.gov (NCT00313677). All patients with *FKRP* mutations were eligible for enrollment from 2006 to the present.

Genetic testing. *FKRP* mutations were confirmed in the University of Iowa Molecular Pathology Laboratory using an amplification-refractory mutation system (ARMS) assay, direct sequencing, or both. The ARMS assay identifies the c.826C>A *FKRP* variant and common point mutations in *SCGA* (c.229C>T in LGMD2D) and *SCGB* (c.341C>T in LGMD2E). Primer sequences and technical details are provided in appendix e-1 on the *Neurology*® Web site at www.neurology.org.

Clinical evaluation. Participants completed a questionnaire and interview regarding medical history and activities. Medical records were reviewed and selected items including age at onset, presenting symptoms, comorbidities, and laboratory testing were abstracted to a secure database. All participants were specifically asked about any episodes of "cola-colored" or brown urine, and details surrounding the event.

Results. Twenty-six LGMD 2I patients have enrolled. Fourteen (54%) are homozygous for the c.826C>A mutation and 12 (46%) are heterozygous with 1 c.826 C>A allele and 1 other pathogenic allele. Self-reported age at onset of weakness was 13.6 ± 8.5 years for c.826C>A homozygotes, compared to 4.3 ± 3.5 years for patients with only 1 copy of the c.826C>A allele. Serum creatine kinase (CK) levels not associated with myoglobinuria ranged from 1,193 to 18,474 IU.

Seven patients (27%) have had at least 1 episode of myoglobinuria by self-report. Six of these 7 (86%) are homozygous for the c.826C>A mutation. Clinical features of those with myoglobinuria are summarized in the table. In 3 cases, the first bout of myoglobinuria led to the diagnosis of muscular dystrophy. In other patients, myoglobinuria occurred many years prior to diagnosis of muscular dystrophy, usually because patients did not report the problem to medical personnel (see the table). The first episode of myoglobinuria occurred before age 18 years in 6 individuals, and was precipitated by physical activity. No one in this group is known to have sustained renal damage. Serum CK as high as 56,000 IU was recorded in association with myoglobinuria.

Sixteen patients (61%) of the cohort reported significant muscle pain or cramps as part of their symptom complex, including 6 with myoglobinuria and 10 without. Eleven patients (69%) with myalgia are homozygous for c.826A>C and 5 (31%) are compound heterozygotes. Muscle pain, like myoglobinuria, was typically associated with physical exertion.

Discussion. Myoglobinuria and muscle pain were common features of the LGMD 2I phenotype in our series. Other series have variably reported myalgia[1-5] but 2 large series found a strikingly similar incidence of myoglobinuria (~25% of patients).[4,5]

Of the patients with myoglobinuria described here and in the literature, all but 2 are homozygous for the common c.826C>A allele. These patients, with a milder phenotype, may engage in more vigor-

Supplemental data at www.neurology.org

Table Clinical characteristics of patients with myoglobinuria

Patient	Genotype	Age at diagnosis of muscular dystrophy, y	Age at onset of pain, y	Age at onset of myoglobinuria, y	No. of lifetime episodes of myoglobinuria	Precipitants of myoglobinuria
1	Homozygous c.826C>A	14	13	13	>15	Swimming, hiking, weight lifting
2	Homozygous c.826C>A	20	6	11	>50[a]	Physical education class, weight lifting
3	Homozygous c.826C>A	7	9	6	4	Swimming, cold exposure
4	Homozygous c.826C>A	9	9	9	1	Wrestling match
5	Homozygous c.826C>A	32	10	16	3	Square dancing, sky diving
6	Homozygous c.826C>A	42	Cannot recall	8	>50[a]	Running, playing basketball, football
7	Compound heterozygous c.826C>A and c.1141delG	11	None	8	2	Squats, step-ups

[a] Two patients reported tea- or cola-colored urine associated with most athletic workouts over many years. (One viewed this as a sign that he had a good workout.)

ous activity (see activities listed in the table) and thus precipitate rhabdomyolysis. A similar pattern is seen in dystrophinopathy, where up to 37% of minimally symptomatic patients reported myoglobinuria,[6] compared to rare reports in typical Duchenne muscular dystrophy.

We note that one of our patients, and at least 3 others in the literature,[5,7] experienced anesthetic reaction suggestive of malignant hyperthermia. It will be important to clarify the frequency of this potential complication in future series.

Myoglobinuria can be precipitated by a wide variety of insults in healthy muscle or can be associated with myopathy. An underlying *FKRP* mutation should be considered in patients presenting with childhood-onset myoglobinuria who have sustained elevation of CK (>5× normal). In addition, management of patients with LGMD 2I should include specific questions about myalgia and myoglobinuria.

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Appendix e-1

The LGMD2 ARMS PCR panel consisted of three pathogenic gene variants: *SGCA* c.229C>T (p.R77C), *SGCB* c.341C>T (p.S114F), and *FKRP* c.826C>A (p.L276I). Nucleotide and amino acid assignments for the *SGCA*, *SGCB* and *FKRP* genes were based on reference sequences NM_000023 (*SGCA*), NM_000232 (*SGCB*), and NM_024301 (*FKRP*) using Genome Browser at UCSC and NCBI Build 36.1.

Genomic DNA was extracted either from whole blood using Puregene reagents (Gentra Systems, Inc., Minneapolis, MN) or from frozen muscle biopsy using proteinase K digestion followed by phenol/chloroform extraction and ethanol precipitation.

All ARMS PCR reaction volumes were 50 µL and *SGC* gene mutations were co-amplified separately from the *FKRP* mutation. Wild-type *SGC* alleles were amplified in 1X PCR buffer (Promega Corp., Madison, WI), 1.5 mM $MgCl_2$, 200 µM dNTPs, 1.0 µM each of c.229C>T wild-type and common primers, 0.5 µM each of c.341C>T wild-type and common primers, 1 unit of taq polymerase (Promega) and 150 ng of patient DNA. Mutant *SGC* PCR was performed in 1X PCR buffer (Promega), 1.5 mM $MgCl_2$, 200 µM dNTPs, 1.5 µM each of c.229C>T mutant and common primers, 1.5 µM each of c.341C>T mutant and common primers, 0.25 µM each of *CFTR*2 and *CFTR*4 control primers, 1 unit of taq polymerase (Promega) and 150 ng of patient DNA.

Wild-type *FKRP* c.826C>A PCR was performed in 1X PCR buffer (Qiagen, Inc., Valencia, CA), 1.5 mM $MgCl_2$, 200 µM dNTPs, 2X Q solution (Qiagen), 1.0 µM each of c.826C>A common and wild-type primers, 1.0 µM each of *CFTR*2 and *CFTR*3 control primers, 1 unit of taq polymerase (Qiagen) and 150 ng of patient DNA. Mutant c.826C>A PCR was performed in 1X PCR buffer (Qiagen), 1.5 mM $MgCl_2$, 200 µM dNTPs, 2X Q solution (Qiagen), 1.0 µM each of c.826C>A common and mutant primers, 0.25 µM each of and *FKRP*2 and *FKRP*4 control primers, 1 unit of taq polymerase (Qiagen) and 150 ng of patient DNA.

Cycling parameters were as follows: 5 min at 94^{o}C; followed by 10 cycles of 30 sec at 94^{o}C, 30 sec at 62^{o}C (decreasing 0.5^{o}C/cycle) and 30 sec at 72^{o}C; followed by 25 cycles of 30 sec at 94^{o}C, 30 sec at 57^{o}C and 30 sec at 72^{o}C; and finally 7 min at 72^{o}C. Approximately 20 µL of each amplification reaction was visualized on a 2% agarose gel stained with ethidium bromide. For size determination, a 100 bp DNA ladder (Invitrogen Corp., Carlsbad, CA) was used.

Amplification of genomic DNA for *FKRP* sequence analysis was performed in 50 µL reaction volumes containing 1X PCR buffer (Qiagen), 1.5 mM $MgCl_2$, 200 µM dNTPs, 2X Q solution (Qiagen), 1.0 µM each of *FKRP* F and R primers (table e-1), 1 unit of taq polymerase (Qiagen) and 150 ng of patient DNA. Cycling parameters were as follows: 2 min at 97^{o}C; followed by 35 cycles of 30 sec at 97^{o}C, 45 sec at 55^{o}C 3 min at 72^{o}C; followed by 10 min at 72^{o}C. The entire reaction volume was separated on a 1% agarose gel stained with ethidium

bromide. The 1.87 kb band was excised to separate it from non-specific PCR products and the DNA was purified from the agarose using a gel extraction kit (Qiagen).

Sequence analysis of *FKRP* exon 4 was performed by cycle sequencing 50 ng of exon 4 template in 10 µL reactions with Big Dye Terminator 3.1 (Applied Biosystems Inc., Foster City, CA) as recommended by the manufacturer. Seven primers, 1F-6R, listed in table e-1 were used to generate bidirectional and overlapping coverage of exon 4. An ABI3130 instrument and Seqscape software (ABI) was used for analysis. Patient results were compared to reference sequence NM_024301 (UCSC Genome Browser, NCBI Build 36.1) to identify sequence variations.

Table e-1. A.R.M.S. PCR and *FKRP* Sequencing Primers

	A.R.M.S.	
SGCA (NM_000023) c.229C>T (p.R77C)		Amplicon size (bp)
common wild type mutant	GCG CTT CTC TCG GCT CCT TAG GGC TGC GCT GGG TGT AGT G GGG GGC TGC GCT GGG TGT AGT A	415
SGCB (NM_000232) c.341C>T (p.S114F)		
common wild type mutant	TCC AAT TAC ATT AAA TGT ATG CGG CCT AC GGT GGA TCA CTC CCA TGT GAG GGT GGA TCA CTC CCA TGT CAA	174

FKRP (NM_024301) c.826C>A (p.L276I)		
common	TAG TCC CAT GGG ATG ATG TCC CCG T	275
wild type	CGC GCG CTG GGC ATC CTC C	
mutant	C CGC GCG CTG GGC ATC CGC A	
	Amplification Controls	
CFTR (NM_000492)		
CFTR-2 reverse	AGA ATA TAT GTG CCA TGG GGC CTG TG	400
CFTR-3 forward	GTG CTA AGA GTT TCA CAT ATG GTA TGA CCC	
CFTR-4 forward	TTG GCC TTC ATC ACA TTG GAA TGC	100
FKRP-2	ACA GCA TCT CCG TCC AGG GCG TCG CAG	485
FKRP-4	GGG CCC CGT CGT GCC TCT GCT G	
	FKRP exon 4 PCR	
FKRP forward	TAG GGA AAA GAA AGG GAA TTG AGA AAG AGC	1,870
FKRP reverse	CTC CCA GTG GGC ATG ACC ATT TGA TAA ATC	
	FKRP Sequencing	
1F	AA AGG GAA TTG AGA AAG AGC	
1R	GCA GGA AGG AGT CTA CCA G	
2F	AAC GTC AGC CTG CGA GAG	
2R	CCG AGA GGT TGA AGA GGT	
3F	CAT CTA CTT GGA GGA CGT G	
4R	CCT TCT CCC ATA CGA AGC	
6R	A GTG GGC ATG ACC ATT TGA T	