SUPPLEMENTAL MATERIAL

OBSCURIN (OBSCN) MUTATIONS ARE ASSOCIATED WITH FAMILIAL DILATED CARDIOMYOPATHY AND HAPLOINSUFFICIENCY

SUPPLEMENTAL MATERIAL AND METHODS

Human heart muscle

All patients and subjects were enrolled to studies with local ethical approval and all investigations conform to the principles of the Declaration of Helsinki.

We used tissue samples from explanted hearts in the Sydney Tissue bank. 30 patients with a diagnosis of familial DCM or idiopathic DCM requiring transplant at less than 25 years old were selected for sequencing and further studies (see supp A). As controls we studied muscle from 6 donor hearts (see Supp B). Donor hearts had no history of cardiac disease and normal ECG and ventricular function and were obtained when no suitable transplant recipient was found. The functional characteristics of some of the donor heart samples have been previously reported.(1, 2).

In addition to the 1000 genomes database and Exon Variants Server (EVS), a control population was used to analyze the frequency of the variants identified. This population consists of a cohort of 460 patients previously treated with doxorubicin but which did not develop heart failure (3).

DNA extraction

25 mg of frozen tissue was equilibrated at room temperature, incubated with a Steel Ball (Qiagen 69989) in a tissue lyser at 30 Hz for 1 minute, lysed overnight using proteinase K and genomic DNA has been isolated according to the manufacturer’s protocol (Qiagen; Dneasy Blood-Tissue extraction).

RNA extraction

Extractions were performed with 25 mg of heart tissue with the RNA easy kit from Qiagen as described by the manufacturer, except for the lysis step. Lysis has been performed using the tissuelyzer at 50Hz during 2 minutes for a complete lysis). After
lysis, RNA extraction has been performed using the Qiagen RNA easy blood and tissue kit according to standard protocol. Reverse transcription was performed using the SuperScript™ II Reverse Transcriptase from Invitrogen as described by the manufacturer with 100ng of input RNA.

q-RT-PCR

cDNA was diluted in water (1:10) and 4mL of diluted cDNA were used as template in each assay. The q-PCR reaction mix is composed of 1mL of reverse and forward primer at an initial concentration of 2.5mM each and 12.5mL of SyberGreen (Invitrogen) and 6.5 mL of RNAse-free water.

The thermal cycle is composed of three steps 10 minute of denaturation at 94C, 35 cycles with a denaturation step of 30 sec at 94, an annealing at 55 for 45 sec, an elongation at 72 for 30 sec. The third step is a melting curve step to ensure the specificity of the reaction. All assays have been done in triplicate.

The analysis was performed with the relative 2\textsuperscript{DCt} method assuming that the reaction is efficient and that the quantity is doubled at each cycle and that GAPDH is the calibrator.

The following were used: Obscurin -F:ACTGGTGTTGTTAAGGGAGG,
Obscurin- R: AGAGCTGCCAGACATTCAGT,
GAPDH- F:AATCCCATCACCATCTTCCA,
GAPDH-R :TGGACTCCACGACGTACTCA

Whole genome sequencing

For whole-exome sequencing, we fragmented 1 µg of DNA with sonification technology (Bioruptor, diagenode, Liège, Belgium). The fragments were end-repaired and adaptor-ligated, including incorporation of sample index barcodes. After size selection, we subjected the library to an enrichment process with the SeqCap EZ Human Exome Library version 2.0 kit (Roche NimbleGen, Madison, WI, USA). The final libraries were sequenced on an Illumina HiSeq 2000 sequencing instrument (Illumina, San Diego, CA, USA) with a paired-end 2×100 bp protocol.
On average, 7 Gb of sequence were produced, resulting in 30-times coverage of more than 80% of target sequences (44Mb). We filtered primary data according to signal purity with the Illumina Realtime Analysis software. We analysed the whole exome data for potential disease causing variants in 58 genes implicated in inherited cardiomyopathies, shown in Supp C using the filters indicated.

1000genomes database and Exon Variants Server, EVS

http://browser.1000genomes.org/Homo_sapiens/Info/Index has been used to analyse the frequency of the variants identified.

The current EVS data release (ESP6500SI-V2) http://evs.gs.washington.edu/EVS/ is taken from 6503 samples (a set of 2203 African-Americans and 4300 European-Americans unrelated individuals, totaling 6503 samples i.e 13,006 alleles). The samples included in the ESP6500 were selected from the populations of different studies and include controls, the extremes of specific traits (LDL and blood pressure), and specific diseases (early onset myocardial infarction and early onset stroke), and lung diseases. The goal of the ESP dataset is to release the frequency counts of specific variants without regard to phenotype.

The fact that patients with heart and lung diseases are the main source of the EVS data base does not allow the exclusion of a disease causing role of a variant if this variant is present in the data base.

Pyrosequencing

We confirmed the predicted mutations in D4, D14, D20 and D21 and tested for their presence in our cohort of 460 samples (3)( see Table 1). SNP genotyping by pyrosequencing was performed on an automatic PSQTM PSQ HS96A pyrosequencing instrument (Qiagen, Hilden, Germany). Pyrosequencing assays for SNP genotyping were designed using PSQ Assay Design software (Qiagen, Hilden, Germany). Primer sequences and assay conditions for the noncommercial pyrosequencing assays are available on request.
Pyrosequencing was performed according to manufacturer's instructions on a PSQ™ 96 HS instrument (Qiagen, Hilden, Germany) and analyzed by pyrosequencing software.
**Gel electrophoresis**

Whole tissue extracts were obtained for gel electrophoresis using T-Per Protein extraction reagent (Pierce, Thermo Scientific) including 1µg/ml E64, chymostatin and leupeptin protease inhibitors according to the manufacturer’s protocol. The myofibrillar fraction of heart muscle was prepared by our standard protocol (1). Proteins were separated by two SDS-PAGE systems. High molecular weight proteins were separated on 2%agarose/2% polyacrylamide gels as described (4, 5). Other proteins were separated on 4-15% Polyacrylamide gradient gels (Criterion, Bio-Rad). Western blots were made in a wet blotting apparatus for 5 hours at 250 milliamps in the cold room. Blotted proteins were reversibly stained with MemCode Reversible stain kit (Thermo Scientific) and then probed with antibodies to Obscurin (OB13, Ob58 and Ob59, a gift from M.Gautel, King’s College London (6)) and visualised with ECL pus (GE Healthcare).

**Immunohistochemistry**

Frozen sections of human heart tissue were cut using a Leica CM 1950 cryostat and were retrieved on poly-L-lysine coated slides. After drying they were fixed for 5 min in acetone at -20°C, washed in PBS and blocked with 5% preimmune goat serum in 1%BSA/GB (GB: 20 mM Tris, 155 mM NaCl, 2 mM EGTA, 2 mM MgCl2, pH7.5) for 30 min at RT. Incubations with primary and secondary antibodies were carried out in a humid chamber at RT, intermittent washing was done in PBS. As primary antibodies polyclonal rabbit anti obscurin antibodies (see above) were used in combination with either monoclonal mouse anti sarcomeric alpha-actinin (clone EA-53, Sigma) or monoclonal mouse anti myomesin (clone B4, Grove et al., 1984 PMID: 6537951). As secondary antibodies multilabelling quality Cy2-goat anti rabbit immunoglobulins and Cy3-goat anti mouse immunoglobulins (from Jackson Immunochemicals via Stratech Scientific) were used in combination with DAPI (Sigma) to visualize the nuclei. Samples were mounted in 0.1M Tris/glycerol (3:7), 50 mg/ml N-propyl gallate pH9.5. Imaging was carried out using a 63x/1.4NA lens on a Leica SP5 confocal microscope equipped with a blue diode and argon and helium neon lasers.
LEGENDS to SUPPLEMENTAL FIGURES

A  Table of muscle samples studied and putative disease-causing mutations found.

B  Patient data for the donor heart muscle used as controls in this study.

C  WES sequencing protocols: left, list of genes tested for mutations, right filter conditions used to find the potential disease-causing mutations

D  Alignment of Obscurin B isoforms in human, mouse, mole rat and turtle. Only the regions of the sequence with mutations are shown

E  Domain diagram of the two large muscle isoforms of obscurin. Location of mutations and the epitopes of Ob13, Ob58 and Ob59 are shown

F  Relative Obscurin mRNA content of heart muscle samples normalised to GAPDH mRNA

G  Relative obscurin protein expressed in whole homogenates of heart muscle

A)  Western blots of whole heart muscle homogenates separated by SDS-PAGE (See Figure 1). Top Memcode protein stain, bottom Ob59 anti-obscurin antibody.

B)  Calculated relative obscurin levels using the method as shown in Figure 2. Results from individual lanes are shown.

C)  Relative proportions of the three obscurin bands in atrial and ventricular muscle homogenates.
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Titin amino acid numbering is based on UniProt Q8WZ42-11 Isoform, Obscurin numbering is based on Q5VST9

Supplement A
Clinical details of the donor heart samples used in this study

SAH = subarachnoid haemorrhage, ICH = intracerebral haemorrhage

Clinical details of the myectomy samples used in this study

HCM = hypertrophic cardiomyopathy
Genes analysed for FDCM mutations

Filter parameters for identifying obscurin gene variants

WES sequencing data: left, list of genes tested for mutations, right filter conditions used to find the potential disease-causing mutations
Alignment of sequences for OBSCN gene from human, mouse, mole rat and turtle (human ObscurinB, UniProt Q5VST9-1,is 7968 amino acids. RefSeq NM-001098623)
Location of mutations and antibody epitopes in the domain structure of the long obscurin isoforms

figure adapted from Ackerman et al (2014) DOI: 10.1371/journal.pone.0088162
Relative Obscurin mRNA content of heart muscle samples normalised to GAPDH mRNA
A

MHC
MyBP-C

obscurin
420kDa
250kDa

obs
iso 1
iso 2

B

Quantity of obscurin protein relative to α-actinin, normalised to D21. Calculated from 5 gels similar to the one shown here.

C

Relative quantities of the three isoforms of obscurin detected in whole human heart muscle homogenates of atrium and ventricle.

Obscurin supp G
REFERENCES


11 Lopes, L.R., Zekavati, A., Syrris, P., Hubank, M., Giambartolomei, C., Dalageorgou, C., Jenkins, S., McKenna, W., Consortium, U.k., Plagnol, V. *et al.*


