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Abstract

Background—Previously we reported a rare X-linked disorder, Uruguay syndrome in a single family. The main features are pugilistic facies, skeletal deformities, muscular hypertrophy despite a lack of exercise and cardiac ventricular hypertrophy leading to premature death.

Methods and Results—An approximately 19 Mb critical region on X chromosome was identified through identity by descent analysis of three affected males. Exome sequencing was conducted on one affected male to identify the disease-causing gene and variant. A splice site variant (c.502-2A>G) in the FHL1 gene was highly suspicious among other candidate genes and variants. FHL1A is the predominant isoform of FHL1 in cardiac and skeletal muscle. Sequencing cDNA showed the splice site variant led to skipping of exons 6 of the FHL1A isoform, equivalent to the FHL1C isoform. Targeted analysis showed that this splice site variant co-segregated with disease in the family. Western-blot and immunohistochemical analysis of muscle from the proband showed a significant decrease in protein expression of FHL1A. RT-PCR analysis of different isoforms of FHL1 demonstrated that the FHL1C is markedly increased.

Conclusions—Mutations in the FHL1 gene have been reported in disorders with skeletal and cardiac myopathy but none has the skeletal or facial phenotype seen in patients with Uruguay syndrome. Our data suggest that a novel FHL1 splice site variant results in the absence of FHL1A.

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Conflict of Interest Disclosures: None.
and the abundance of FHL1C, which may contribute to the complex and severe phenotype. Mutation screening of the FHL1 gene should be considered for patients with uncharacterized myopathies and cardiomyopathies.

Keywords
candidate genes; cardiac hypertrophy; gene; gene mutation; single nucleotide polymorphism; exome sequencing; gene discovery; Uruguay syndrome; skeletal deformities; cardiac ventricular hypertrophy

Introduction

We previously described the Uruguay facio-cardio-musculo-skeletal syndrome, a rare X-linked disorder found in a single family (OMIM#300280). No other families with this disorder have been reported. The main features in affected males are skeletal muscular hypertrophy despite of lack of exercise and cardiac ventricular hypertrophy leading to premature death. Other findings include a distinctive facial appearance and skeletal abnormalities consisting of large, deformed hands and feet, congenital hip dislocations, and scoliosis. The mother of the proband has a milder phenotype while other women in the family are asymptomatic.

In this study, we performed an identity-by-descent genetic analysis and exome sequencing to identify the causal gene of Uruguay syndrome, FHL1, broadening the significant phenotypic variability found with mutations in FHL1. Further expression studies on muscle and cultured myocytes demonstrate how the identified mutation may be causing some aspects of the phenotype.

Materials and Methods

Patients

Informed consent was obtained from the patients for participation in research. This study was approved by the human subjects research committees at Cedars-Sinai Medical Center (where YX and WRW were through 2013) and the Facultad de Medicina, Universidad de la República Oriental del Uruguay. Blood and muscle (trapezius) samples were taken from the proband VI-2. Other family members only provided a blood sample from which DNA was isolated.

An abbreviated, updated pedigree as of 2014 is presented in Figure 1, retaining the numbering in the original pedigree. The proband VI-2 graduated from college and is teaching school. His hypertrophic cardiomyopathy remains stable on a beta blocker and he has developed no new orthopedic problems than those originally defined. VI-1 remains healthy and has had a normal daughter. VI-3 also remains healthy and has a normal son. The proband’s affected uncle, V-7, died at 48 years of age from heart failure. The mother of the proband who had minor features of the disorder, V-2 is being evaluated for a myopathic syndrome associated with a polyneuropathy.
Identification of the gene and the mutation

**Genome-wide SNP analysis**—A genome-wide 250K NspI Affymetrix SNP microarray genotype analysis (Affymetrix, CA, USA) analysis was performed on three affected males (see Figure 1, pedigree members V-7, V-12 and VI-2) at the Microarray facility at UCLA (University of California, Los Angeles). SNP microarray gene chip data were subsequently analyzed with dCHIP software (http://www.hsph.harvard.edu/cli/complab/dchip/).

**Exome sequencing and bioinformatics analysis**—Whole-exome sequencing (WES) was performed at BGI (Beijing Genomics Institute; Shenzhen, China) on one affected male (patient VI-2; see Figure 1). Briefly, the Agilent Sure Select Human All Exon Kit was used to enrich for 50 megabases of coding sequences. A capture kit restricted to the X chromosome was not available at the time when the experiment was being performed. The captured and amplified library was loaded onto an Illumina HiSeq 2000 platform for sequencing at an estimated 50× coverage. Raw reads were aligned to the human reference genome GRCh37.69 using SOAP (Short Oligonucleotide Analysis Package) which was also used for variant calling. We identified regions shared by all three affected patients based on the genotyping array analysis and selected regions on the X chromosome that did not have a breakpoint for at least 1.5 million base pairs for further investigation in the exomic data. Variants were annotated using the software tool Variant Annotator X, which queries databases such as the Ensembl Variant Effect Predictor. Public population databases [dbSNP135 (http://www.ncbi.nlm.nih.gov/SNP/), 1000 Genomes Project, EVS database (http://evs.gs.washington.edu/EVS)] and in-house databases were used to filter sequencing variants and identify novel single-nucleotide variants (SNVs) that are predicted to alter protein function.

**Sanger sequencing**—Sanger sequencing was conducted for validation of the variants found in candidate genes identified through WES within the critical region in patient VI-2. After identifying the candidate gene, *FHL1*, which contained a novel splice site variant, DNA from 15 available family members were analyzed by Sanger sequencing for family segregation analysis. Sanger sequencing was also performed on cDNA from patient VI-2 to analyze the end products of splice site change. Primers were designed with the Primer3 program (primer sequences available on request). PCR products were sequenced with the BigDye Terminator 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). Sequencing reactions were size-separated on the ABI Prism 3100 DNA Analyzer (Applied Biosystems, Foster City, CA, USA), and sequence data was collected with the ABI Data Collection software version 1.1 and subsequently analyzed with the ABI DNA Sequencing Analysis version 3.6 software. The transcript of *FHL1* NM_001449.4 was used for sequence alignment.

**Functional study of the gene and variant**

**Western blot analysis**—For western-blot studies, proteins were extracted from 50 mg of skeletal muscle from five normal controls, and two disease controls (Becker muscular dystrophy, Duchenne muscular dystrophy). Protein concentrations of the supernatants were measured with the BCA Protein Assay Kit (Pierce). Samples were stored frozen at −80°C. Aliquots of protein (2 mg) were loaded onto a sodium dodecyl sulfate (SDS)-polyacrylamide
gel, composed of 4% stacking (0.5 M Tris-HCl [pH 6.8] and 10% SDS) and 10% resolving (1.5 M Tris-HCL [pH 8.8] and 12% SDS) gels. The protein was then transferred to nitrocellulose membranes for 1 hr (110V). Nonspecific binding was blocked for 2 hr with TBST (10 mM Tris-HCL [pH 7.4], 140 mM NaCl, and 0.1% Tween-20) containing 5% skimmed milk; this was followed by incubation with primary antibodies for 4 hr at room temperature. Rabbit polyclonal antibody anti-FHL1 (AVIVA Systems Biology) was used at a dilution of 1:5000 in TBST-5% skim milk. After washing, the membranes were incubated with the corresponding anti-rabbit or anti-mouse IgG secondary antibodies (Dako) at a dilution of 1:1000 for 1 hr at room temperature. The membranes were developed with the chemiluminescence ECL system (Pharmacia) and then exposed to autoradiographic film. Analysis of immunoreactive bands was performed semiquantitatively. The used anti-rabbit polyclonal FHL1 antibody (dilution 1:2000 AVIVA Systems Biology) recognizes the C-terminal domain of the protein and detects only FHL1A. As control maker we used caveolin-3 at 21 kDa. The antibody is only able to bind isoform FHL1A. Even though FHL1 different isoforms have common last coding exon but the C terminus actually are quite different which is demonstrated nicely in figure 1C of review paper by Cowling (2011).

**Immunohistochemistry**—In brief, after fixation with 2% paraformaldehyde and blocking with 4% BSA in PBS, samples were treated with a polyclonal anti-rabbit FHL1 antiserum (dilution 1:1000 AVIVA Systems Biology) and monoclonal anti-mouse skeletal slow myosin antiserum (dilution 1:5000, Sigma, Germany) overnight at 4°C. After rinsing for 15 min in PBS, the sections were incubated with Cy3-conjugated streptavidin goat anti-rabbit serum (Jackson-Immuno Research, dilution 1:1000) and with Alexa-Fluor 488 goat anti-mouse serum (Invitrogen). After the sections were mounted onto slides, a Leica epifluorescent microscope with a Zeiss Axiovert imaging system was used for visualization. For comparison with normal controls, as well as limb-girdle muscular-dystrophy muscle, sections were prepared using identical procedures as described above.

**RNA Isolation and Real-time PCR**—Patient myoblasts were cultured in Skeletal Muscle Cell Growth Medium Supplement (PromoCell) with 10% FBS (GIBCO), 50 µg/ml Gentamicin (GIBCO) and 7.5 ml Glutamax (per 500 ml of media) (GIBCO) at 37°C with 5% CO₂. Cells were split 1:4 after reaching ~70% confluence to avoid the generation of myotubes.

Cells were trypsinized with 0.05% Trypsin-EDTA (GIBCO) and cell pellets were washed 2× with 1× DPBS without calcium and magnesium (Life Technologies, GIBCO). RNA was extracted using RNaseasy Mini Kit (Qiagen) according to the manufacturer’s protocol and eluted in 14 µl RNase free H₂O. 800 ng RNA of each sample were transcribed using Long-Range 2 Step RT-PCR Kit (Qiagen). cDNA concentrations were determined by NanoDrop measurement and 50 ng of each cDNA were then added to Fast Sybr Green Master Mix (Applied Biosystems, Life Technologies) for semiquantitative real-time PCR. All qRT-PCR experiments were performed on the Step One PLUS Real time PCR System from (Applied Biosystems, Life Technologies). Data were analyzed by using the Step One Plus Real time PCR software (Applied Biosystems) and data were normalized to GAPDH. Numbering of the exons corresponds with NM_001159702 (FHL1B, shown in Fig.1A) beginning with
exon 3 as first coding exon. Primers for FHL1 isoform expression analysis are listed in supplementary table 1. In general, quantification experiments were performed in triplicates for each sample, except for the expression of FHL1 A/B/C of the sample with the c.688+1G>A mutation, where due to a technical error one dataset was excluded.

We performed exact Kruskal-Wallis tests to compare ΔCt values of FHL1 isoforms between the three groups (healthy control, Uruguay patient, and patient with a c.688+1G>A mutation). In case of significant results we performed post-hoc pairwise comparison between the healthy control and the patients with mutation using one-sided exact Mann-Whitney U tests.

Results

Hemizygosity mapping with dChip software detected an approximate 19 Mb shared disease haplotype for all three affected males on Xq24-q26.3, which encompasses 708 consecutive SNPs (rs1716767 [117,442,440; Xq24] to rs4829618 [136,780,188; Xq26.3]). Genomic coordinates are based on the hg19/GRCh37 genome assembly.

Based on exome sequencing data, candidate genes located within the critical region had single nucleotide change or insertion/deletion (see supplementary table 2). All the single base pair nucleotide changes detected through exome sequencing were confirmed by Sanger sequencing. Neither the insertion nor deletion was confirmed by Sanger, which might be due to the limitation of next-generation sequencing technology on detecting insertions and deletions at the time. All the single nucleotide changes except for a splice site variant in the FHL1 gene (c.502-2A>G) were found with high allele frequency in the population databases, indicating they are most likely benign polymorphisms. Sequencing of all available family members proved the segregation of the FHL1 gene (c.502-2A>G) splice variant in the family according to a X-chromosomal recessive inheritance pattern (see Figure 1).

Sequencing of cDNA derived from myoblasts of the Uruguay patient VI-2 proved skipping of the entire exon 6. The resulting primary structure is identical to the protein isoform FHL1C (figure 2B).

In Western blot experiments we showed that compared to a healthy control there is almost complete absence of the FHL1A-protein (expected at 29 kDa) in our patients’ muscle (Figure 3A). See supplemental figure for the other 4 controls and the Duchenne muscular dystrophy and Becker muscular dystrophy controls. This is consistent with the observation that we do not see any signal in the IF of tissue sections in muscle from the proband using an antibody that detects the C-terminus of FHL1A (Figure 3B).

We tested the expression of three FHL1 isoforms (FHL1A, FHL1B and FHL1C) in myoblasts from healthy individuals (control), patients with a c.688+1G>A mutation (the donor splice site mutation seen in XMPMA patients) and the Uruguay patient (proband VI-2). By using primers that span the exon junction of exon 5/6 we were also able to prove that exon 6 is missing in our patients’ myoblasts and thus the expression of isoform A and B is markedly decreased. The expression of isoform FHL1C, in which exon 6 and 7 are
missing, is highly increased in XMPMA and Uruguay patients. Interestingly, compared to the expression in the XMPMA patient the level of FHL1C in the Uruguay patient is almost 4 fold increased. Exact Kruskal-Wallis tests were significant for all isoforms (i.e. p=0.01, p=0.0035, and p=0.05 for FHL1 A/B/C (exon 3–4), FHL1C (exon 5–8), and FHL1A/B (exon 5–6), respectively). All post-hoc one-sided pairwise comparisons between the healthy control and the Uruguay patient were significant. Post-hoc one-sided pairwise comparisons between the healthy control and the patient with c.688+1 mutation were significant for FHL1C (exon 5–8) and FHL1A/B (exon 5–6) (Figure 4).

Discussion

In this study, we identified FHL1 as the causative gene for the rare X-linked Mendelian disorder, Uruguay syndrome, characterized in a previous publication,\textsuperscript{1} utilizing identity by descent mapping, exome sequencing, and further genetic and expression studies. The c. 502-2A>G splice site mutation in FHL1 identified in Uruguay syndrome patients causes the skipping of exon 6 resulting in an isoform identical to FHL1C (Figure 2B). The abundance of isoform FHL1C in the absence of FHL1A and B may explain the skeletal and cardiac muscle anomalies seen in Uruguay patients.

Four and a half LIM domain protein 1 (FHL1) encodes a transcription factor protein with highly conserved four-and-a-half tandem repeated LIM domains. The LIM domain is a protein-interaction motif and is involved in linking proteins with both the actin cytoskeleton and transcriptional machinery. Proteins containing LIM domain play important roles in various cellular processes, such as cytoskeleton organization, signal transduction, gene expression and cell differentiation.\textsuperscript{5} FHL1 (MIM#300163), located on Xq26.3, consists of 4–6 coding exons (dependent on the isoform) and is expressed in skeletal and cardiac muscle cells,\textsuperscript{6} where it is suggested to play a role in sarcomere synthesis and assembly. FHL1 has been identified as the causative gene for six different X-linked myopathies, with patients often presenting with cardiovascular disease.\textsuperscript{7} Different mutations in the FHL1 gene lead to different myopathies. Since 2008, there are about 40 mutations reported in FHL1 related myopathies with a wide spectrum of clinical phenotypes. It appears that different phenotype and severity may be due to which LIM domain that FHL1 the mutation occurs and if all isoforms are affected (See review by Schessl et al\textsuperscript{8} for more detail). Although these myopathies share some overlapping clinical features, they differ with respect to age of onset, distribution and severity of the disease (see review by Cowling et al\textsuperscript{9} and, Schessl et al\textsuperscript{8}). It is unclear how different FHL1 mutations lead to distinct muscle diseases.

There are at least three isoforms of FHL1 (FHL1A, 1B, 1C; see Figure 2A) generated by alternative splicing. These isoforms differ in primary structure, expression pattern, binding partners and subcellular locations. The FHL1A (commonly referred as FHL1) isoform contains all exons except exon 7. The C-terminal sequence of full-length isoform FHL1B binds RBP-Jk. Isoform FHL1C, with two and a half LIM domains, skips exons 6 and 7 with a similar C-terminus as FHL1B. FHL1A is the predominant isoform in heart and skeletal muscle; FHL1B and C are also expressed at a much lower level.\textsuperscript{6,10} Each of the FHL1 protein isoforms appears to exhibit distinct functions. However, in striated muscle much less
is known of the functions of FHL1B and FHL1C. Both proteins are hypothesized to play an important role during myogenesis via regulation of RBP-Jκ function. The toxic accumulation of protein aggregates (i.e. Toxic gain of function) is suggested to be responsible for the diagnostic feature of severe FHL1 associated reducing body-myopathy. Reducing body aggregates are observed in skeletal muscle from a few patients with XMPMA, however not in EDMD Plus, or Uruguay syndrome at least by light microscopy. The majority of FHL1 mutations in EDMD patients are frameshifts, which are predicted to result in premature stop codons. These novel mRNA transcripts serve either as templates for the expression of truncated protein products or loss of expression due to nonsense mediated-decay. Loss of normal FHL1A protein is present in all the disorders. XMPMA and Uruguay syndrome have muscular hypertrophy and elevated FHL1C protein levels. Although FHL1C expression is also increased in EDMD, muscular hypertrophy has not been reported. Why the phenotypes of the 3 disorders are significantly different in many respects cannot be explained on the basis of gain of function due to overexpression of FHL1C, unless there is a significant difference in the amount of FHL1C protein produced among the patients.
disorders. It is interesting to note that there is a missense variant in the BCORL1 gene identified in Uruguay patient (see suppl. Table 2). BCORL1 can act as a corepressor when tethered to DNA. To date, this gene has not been associated with any disease. Variants in this gene have only been found in individuals with autism and intellectual disability. Even though the variant in this gene is predicted to be deleterious by computational algorithms and this gene is expressed in heart and muscle, we are not sure how the variant in this gene can contribute to the phenotype seen in Uruguay patients or any patients with muscular disorders.

In conclusion, we have identified the FH1L1 gene as the causative gene for Uruguay syndrome. Our study demonstrated that the splice site mutation of FH1L1 detected in patients with Uruguay syndrome leads to abundance of isoform FH1L1C and absence of other isoforms, especially FH1L1A, in skeletal muscle what might explain skeletal muscle hypertrophy and cardiac dysfunction. The findings of the current study have important implications for diagnostic evaluation, screening, and genetic counseling of patients (also carriers) with myopathy of an unknown genetic cause, particularly in cases where pedigree information would suggest X-linked inheritance.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

**Acknowledgments**

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Clinical Perspective

Mutations in the FHL1 gene have been reported in disorders with skeletal and cardiac myopathy but none has the skeletal or facial phenotype seen in patients with Uruguay syndrome. Our data suggest that a novel FHL1 splice site variant results in the absence of FHL1A and the abundance of FHL1C, which may contribute to the complex and severe phenotype. Mutation screening of the FHL1 gene should be considered for patients with uncharacterized myopathies and cardiomyopathies. The findings of the current study have important implications for diagnostic evaluation, screening, and genetic counseling of patients (also carriers) with myopathy of an unknown genetic cause, particularly in cases where pedigree information would suggest X-linked inheritance.
Figure 1.
Updated pedigree with segregation analysis of available family members.
Figure 2.
A. Isoforms of FHL1. B. Splice site changes of exon 6 in Uruguay syndrome, XMPMA and EDMD plus patients.
Figure 3.
A. Western blot for FHL1A. Different dilutions (1:5 and 1:2) and different amounts (10uL and 5uL) of antibody to FHL1A and duplicates of Uruguay patient (A and B) were used. In all patient samples there is no normal FHL1 band at 37 kDa, but a minor degraded band at ~28 kDa. As a loading cytoskeletal control marker at the bottom of the western blot, caveolin-3 was used with a band at 21 kDa. B. Immunohistochemistry for FHL1A. Immunohistochemistry was performed in muscles from Uruguay syndrome patient, healthy
controls, and a Becker muscular dystrophy patient. In the Uruguay syndrome patient the FHL1 antibody shows no staining compared to the normal membrane staining in the muscles of both controls and the Becker muscular dystrophy patient. All internal non-membrane bound staining is an artifact. Bars adjusted to 50 µm.
Figure 4.
Expression of FHL1 isoforms in myoblasts. The expression of all FHL1 isoforms (FHL1A, FHL1B and FHL1C) was tested in myoblasts from healthy individuals (control), from patients with a c.688+1G>A mutation (donor splice site mutation) and the Uruguay patient. In general, quantification experiments were performed in triplicates for each sample, except for the expression of FHL1 A/B/C of the sample with the c.688+1G>A mutation, where due to a technical error one dataset was excluded. The error bars in the three panels display the calculated mean, maximum and minimum expression levels presented as RQ, RQmin and RQmax. RQmax and RQmin are based on the 95% confidence interval of ΔCt.
Table 1
Phenotype comparison among Uruguay, XMPMA and EDMD Plus and reducing body myopathy

|                  | Uruguay syndrome (1 family) | XMPMA (7 families) | EDMD Plus phenotype (1 family) | Reducing body myopathy (RBM) |
|------------------|-----------------------------|--------------------|--------------------------------|-----------------------------|
| **Age onset**    | Male: 1 – 5 yr, Female: mild, most unaffected | Male: 24 yr, Female: 44 yr | Male: as early as birth, Female: unaffected | Male: 5 yr, Female: 13 yr |
| **Skeletal**     | Scoliosis, toe and hip dislocation, pes cavus, broad hands, No rigid spine | Rigid spine; scapular winging; contractures | Kyphosis, contractures, pectus deformity, brachydactyly, wide feet | Rigid spine; scapular winging; contractures; scoliosis |
| **Cardiac**      | Hypertrophy                 | Hypertrophy        | Hypertrophy and arrhythmia      | Less common, dilated        |
| **Muscle**       | Hypertrophy overall         | Atrophy of postural muscles, hypertrophy of proximal upper limb | Proximal muscle weakness      | Proximal muscle weakness    |
| **Dysmorphic facial features** | Yes                         | No                 | Yes                            | No                          |
| **Other**        | No                          | Respiratory involvement | Pulmonary artery hypoplasia, learning disability | Respiratory involvement |
| **Death**        | Young adult (~30) due to cardiac failure (untreated) | Adult (42 – 70) cardiac and respiratory failure | One age 16 (? arrhythmia) | Infantile and childhood respiratory failure |
| **Reference**    | 1                           | 14                 | 12                             | 8                           |