Isolated X-Linked Hypertrophic Cardiomyopathy Caused by a Novel Mutation of the Four-and-a-Half LIM Domain 1 Gene

Running title: Hartmannova et al.; Isolated HCM due to FHL1 mutation

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Abstract:

Background - Hypertrophic cardiomyopathy (HCM) with severe left ventricular diastolic dysfunction (LVDD) has been associated with marked exercise intolerance and poor prognosis. However, molecular etiology of this phenotype remains unexplained in a large proportion of cases.

Methods and Results - We performed whole exome sequencing as an initial genetic test in a large Czech family with three males affected by non-obstructive HCM with severe LVDD in end-stage disease. A novel frameshift mutation of four-and-a-half LIM domain 1 gene (FHL1) (c.599_600insT; p.F200fs32X) was detected in these individuals. The mutation does not affect transcription, splicing and stability of FHL1 mRNA and results into production of truncated FHL1 protein, which is contrary to heart tissue homogenate not detectable in frozen tissue sections of myocardial biopsy of affected males. The identified mutation co-segregated also with abnormal electrocardiogram and with one case of apical HCM in heterozygous females. Although skeletal muscle involvement is a common finding in FHL1-related diseases, we could exclude myopathy in all mutation carriers.

Conclusions - We identified a novel FHL1 mutation causing isolated HCM with X-chromosomal inheritance.

Key words: hypertrophic cardiomyopathy, diastolic function cardiomyopathy, diastolic heart failure, exome, gene mutation, four-and-a-half LIM domain 1 gene
Introduction:

Severe left ventricular diastolic dysfunction (LVDD) in individuals with hypertrophic cardiomyopathy (HCM) has been associated with a poor exercise tolerance and prognosis both in adults and children with this disease (1-7). Severe LVDD may occur either as an initial manifestation of HCM with limited left ventricular hypertrophy or it may represent advanced disease, an alternative to end-stage dilated HCM (6). Recently, mutations of beta-myosin heavy chain, cardiac troponin I (7,8) and cardiac troponin T (9) have been identified as disease genes for HCM with severe LVDD.

We report on a large family with isolated X-linked HCM with severe LVDD co-segregating with a novel mutation of FHL1 gene encoding four-and-a-half LIM domain protein 1 (FHL1). Over 30 mutations of FHL1 have been associated with five different X-linked myopathies – reducing body myopathy [MIM 300718], scapuloperoneal myopathy [MIM 300695], X-linked myopathy with postural muscle atrophy [MIM 300696], rigid spine syndrome and Emery-Dreifuss muscular dystrophy [MIM 310300] with a variable cardiac involvement presenting either as dilated or hypertrophic cardiomyopathy (10-14). In addition, a recent report described two novel variants of FHL1 causing isolated HCM (15). This report extends the spectrum of FHL1-related diseases by description of FHL1 as a causal gene for isolated HCM with severe LVDD in advanced disease.

Methods:

1. Study population

The index patient was identified in a large Czech family with three males affected by HCM who developed severe LVDD and advanced heart failure in two cases. A detailed family history was obtained, and an extended pedigree was constructed (Figure 1) according to the guidelines (16).
Further investigations were approved by participating center's Institutional Review Boards, and were conducted according to the Declaration of Helsinki principles. Written, informed consent was obtained from all subjects.

2. Cardiologic screening

We examined 20 members of the above family. Medical records were acquired in the proband’s uncle who was treated in our institution and died after heart transplantation. The cardiologic screening included physical examination, electrocardiography and echocardiography.

Echocardiography was performed by a single operator (T.M.) in accordance with guidelines of the American Society of Echocardiography (17,18). M-mode, 2D images, conventional and tissue Doppler recordings were obtained using a Vivid 7 (GE Healthcare, Chalfont St Giles, UK). Left ventricular ejection fraction was assessed using Simpson’s biplane method. Mitral inflow pattern was classified as restrictive in the presence of E-wave deceleration time < 120 ms or a ratio of early transmitral flow velocity to atrial flow velocity ≥ 2 associated with an E-wave deceleration time ≤ 150 ms (19).

The proband and his brother underwent cardiovascular magnetic resonance (Siemens Trio scanner, Siemens Medical Solutions, Erlangen, Germany). Ventricular volumes, ventricular mass and ejection fractions were obtained from standard cine images in short-axis (repetition time/echo time 65/1.2 ms; slice thickness of 8 mm without inter-slice gap) using a dedicated software Segment 1.8 (http://segment.heiberg.se) (20). Images for visualization of delayed contrast enhancement were acquired 10-15 min after intravenous bolus injection of 0.2 mmol/kg of Gadobutrol (Gadovist, Bayer Schering, Germany) in short-axis and orthogonal long-axis planes using phase-sensitive inversion-recovery sequences (repetition time/echo time 690-850/3.2 ms; adjusted inversion time; slice thickness of 8 mm; inter-slice gap of 0.8 mm; in-plane...
resolution of 1.7 x 1.7 mm). A similar magnetic resonance study was available also in the
proband’s uncle (II/3).

3. Molecular genetic analysis

Genomic DNA of all available individuals was extracted from whole blood samples using a
standard technology. Genomic DNA of the proband’s uncle was available from a previous
research project (21). With respect to known genetic heterogeneity of familiar cardiomyopathies,
the whole exome sequencing was performed as an initial genetic test. Exome sequencing was
performed using 2 μg of DNA from three affected individuals (II/3, III/4, III/5). For DNA
enrichment, individually bar-coded DNA libraries (22) and SureSelect All Exome Kit V4 (Agilent,
Santa Clara, USA) were used according to the manufacturer’s protocol. DNA sequencing was
performed on the captured barcoded DNA library using SOLiD™ 4 System (Applied
Biosystems, Carlsbad, USA) at the Institute for Inherited Metabolic Disorders (Prague, Czech
Republic). A 50 base pair reads were aligned in color space to the reference genome (hg19) using
NovoalignCS version 1.08 (Novocraft, Malaysia) allowing for up to six mismatches. Sequence
variants in analyzed samples were identified using SAMtools package (version 0.1.8) (23). The
high confidence variants, (quality >50 and coverage >10X), were annotated using ANNOVAR
Annotation tool (hg19). Only the sequence variants present in all three affected individuals and
having frequency lower than 0.001 in the dbSNP, 1000 Genomes, Exome Variant Server
(http://evs.gs.washington.edu/EVS/) and internal exome database were prioritized for further
analysis. Identified genetic variants were filtered according to autosomal dominant or X-linked
genetic model of the disease which was inferred from the pedigree structure and the resulting 26
candidate coding variants were evaluated in GeneDistiller (24), according to biological relevance
of corresponding genes to cardiomyopathy. Candidate variants were visualized in Integrative
Genomics Viewer (IGV) - version 1.5.65. Mutation-bearing fragment of \textit{FHL1} (NM_001159702) was PCR amplified from genomic DNA of all available individuals from the family and sequenced using version 3.1 Dye Terminator cycle sequencing kit (Applied Biosystems, Foster City, CA) with electrophoresis on an ABI 3500XL Avant Genetic Analyzer (Applied Biosystems). Data were analyzed using Sequencing Analysis software, and the segregation of the candidate \textit{FHL1} mutation with the phenotype was assessed.

4. Analysis of myocardial samples

\textit{Material collection}

Myocardial specimens from hearts explanted during transplantation were available in the proband (III/4) and his uncle (II/3). Samples from all major anatomic locations of the proband’s heart were prospectively saved for histopathology, immunohistochemistry, electron microscopy and were also snap-frozen for molecular analyzes. Explanted heart from the male patient II/3 was available as formaldehyde fixed paraffin embedded tissue (FFPE) from all major anatomic heart locations and small snap-frozen samples for molecular analyzes from another research project (21).

\textit{Control samples}

Snap-frozen myocardial specimens were obtained from four hearts explanted during heart transplantation. Underlying cardiac disease was dilated cardiomyopathy in control 1 a 18-year-old female and control 2 a 52-year-old male. Ischemic heart disease had control 3 a 53-year-old male and control 4 a 52-year-old female.

\textit{Histology and immunohistochemistry}

FFPE samples were sectioned (4 \( \mu \text{m} \)) and processed for histology and immunohistochemistry.

The paraffin sections were stained by standard histological techniques (hematoxylin-
eosin, trichrom stain). Immunohistochemistry of sarcomeric actin, desmin, lysosomal-associated membrane proteins (LAMP) 1 and 2, and beta subunit of mitochondrial ATP synthase (ATP B) was applied on selected paraffin sections. Immunohistochemical findings were evaluated in comparison with those seen in a set of previously examined myocardial specimens diagnosed for cardiomyopathies based on mitochondrial disorders, lysosomal storage disorders and amyloidosis, and for dilated or hypertrophic cardiomyopathy not otherwise specified for a genetic defect.

**Immunohistochemical analysis of FHL1 protein**

The monoclonal antibody detecting FHL1 (Ab58067, Abcam, Cambridge, UK) was tested in both paraffin and cryostat sections of myocardium and skeletal muscle including demasking of antigen epitopes in FFPE material. Specific and sensitive results were obtained only in unfixed frozen tissues. Immunohistochemical detection of FHL1 was therefore performed in cryostat sections of myocardium from the proband III/4. Frozen samples from male patient II/3 were no longer available for immunohistochemical analysis. Cryostat sections (5 μm) fixed in cold anhydrous acetone for 10 min at -20°C were dried for 20 min at room temperature, then underwent standard blocking procedures. Primary mouse monoclonal antibody (Ab58067, Abcam, Cambridge, UK) was applied diluted 1: 100 in 5% BSA, in PBS. Sections were incubated for 1 hour at 37°C with the primary antibody, then washed in PBS. Detection of bound primary antibody was achieved using Dako EnVision+ System-HRP Mouse (Dako, Glostrup, Denmark) with 3,3’- diaminobenzidine as substrate.

**Electron microscopy**

Samples fixed in 10% paraformaldehyde were subsequently embedded into an Epon-Araldite mixture, double stained, and examined using a Jeol 1200 electron microscope.
**Qualitative (RT-PCR) analysis of FHL1 mRNA**

Total RNA was isolated from snap-frozen myocardial specimens using the TRIZOL solution (Invitrogen, Carlsbad, Ca). RNA concentration was determined spectrophotometrically at 260 nm by NanoDrop (NanoDrop Technologies, Wilmington, USA) and quality was checked on Agilent 2100 bioanalyzer - RNA Lab-On-a-Chip (Agilent Technologies, Santa Clara, USA). Aliquots of isolated RNA were stored at –80°C until analysis. The first-strand cDNA synthesis was carried out using oligo-dT primer and SuperScript® III Reverse Transcriptase (Life Technologies, Carlsbad, USA). FHL1 cDNA was PCR-amplified from the synthesised first-strand cDNA using oligonucleotide primers cFHL1_135U 5´-CCCATCGGTGCGGACTCCAAG-3´ and cFHL1_1013L 5´-TTTGGCACAGTCGGGACAATA-3´ designed to span and amplify all three FHL1 mRNA isoforms in parallel. Obtained PCR products were sequenced using version 3.1 Dye Terminator cycle sequencing kit (Applied Biosystems, FosterCity, CA) with electrophoresis on an ABI 3500XL Avant Genetic Analyzer (Applied Biosystems).

**Quantitative (qPCR) analysis of FHL1 mRNA.**

The reactions were carried out in a 96-well plate in a 20-µl reaction volume containing 10 µl of 2×Maxima SYBR Green qPCR Master Mix (Thermo Scientific); 0.2 µM of cFHL1_135U and cFHL1_1013L primers and 5 ng of cDNA on a StepOne Plus Real Time System (Applied Biosystems). Data were analyzed by StepOne Software v 2.0. The comparative Ct (ΔΔCt) method was used to normalize FHL1 mRNA to GAPDH mRNA amounts.

**Western blot analysis of FHL1 protein**

Frozen myocardial specimens were homogenized under liquid nitrogen, dissolved in 10mM Tris, 10mM KCl, 2mM EDTA, 4% glycerol, 1mM DTT, Complete Protease Inhibitor Cocktail (Roche
Diagnostics, Basel, Switzerland), centrifuged at 15,000 g for 30 min at 4°C, and assessed for protein content in the supernatant using the Bradford assay. Homogenate aliquots corresponding to 30 μg of total protein were resolved on 10% SDS-PAGE and transferred to PVDF membrane. Membranes were blocked by 5% BSA, 0.05% Tween 20 in PBS. FHL1 was visualised by incubation with mouse monoclonal antibodies potentially detecting all three FHL1 isoforms (Ab58067, Abcam, Cambridge, UK) diluted 1:2000 in 5% BSA, 0.05% Tween 20 in PBS for 90 min, followed by incubation with goat anti-mouse HRP (Pierce), 1:20000 in 0.05% Tween 20 in PBS for 60 min and detection by SuperSignal West Pico Maximum Sensitivity Substrate (Pierce).

5. Neurological assessment

All living individuals with FHL1 mutation underwent neurological assessment focused on detection of possible muscle disability (muscle atrophy, muscle strength testing), including standard routine examination of the central and peripheral nervous systems. In adult patients with FHL1 mutation, nerve conduction studies of the motor and sensory nerves of lower extremities were performed to exclude neuropathy. Two muscles (left vastus medialis and left deltoid muscle) were investigated by needle electromyography. Electromyographic signals were evaluated using commercially available analyzer Medelec Synergy (Viasis Neurocare, Madison, Wisconsin, US) to detect possible muscle disease. In addition, all living individuals with FHL1 mutation had a biochemical analysis to assess creatine phosphokinase activity and myoglobin concentration in serum.

Results

Clinical features of the proband

The proband was a 31-year-old male (III/4) with a history of non-obstructive HCM since 18
years of age who presented with dyspnea in New York Heart Association (NYHA) functional class IV, right heart failure and new-onset atrial fibrillation with a rapid ventricular response. Symptoms of end-stage heart failure persisted even after successful cardioversion to sinus rhythm. Proband’s electrocardiogram showed signs of biatrial enlargement and left ventricular (LV) hypertrophy (Figure 2). In comparison with previous data (Table 1), the most recent echocardiography study demonstrated non-obstructive HCM with less prominent LV hypertrophy, preserved LV systolic function and severe LVDD. Cardiac catheterization revealed normal coronary arteries, low cardiac output, elevated filling pressures of both ventricles and borderline pulmonary artery pressures (Table 1). These findings were compatible with advanced heart failure due to non-obstructive HCM with severe LVDD. The patient underwent an uneventful heart transplantation six months later. His family history revealed diagnosis of HCM in his brother and uncle.

**Pedigree analysis**

**Male patient III/5**

Brother of the proband was diagnosed with non-obstructive HCM aged 17. At 30 years of age, he reported mild breathlessness. ECG showed 1st degree of atrioventricular block, signs of biatrial enlargement and LV hypertrophy (Figure 2). Echocardiographic findings were compatible with non-obstructive HCM with severe LVDD (Table 1).

**Male patient II/3**

Uncle of the proband experienced bilateral decompensation of heart failure aged 55 years. Subsequent cardiologic examination revealed non-obstructive HCM with severe LVDD disproportional to mild to moderate LV systolic dysfunction (ejection fraction 40-45%) (Table 1, Figure 3). Aged 59 years, he was admitted to our institution due to end-stage heart failure and
underwent an urgent heart transplantation. Unfortunately, the postoperative course was complicated by severe right ventricular failure, hepatorenal failure and septicemia. The patient died 4 months after transplantation.

**Female patient I/2**

Grandmother of the proband was diagnosed with apical form of HCM during cardiologic screening in the family. This 83-year-old lady reported a history of myocardial infarction, diabetes and breathlessness NYHA class III; however, she did not experience any episode of heart failure. Echocardiography revealed apical hypertrophy of the left ventricle beginning in the mid-ventricular segment (septum 14 mm, lateral wall 12 mm) reaching 15-16 mm in the apical third (Figure 3). The patient refused magnetic resonance imaging and any other examinations.

**Results of the cardiologic screening**

Cardiologic examination in the remaining family members revealed electrocardiographic signs of LV hypertrophy (Figure 2) in two asymptomatic females heterozygous for FHL1 mutation (II/1-aunt of the proband, age 63y; II/2- mother of the proband, age 53y). Echocardiography demonstrated normal findings in both females except of modest increase in LV mass (98g/m² and 90/m², respectively). Mild electrocardiographic abnormalities, such as mild depression of ST segments and either negative or biphasic T waves in leads III and aVF, were detected in two other females heterozygous for FHL1 mutation (III/1, age 41y; III/8, age 34y) and one male hemizygous for FHL1 mutation (IV/2, age 17y). All these subjects were asymptomatic and had normal echocardiographic findings. The results of the cardiologic screening were normal in the remaining members of the family including a female heterozygous for FHL1 mutation (IV/1, age 20y) and a male heterozygous for FHL1 mutation (IV/9, age 2y).
Genetic and molecular biology analyses

To directly identify possible disease-causing mutation we performed exome sequencing in three affected individuals - (II/3, III/4, III/5). From the sequencing run we obtained from these samples 128, 151 and 109M of sequencing reads, respectively, of which on average 57% we were able to map uniquely on the human genome reference sequence (hg19). Compared to the reference sequence we identified in these data 16486, 17953 and 16591 variants, respectively. Among them we identified 8515 single nucleotide variants (SNVs), (SNP/indel qual > 50) that were present in all three affected probands. From these variants, 69 SNVs (2 stop-gain, 46 non-synonymous SNVs and 21 synonymous SNVs) and 3 indels were either novel or present at frequencies lower than 0.01 in the 1000 Genomes, Exome Variant Server (http://evs.gs.washington.edu/EVS/) and internal exome database (> 200 exomes). Resulting 51 rare coding variants, 48 SNVs and 3 indels (Supplemental Table 1), were further evaluated in GeneDistiller (24) according to potential biological and clinical relevance of corresponding genes to cardiomyopathy. From this evaluation emerged as the only potentially causative variant the frameshift insertion c.599_600insT in exon 6 of FHL1 (NCBI reference sequence: NM_001159702). FHL1 encodes the four-and-a-half-LIM-domain protein 1, (FHL1). The gene is transcribed into three alternatively spliced mRNA isoforms FHL1A, FHL1B and FHL1C, encoding FHL1A, FHL1B and FHL1C proteins, respectively. FHL1A is by far the most abundant form. It is highly expressed in skeletal muscle, moderately in heart and to a much lesser extent in a wide array of other tissues (10,11). Compared to FHL1A the other two isoforms are expressed at much lower abundance and demonstrate tissue specific expression patterns. FHL1B is specifically expressed in brain, whereas FHL1C is expressed in skeletal muscle and at lower levels in aorta, left atrium, left, and right ventricles of human heart (10,11,25). The insertion encodes for a frameshifts in
translation of *FHL1A* and *FHL1B* isoforms, that are in both cases followed soon thereafter by a novel stop codon predicting proteosynthesis of one sequentially identical truncated form of the FHL1A containing a neo-peptide composed of 32 aminoacids (200 HRCGGPVLLRGLLQELCGQEVC WMQEPHHWVW-232) on the C-terminal end (p.F200fs32X). Located in alternatively spliced exon 6, the identified insertion is not predicted to affect proteosynthesis of the FHL1C variant (Figure 4A). We confirmed the presence of the c.599_600insT variant of *FHL1* in genomic DNA of probands by Sanger sequencing (Figure 4B) and using the same technique we subsequently demonstrated segregation of the mutation with the phenotype of cardiac disease in the family (Figure 1).

To characterize molecular consequences of the identified mutation, we assessed expression of *FHL1* mRNA isoforms and presence of FHL1 proteins in patients and control tissues obtained from two females (C1F and C4F) and two males (C2M and C3M). We performed quantitative PCR and RT-PCR analysis of total RNA extracts and Western blot analysis of protein homogenates prepared from the snap-frozen myocardial specimens. Quantitative PCR analysis suggested that FHL1 transcription may be generally higher in females than in males and that the mutation and related dysfunction of FHL1 protein may stimulate FHL1 transcription in affected males. (Figure 4C). RT-PCR analysis revealed in all analyzed myocardial specimens presence of a single RT-PCR product of the size expected for *FHL1A* isoform (Figure 4D). The Sanger sequencing and sequence analysis demonstrated that the obtained RT-PCR products correspond to *FHL1A* mRNA isoform and independently confirmed in patients’ samples the presence of the frameshift insertion c.599_600insT identified previously in corresponding genomic DNA (not shown). In accordance with this finding, Western blot analysis of myocardial homogenates revealed in patient’s samples presence of the immune-
reactive protein of a molecular weight ~ 27 kDa corresponding to predicted molecular weight of
the p.F200fs32X FHL1 protein. Immunoreactive protein of a molecular weight ~ 32 kDa,
corresponding to predicted molecular weight of the FHL1A identified in control samples, was in
patients’ samples absent. Immunoreactive protein of a molecular weight ~ 22 kDa,
corresponding to predicted molecular weight of the FHL1C was not detected. Instead, the
analysis revealed in all control’s and patient’s samples presence of the immunoreactive protein
of a molecular weight ~ 25 kDa, which identity is unknown (Figure 4E). In contrast to Western
blot, the immunohistochemical staining of cryostat sections of myocardium revealed in the
proband III/4 absence of any immunoreactive FHL1 protein (Figure 4F) in comparison to
distinct cross-striation pattern observed in control myocardium (Figure 4G).

**Morphological findings in two explanted hearts**

**Male patient III/4 (proband):**

The explanted heart weighted 346 grams (less than the upper limit of normal 400 grams). On
gross pathology, both ventricles were non-dilated with relatively thin walls (left ventricle 9-12
mm, right ventricle 5 mm) and discrete focuses of fibrosis on incision. Both atria were dilated
with limited amounts of tissue missing due to surgical reasons. Coronary vessels showed mild
intimal thickening. Histopathology revealed hypertrophy of cardiomyocytes with anisonucleosis
and hyperchromasia, interstitial and replacing fibrosis, subendocardial scars and vacuolization of
cardiocytes, and mild sclerosing arteriosclerosis. Disarray of cardiomyocytes was present,
involving 5-15% of the tissue section in the left ventricular wall (Figure 5A, C, E). Sarcomeric
actin and desmin detected immunohistochemically revealed an usual cross-striation pattern
without detectable inclusions, being slightly disordered in focuses of myocyte disarray (Figure
5F). There were no abnormalities of lysosomal and mitochondrial compartments, based on
immunohistochemistry. Electron microscopy showed prevalent regular organization of myofibrils and increased number of mitochondria, in subsarcolemmal localization or focally replacing myofibrils. Mitochondria did not show any substantial abnormalities in size, or shape and organization of their crists. Disorganized myofibrils in cardiocytes were found exceptionally (Figure 5G, H). Morphological findings were comparable in both ventricles.

**Male patient II/3 (proband’s uncle)**

The explanted heart weighted 578 grams (the upper limit of normal 400 grams). Gross pathology showed mild dilatation of both ventricles with thickened LV wall (10-18 mm), dilated atria and mild atherosclerotic plaques of coronary arteries. Histopathology demonstrated hypertrophy of cardiomyocytes, focal myocyte disarray less frequent than in the proband and marked interstitial and replacing fibrosis (Figure 5B, D). Immunohistochemical staining for sarcomeric actin and desmin showed patterns comparable with that in the proband. No abnormalities of lysosomal and mitochondrial systems were detected using immunohistochemistry. The histopathological changes involved both ventricles.

In summary, both patients, the proband (III/4) and his uncle (II/3) showed to a different degree of hypertrophy and disarray of cardiocytes together with interstitial fibrosis, which are features consistent with HCM at the histological level.

**Neurological assessment**

Mutations in FHL1 have been identified as the cause of several skeletal muscle diseases. In this respect, carriers of the c.599_600insT mutation underwent neurological examination.

Neurological status of individuals with *FHL1* mutation was unremarkable, except of mild muscle hypotrophy in the proband. However, needle electromyographic recordings as well as nerve conduction studies showed normal findings. Similarly, biochemical markers of muscle injury in
peripheral blood had normal values in all individuals with FHL1 mutation. Taken together, there were no signs of muscle disease in these individuals.

**Discussion:**

In this report, we describe a Czech family in which frameshift mutation c.599_600insT in exon 6 of FHL1, a gene located on chromosome X, co-segregated with non-obstructive HCM with severe LVDD in advanced disease in three hemizygous males. Penetrance of this mutation was age-dependent and included manifestation of HCM in hemizygous males in the second to sixth life decade and progression into advanced heart failure in two of these individuals in the fourth to sixth decennium. On the other hand, mild cardiac involvement in heterozygous females, consistent with random X-inactivation of mutated FHL1, included asymptomatic abnormalities of electrocardiogram with a modest increase in LV mass since the sixth decennium and one case of apical HCM in the ninth decennium. Microscopic features compatible with HCM were detected in both cases available for histopathological analyses. None of the individuals showed signs of skeletal muscle disease.

**Molecular consequences of c.599_600insT FHL1 mutation**

The above mutation is either very rare or even private, as it has not been observed in any of the publically searchable human genome, exome or polymorphism and mutation databases as well as in our internal population specific human exome sequence database (> 200 exomes). The mutation does not affect synthesis and stability of FHL1A mRNA in heart tissue and the transcript seems to be translated into truncated 27 kDa form of the FHL1 protein which is probably missing LIM3 and LIM4 domains, and containing the neo-peptide composed of 32 aminoacids on C-terminus. In parallel, the mutation which is predicted to abolish proteosynthesis of FHL1A and FHL1B proteins, should not have impact on proteosynthesis of FHL1C. In our
study, we have not found any evidence for expression of FHL1C mRNA isoform and production of FHL1C protein (of predicted molecular weight) in analyzed control and patient derived myocardial specimens. Instead, we detected in all these samples presence of an immunoreactive protein of a molecular weight ~ 25 kDa. Origin of this protein is unknown and it remains to be investigated whether it represents post-translationally modified FHL1C isoform, proteolytically processed FHL1 protein, or simply nonspecificity of the antibodies used in this study. The amounts of the p.F200fs32X FHL1 protein in homogenates of patients myocardial specimens were comparable to amounts of FHL1A in control samples. Contrary to this observation, we found reduction of FHL1 signal in frozen tissue sections from patients specimens. Decrease in FHL1 staining in this material thus suggests that p.F200fs32X mutation results in proteosynthesis of truncated neo-protein, which has, either due to loss of LIM 3 and LIM4 domains and/or presence of the neo-peptide on its C-terminal end, altered structural properties limiting function and immunodetection of the mutant protein in the heart tissue.

**Reasons for preserved skeletal muscle function in p.F200fs32X FHL1 mutation**

Our finding of isolated HCM resulting from complete structural and functional deficiency of FHL1A demonstrates different biological and functional roles of FHL1 proteins in skeletal and heart muscle. In skeletal muscle, it has been shown that FHL1 may have multiple roles in myoblast migration and elongation, satellite cell activation, inhibition of myoblast apoptosis, regulation of skeletal muscle mass, sarcomere formation and Notch signaling (10,11). Clinical studies revealed that C-terminal FHL1 mutations preserving synthesis of FHL1C are responsible for less severe myopathic phenotype than the N-terminal ones (10,26). Importantly, FHL1C isoform seems to be expressed much stronger in human skeletal muscle than in myocardium (25). Preserved expression of the FHL1C isoform in skeletal muscle may explain absence of
myopathy in our case. Unfortunately, we had no opportunity to analyze skeletal muscle specimens in our patients.

In addition, missense mutations disrupting zinc-binding residues critical for tertiary structure of FHL1 result in more severe myopathy than FHL1 truncations (10). This can be illustrated by replacement of highly conserved cysteine binding a zinc-ion within LIM3 domain (c. 625T>C; p.C209R). Although this mutation affects the same exon and LIM domain as in our case, it causes not only hypertrophic cardiomyopathy but also Emery-Dreifuss myopathy with cytoplasmatic bodies suggesting misfolding of mutated FHL1 protein (13). This observation provides an alternative hypothesis, that mutations leading to reduced amounts (or even absence) of FHL1 proteins are less deleterious (or even benign, as in our case) for skeletal muscle than missense mutations exerting their pathogenic effects through misfolding, self-aggregation and co-aggregation of FHL1-binding partners (10,11,14).

Practical implications

HCM with severe LVDD has been associated with mutations of myofilament proteins (beta-myosin heavy chain, cardiac troponin I, cardiac troponin T, alpha-cardiac actin) (7,8) and Z-disk proteins (myopalladin) (27). However, myofilament mutations explained in the above mentioned study (7) approximately 50% cases of HCM with severe LVDD. In our study, phenotype of HCM with severe LVDD seemed to be associated rather with advanced disease than to be a primary phenotype of the above mentioned FHL1 mutation. As shown by Friedrich et al., FHL1 mutations may explain etiology of isolated HCM in 2.5% of cases unexplained by traditional sarcomeric mutations (15). In addition, a recent report of Binder et al. extends the spectrum of FHL1-related diseases by description of spongious HCM in individuals with X-linked myopathy with postural muscle atrophy (28). In summary, mutation of FHL1 should be suspected in
individuals with X-linked HCM. FHL1 mutations thus extend the spectrum of X-linked HCM where belong Barth syndrome, Danon’s and Fabry’s disease. Exome sequencing seems to be the most practical approach in genetic dissection of familiar cardiomyopathies.

**Study limitations:**

Unfortunately, we did not have an opportunity to study the pathophysiology of p.F200fs32X FHL1 mutation in experimental models. However, perfect segregation of the mutation in 12 individuals, available evidence in other FHL1-related diseases, and absence of wild-type FHL1A protein in myocardial samples support involvement of the above mutation in the pathophysiology of HCM in our case. Finally, the disease severity may be determined not only by the gene mutation itself, but also by gene dosage and age. Further studies are needed to evaluate relationship between the above gene mutation and LVDD observed in our patients.

**Conclusions:**

We identified a novel mutation of \textit{FHL1} (c.647_648 ins.T) causing isolated HCM with X-chromosomal inheritance and severe LVDD in advanced disease in three hemizygous males. Mild cardiac involvement in heterozygous females included asymptomatic abnormalities of electrocardiogram with a modest increase in LV mass since the sixth decennium and one case of apical HCM in the ninth decennium. None of the individuals showed signs of skeletal muscle disease. Mutations of \textit{FHL1} should be suspected in individuals with X-linked HCM.

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**Conflict of Interest Disclosures:** None.

**References:**


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## Table 1: Clinical, electrocardiographic, echocardiographic, hemodynamic and laboratory characteristics of individuals with FHL1 mutation associated hypertrophic cardiomyopathy. To demonstrate progression of the disease, available previous observations in each individual were added into this table and labeled with age.

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<th>Proband</th>
<th>Proband’s brother</th>
<th>Proband’s uncle</th>
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**History of myocardial infarction, diabetes**
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**Abbreviations:** AF- atrial fibrillation, BAE- biatrial enlargement, E/Em ratio- early mitral inflow velocity to peak mitral annulus velocity ratio, ICD-implantable cardioverter-defibrillator, HF- heart failure, HTx- heart transplantation, LAVI- left atrium volume index, LBBB- left bundle branch block, LVS- left ventricular strain, LVEF- left ventricular ejection fraction, LVH- left ventricular hypertrophy, LVOT- left ventricular outflow tract, NSVT- non-sustained ventricular tachycardia, SR- sinus rhythm.

**The upper limit of normal:** creatine phosphokinase 3.33 μkat/l, myoglobin 140 μg/l.
Figure Legends:

**Figure 1:** Pedigree. Black symbols indicate affected males with hypertrophic cardiomyopathy with restrictive physiology and one affected female with apical hypertrophic cardiomyopathy. Half-filled symbols depicts individuals with an abnormal electrocardiogram and a normal echocardiogram. Plus signs indicate presence of the FHL1 c.599_600 insT mutation. Minus signs indicate absence of this mutation. Circles, females; squares, males; diagonal line, deceased; arrow, the proband.

**Figure 2:** Electrocardiograms in individuals with FHL1 mutation (labeled by patient numbers).

**Figure 3:** Images from magnetic resonance in three males with restrictive HCM and transoesophageal echocardiogram in female with apical HCM (labeled by patient numbers).

**Figure 4:** Effects of the identified FHL1 mutation. (A) Schematic representations of FHL1 genomic structure (FHL1 gene), FHL1 mRNA isoforms (FHL1A, FHL1B and FHL1C) and corresponding FHL1 protein variants. Light gray and dark gray boxes demonstrate alternatively and constitutively spliced exons, respectively. ATG – position of the initiation codon; TAA, TGA – position of the stop codons. Positions of the c.599_600 insT mutation and of the primers (U primer and L primer) used for RT-PCR and qPCR amplification are indicated. Blue boxes demonstrate FHL1 domains. LIM1/2, LIM1, LIM2 and LIM4 denotes individual LIM domains; NLS - nuclear localization signal; NES – nuclear export sequence; RBP-JK - recombining binding protein immunoglobulin J kappa binding site. Protein domain recognized by employed
FHL1 antibody and predicted effects of the p.F200fs32X mutation on expression of FHL1 variants in FHL1 are indicated. (B) Chromatograms of FHL1 genomic DNA sequences showing identified mutations in the Czech family. (Upper panel) Sequence of the proband, (middle panels) sequence showing heterozygous mutation in the mother, and (lower panel) sequence of an unaffected individual. (C) Relative amounts of FHL1 mRNA normalized to amounts of Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA in myocardial specimens; C1-C4 denotes specimens from controls, P1 and P2 denotes specimens from probands III/4 and II/3, respectively; adjoined F or M denotes gender (female of male, respectively) of control individuals. The means ± SD of three experiments performed in triplicates are shown. (D) FHL1A cDNA analysis showing profiles of RT-PCR products amplified from total RNA isolated from snap-frozen myocardial specimens of control (C1-C4) and probands III/4 (P1) and II/3 (P2). A single RT-PCR product of the size of 699 base pairs expected for FHL1A isoform is detected. RT-PCR product of the size 512 bp expected for FHL1C isoform is absent; M - is a 100 base pair DNA ladder, B-blank. (E) Western blot analysis of homogenates prepared from snap-frozen myocardial specimens showing presence of the immune-reactive protein of a molecular weight ~ 27 kDa corresponding to predicted molecular weight of the p.F200fs32X FHL1 protein in samples from probands P1 and P2. Immunoreactive protein of a molecular weight ~ 32 kDa, corresponding to predicted molecular weight of the FHL1A identified in high abundance in control samples (C1-C4), is in patients’ samples (P1, P2) absent. Specific immunoreactive protein of a molecular weight ~ 22 kDa corresponding to predicted molecular weight of the FHL1C is not detected in patients’ and control samples (C1-C4). Comparable staining intensity of Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) demonstrate that approximately equal protein amounts have been analysed. (F,G) Immunofluorescence analysis of cryostat sections of
myocardium showing absence of immuno-reactive FHL1 in the proband III/4, (F) in comparison to distinct cross-striation pattern in control cardiocytes (G).

**Figure 5:** Histopathology and electron microscopy. (A) Hypertrophy and disarray of cardiocytes in patient III/4, H&E stain. (B) Combination of hypertrophic and regressive changes in myocardium in patient II/3, H&E stain. (C) Interstitial fibrosis in myocardium in patient III/4, trichrome stain. (D) Interstitial and replacing fibrosis in myocardium in patient II/3, trichrome stain. (E) Detail of enlarged hyperchromatic bizarre-shaped nuclei of hypertrophied cardiocytes. Patient III/4, H&E stain. (F) Immunohistochemical detection of desmin demonstrated in patient III/4. Normal cross-striation pattern was a prevalent feature with slightly disordered appearance in branched cardiocytes. (G, H) Electronograms showing findings in myocardium from patient III/4. Disoriented myofibrils in cardiocytes with extensive mechanical junctions are demonstrated in (G). Mitochondria are increased in number, replacing focally myofibrils (H).
Cardiomyopathy: ●
Abnormal electrocardiogram: ○
FHL1 mutation present/absent: (+)/(-)
Isolated X-Linked Hypertrophic Cardiomyopathy Caused by a Novel Mutation of the Four-and-a-Half LIM Domain 1 Gene

Hana Hartmannova, Milos Kubanek, Marek Sramko, Lenka Piberova, Lenka Noskova, Katerina Hodanova, Viktor Stranecky, Anna Pristoupilova, Jana Sovova, Tomas Marek, Jana Maluskova, Petr Ridzon, Josef Kautzner, Helena Hulкова and Stanislav Kmoch

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Supplemental Material
**Supplemental Table 1:** Novel and rare genetic variants identified by exome sequencing as present in all three analyzed probands.

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Individual variants are defined by chromosomal localization (Chr), their chromosomal position (Position), reference (Ref) and observed (Ob) alleles, gene symbol (Gene), accession number of the corresponding cDNA (Accession), resulting cDNA and amino acid changes, population frequency in exome sequence project (ESP5400) and predicted effect on protein function according to SIFT and Polyphen2 algorithms. In SIFT prediction D denotes
damaging effect and T denotes tolerating effect. In PolyPhen2 prediction B denotes benign effect, P denotes possibly damaging effect and D denotes probably damaging effect. All coordinates refer to hg19.