Mutations in the Z-band protein myopalladin gene and idiopathic dilated cardiomyopathy

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1. Introduction

Idiopathic dilated cardiomyopathy (DCM) is a major cause of heart failure and represents one of the first two causes of heart transplantation. In USA, the prevalence of DCM is 36.5 per 100 000 and the mortality is estimated to be 30–50% at 5 years.¹,² DCM usually presents with an isolated cardiac dysfunction and dilatation, but may be associated with additional conduction and/or skeletal muscular disorders in some families. A familial transmission is observed in 25–35% of the cases.³–⁵ Positional cloning or candidate gene screening led to the identification of heterozygous mutations in at least 11 different genes encoding sarcomeric, cytoskeletal or cardiac calcium cycle regulatory proteins responsible for familial DCM. To date, phenotypically isolated DCM has proven to be related to mutations in the α-cardiac actin (ACTC), desmin (DES), δ-sarcoglycan (SCGD), vinculin (VCL), alpha-actinin 2 (ACTN2), titin (TTN), T troponin (TNNT), α-tropomyosin 1 (TPM1), phospho-lamban (PLN), ZASP (LRP5) and β-myosin heavy chain (MYH7) genes. Moreover, mutations in the lamin (LMNA) or the two X-linked dystrophin (DMD) and tafazzin (TAZ) genes are responsible for familial DCM associated with conduction system and/or muscular skeletal dystrophy.⁶ Including DCM associated with other phenotypes such as arrhythmia, atrial fibrillation, hearing loss, or other complex syndromes, up to 20 genes have been identified with causative mutations. Interestingly, only a limited number of mutations have been found in the reported disease genes and this was observed...
in only a few families. Furthermore, up to seven genetic loci have also been identified but without subsequent positional identification of disease gene. All these data suggest that familial DCM is associated with a large number of different genes with low prevalence.

A few years ago, myopalladin was identified as an important structural member of the Z/I line of the sarcomeric apparatus of striated muscle. It interacts with actn2 and nebulette7,8 two cardiac proteins encoded by genes associated with DCM.9,10 Myopalladin is also interacting with the CARP factor, a factor activated in rat and mouse models of left ventricular hypertrophy.11,12 Human heart failure,13 dilated cardiomyopathy13 and adriamycin induced cardiomyopathy.14 Finally, the myopalladin/titin/CARP complex is involved in mechanical stretch response of myofibrils,15 as protein factors such as MLP, which gene targeting invalidates in mice is associated to a DCM phenotype.16 We therefore hypothesized that myopalladin gene (MYPN) mutations could be responsible for DCM and identified four mutations through a mutational screening of DCM cases and subsequent tissue immunolabelling and functional analysis in transfected rat neonate cardiomyocytes (RNC) in cell culture.

2. Methods

2.1. Clinical evaluation

Patients and relatives were included in the study after written informed consent in accordance with the study protocol approved by the local ethical committee. The investigation conforms the principles outlined in the Declaration of Helsinki. DCM was assessed according to previously published criteria.17 Patients were classified as familial cases (n = 65) when at least two first-degree relatives in the same family were affected. The ‘sporadic’ term is related to patients (n = 49) with no identified relatives with DCM after family history recording and clinical examination of identified relatives. It does not implicate that both parents’ DNA were accessible for genotyping.

2.2. Molecular and cellular biology

2.2.1. Genetic analysis

Most of the patients were previously genetically screened for mutations in the following DCM responsible genes: LMNA, ACTC, DCM2, MYPN, MYH7, TNNT2, PLN and the cardiосpecific exon 16 of VCL, and no mutation was identified.18–21 Blood samples were collected and DNA was extracted from peripheral lymphocytes using a standard protocol at the Génethon Bank (Evry, France). PCR amplification from genomic DNA and direct sequencing was performed at ‘Centre National de Génotypage’ (Evry, France) with primers specific for each exons of MYPN and designed to amplify and sequence flanking intronic sequences (> 60 nt). Amplifications were conducted following standard PCR protocol (ExTaq Kit, Biothiaker, Verviers, Belgium). PCR conditions and oligonucleotide sequences are available as supplementary material (see Supplementary material online, Table S1). Each amplified DNA fragment was sequenced (Big-Dye v3.0) on an ABI Prism 3100 or 3700 Genetic Analyser (Applied Biosystems, Courtaboeuf, France). Molecular variant detection was achieved with the Genalys software developed at CNB (www.cnbg.fr). Identified mutations were confirmed by sequencing on an independent PCR product as well as in at least one other affected member of each family (when applicable). All suspected mutations were genotyped by PCR-RFLP (restriction fragment length polymorphism) in all available family members and in a control population consisting of 400 subjects (800 chromosomes) from Western European descent aged 18–64 years with no identified cardiovascular pathology. The LINKAGE v5.1 program was used to calculate two point Lod score. Significant linkage (P < 0.05) is reached for Z > 1.3 as only one genetic marker (the mutation) was studied.

2.2.2. Cloning and mutagenesis

Mutant vectors were obtained by PCR-based site directed mutagenesis (QuickChange, Stratagene) from MYPN cDNA cloned in pGFP-C1 (from S Labeit, Heidelberg, Germany). At least two independent plasmid preparations purified with Qiagen columns (Qiagen, Courtaboeuf, France) were used for independent transfection experiments in RNC. All cDNAs inserted in plasmids were sequenced to confirm site-directed mutagenesis and absence of random mutation.

2.2.3. RT–PCR and allele specific quantification

We take advantage of the illegitimate transcription that takes place in easily accessible lymphoblastoid cells22 to measure allele specific mRNA levels from heterozygous patient carrying the I83SX105 mutation. Total mRNA was extracted from index case A-II-1 EBV-lymphoblastoid cell line immortalized at Génethon with Trizol reagent (Invitrogen, CERGY Pontoise, France) according to manufacturer instructions. One μg total RNA was used for reverse transcription primed with 10 pmol oligo d(T)12–18 with the Superscript II Kit (Invitrogen) following manufacturer instructions. cDNA (10% of RT reaction) was amplified in a final volume of 20 μL with the following primers: Forward 5'-ccatttcggcagtccttct-3' and Reverse 5'-gagggtagctgctgccct-3'. The amplified DNA fragment (898 bp) spans two exons (ex2 and ex3) to avoid potential genomic DNA contaminant amplification. PCR products digested with the restriction endonuclease Bcl1 produce different and specific patterns from the mutated (518 + 380 bp) or the wild-type (WT) allele (128 + 390 + 380). The common 380 bp band was digested with Xhol (380 > 208 + 172). Relative band densities at 518 (mutant allele) and 390 bp (WT allele) were compared after 1% agarose gel electrophoresis and ethidium bromide staining. The control experiment was performed by amplification or coamplifications of equal amount (0.5 ng) of vectors I83SX105-GFP-MYPN and WT-GFP-MYPN. Sub-cloning of RT–PCR product obtained from the same mRNA (patient A-II-1) was done in pGem-T Easy according to manufacturer’s instructions (Promega corp., Charbonnières, France). Twenty independent bacterial clones were grown and plasmids were purified before sequencing with primer T7 in order to determine the cloned allele frequency which is representative of allelic population in the mRNA sample.

2.2.4. Cell culture and transfection

The investigation conforms to the ‘guide for the care and use of laboratory animals’ (NIH Publication No. 85/23, revised 1996). Neonate 1-day-old rat hearts were dissected, digested with collagenase A (Roche Diagnostics, Meylan, France) and incubated in culture medium after 1.5 h preloading on 60 mm plastic dishes in order to remove fibroblasts. Non-adherent cells were plated at a density of 4 × 10^5 cells/well on 35 mm dishes containing glass coverslips coated with 10 μg/mL laminin (Roche Diagnostics) in culture medium DMEM (High Glucose/L-glutamine; Gibco ref 41965 039), supplemented with 10% horse serum, 5% FBS, 1% penicillin/streptomycin, Cytosine B-D arabinofuranoside 25 μg/mL and incubated for 24 h (37 C, 5% CO2). Cells were transfected with 10 μL lipopectin 2000 (Invitrogen) and 2 μg expression vectors. At dedicated times after transfection, cells were fixed in 3% paraformaldehyde, washed with PBS, permeabilized in 0.2% Triton X-100 and blocked in 10% goat serum diluted in 1X PBS before immunolabelling with specific primary antibodies (in 10% goat serum/1X PBS) directed against green fluorescent protein (GFP) (ref: TP401; 1/300 dilution; Clinscience, Montrouge, France) or actn2 (ref: EA-53; 1/300 dilution, Sigma-Aldrich, France). Cell survival was obtained by counting all GFP immunolabelled cells in a 22 × 22 mm² coverslip from four independent transfections. Cells observation and counting were performed with a fluorescent microscope eclipse E800 (Nikon) with a 40X oil objective.
2.2.5. Confocal microscopy and image analysis

Explanted myocardial tissues were obtained with informed consent from patients. In addition to the cardiac tissue from the patient carrying the R1088H mutation, we obtained one normal heart left ventricle sample and four independents left ventricle samples from patients with NYHA class IV DCM at time of transplantation. Sections of fresh muscle were frozen in isopentane/liquid nitrogen and stored in liquid nitrogen until cryosectioning. Immunohistochemical labelling was performed on 7 μm cryostat sections from independent tissue pieces (n = 3) of the ventricular free wall with specific antibodies directed against myopalladin (from Dr S Labeit) at 1:100 dilution in Dako/5% goat serum or against actn2 (dilution 1:200, EA-53, Sigma-Aldrich). Confocal microscopy analysis was performed with a Leica SP2 AOBS. Images were analysed using Meta-morph software (Roper Scientific). For each image, the background was measured on three independent tissue-free areas and subtracted prior to subsequent analysis. The ratio of green and red emitting pixels (myopalladin and actn2 present) to the total number of green emitting pixels (myopalladin present) was calculated, indicating the percentage of myopalladin localized with actn2.

3. Results

We performed a systematic screening for mutations in the coding sequence and intron–exon boundaries of MYPN in 114 consecutive and unrelated index-cases essentially from European descent (93%). Ninety per cent of cases had an ‘isolated’ DCM phenotype and the remaining patients presented with associated conduction defects or mild skeletal muscular dystrophy.

We identified four heterozygous mutations (Figure 1) in MYPN coding sequence: three were missense mutations [one familial (R1088H) and two apparently sporadic cases in the absence of extensive familial cardiac examination (V1195M, P1112L)] and one was an insertion-deletion [one familial case (I83fsX105)]. All mutations were absent from 400 healthy control subjects from European descent, ethnically matching the mutation carrier subjects. MYPN mutations were involved in two of 65 familial DCM (3%) and in two of 49 sporadic DCM (4%).

Both familial mutations were cosegregating with the disease with variable estimated penetrance (Figure 1A). They were identified in families from European descent. As commonly observed in familial DCM, the small size of the families and the age related incomplete penetrance did not allow to reach the commonly accepted significant threshold for genetic linkage in whole-genome scan strategy (Z = 3). However two-point linkage analysis for family MYPN-R1088H with the unique mutation marker, thus reducing the Lod score threshold for positive linkage to Z = 1.3, was strongly suggestive of linkage of DCM with the MYPN locus (Lod score = 2.1, θ = 0, LINKAGE v5.1).

Detailed phenotypic data about the heterozygous subjects carrying a mutation are reported in Table 1. The phenotype of patients was characterized by isolated DCM. Mean age at diagnosis was 40.2 ± 18.3 years. Prognosis, as assessed by the follow-up of mutation carriers and by family history, was characterized by three cardiac deaths (including two...
### Table 1 Clinical features of affected patients and mutation carriers at genetic inquest

<table>
<thead>
<tr>
<th>Subject</th>
<th>Age (year)/sex</th>
<th>Age at diagnosis (year)</th>
<th>NYHA class</th>
<th>ECG</th>
<th>LVEDD (mm)</th>
<th>EF (%)</th>
<th>IVS (mm)</th>
<th>Muscular clin./CPK</th>
<th>Clinical status</th>
<th>Comments</th>
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<tbody>
<tr>
<td>Pedigree A</td>
<td></td>
<td></td>
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<tr>
<td>II-1</td>
<td>65/F</td>
<td>62</td>
<td>IV</td>
<td>SR, cLBB</td>
<td>74&lt;sup&gt;b&lt;/sup&gt;</td>
<td>29</td>
<td>9</td>
<td>NL/NL</td>
<td>Affected</td>
<td></td>
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<tr>
<td>II-1</td>
<td>38/F</td>
<td>–</td>
<td>I</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>Unknown</td>
<td></td>
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</tr>
<tr>
<td>III-2</td>
<td>Death at 20/M</td>
<td>20</td>
<td>IV</td>
<td>SR, LVH</td>
<td>Dilated</td>
<td>Low</td>
<td>Normal</td>
<td>NL/NA</td>
<td>Affected</td>
<td>DCM and mitral valve prolapse with important mitral regurgitation; CHF at 20 year while waiting for HT</td>
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<tr>
<td>III-4</td>
<td>37/M</td>
<td>–</td>
<td>I</td>
<td>SR</td>
<td>51</td>
<td>72</td>
<td>10</td>
<td>NL/NA</td>
<td>Healthy</td>
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<tr>
<td>II-2</td>
<td>Death at 55&lt;sup&gt;a&lt;/sup&gt;/M</td>
<td>51</td>
<td>III</td>
<td>SR, LVH, microvoltage</td>
<td>64&lt;sup&gt;b&lt;/sup&gt;</td>
<td>37</td>
<td>8</td>
<td>NL/NA</td>
<td>Affected</td>
<td>Alcoholism, CHF, and atrial fibrillation at 52 year; Ischaemic CVA at 55; CHF, and cardiac death at 55 year</td>
</tr>
<tr>
<td>II-3</td>
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<td>I</td>
<td>SR, iLBB</td>
<td>60&lt;sup&gt;b&lt;/sup&gt;</td>
<td>40</td>
<td>8</td>
<td>NL/NA</td>
<td>Affected</td>
<td>Alcoholism, DCM diagnosed during genetic inquest</td>
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<tr>
<td>III-1</td>
<td>Death at 29&lt;sup&gt;a&lt;/sup&gt;/M</td>
<td>29</td>
<td>IV</td>
<td>SR, rBBB, microvoltage</td>
<td>67&lt;sup&gt;b&lt;/sup&gt;</td>
<td>20</td>
<td>NA</td>
<td>NL/NA</td>
<td>Affected</td>
<td>Initial diagnosis of myocarditis with CHF; Death at 29 year (refractory CHF)</td>
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<tr>
<td>IV-1</td>
<td>18/F</td>
<td>13</td>
<td>I</td>
<td>SR, iLBB</td>
<td>64&lt;sup&gt;b&lt;/sup&gt;</td>
<td>35</td>
<td>7</td>
<td>NL/NA</td>
<td>Affected</td>
<td>IAD</td>
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<td>Pedigree C</td>
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<tr>
<td>II-1</td>
<td>38/M</td>
<td>36</td>
<td>II</td>
<td>SR, iLBB, nsVT</td>
<td>62&lt;sup&gt;b&lt;/sup&gt;</td>
<td>38</td>
<td>11</td>
<td>NL/NL</td>
<td>Affected</td>
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<tr>
<td>Pedigree D</td>
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<tr>
<td>II-1</td>
<td>58/M</td>
<td>58</td>
<td>I</td>
<td>SR, iLBB, LVH</td>
<td>63&lt;sup&gt;b&lt;/sup&gt;</td>
<td>34</td>
<td>8</td>
<td>NL/NA</td>
<td>Affected</td>
<td>Mild alcoholism</td>
</tr>
</tbody>
</table>

*Age is age at genetic inquest; NYHA, New York Heart Association functional class; LVEDD, left ventricular end diastolic diameter; EF, ejection fraction; IVS, interventricular septum thickness (echography); Muscular clin., abnormality in clinical muscular testing; CPK, elevated serum creatine kinase level; M, male; F, female; SR, sinus rhythm; iLBB or cLBB, incomplete or complete left bundle branch block; rBBB, right bundle branch block; nsVT, non-sustained ventricular tachycardia; LVH, left ventricular hypertrophy on ECG; NL, normal; NA, not available; HT, heart transplantation; IAD: interatrial defect.*

*<sup>a</sup>No DNA available for genotyping but obligate carrier.*

*<sup>b</sup>LVEDD above theoretical value accounting for age and body surface area.*
in obligate mutation carriers) and mean age at death was 34.6 ± 18.1 years.

We also identified 18 polymorphisms (see Supplementary material online, Table S2) in the coding sequence including eight non-synonymous single nucleotide polymorphism (nsSNP). All nsSNPs with a rare allele frequency higher than 5%, except Ser691Asn which is in almost complete linkage disequilibrium with Ser707Asn, were evaluated for genotype association with DCM in the 420 sporadic DCM cases and 413 controls matched for age, sex, and body mass index of the CARDIGENE Study.23 None of the nsSNPs (Phe268Leu, Ser707Asn, Ser803Arg, and Pro1135Thr) did indicate a significant association with DCM (P > 0.05, χ²-test, DF = 2) suggesting the absence of susceptibility allele and supporting the neutral status of these nsSNPs.

Molecular alignment of the sequences surrounding mutations with myopalladin from other species showed a high conservation of the mutated residues through evolution suggesting a positive selection pressure and functional importance of the mutated residues (Figure 1B). We explored a possible effect of the identified mutations on the ability of myopalladin to interact with the cardiac sarcomeric structure.

During the course of this study, one of the patient (IV-1 in pedigree B in Figure 1) carrying the heterozygous R1088H mutation underwent urgent heart transplantation. This provided a unique opportunity to study the effect of the mutation at the tissue level. We analysed the explanted cardiac tissue by immunofluorescence using antibodies directed against myopalladin and actn2 and compare labelling to normal samples or control samples with IDCM from MYPN independent causes. In each heart slice analysed, the actn2 signal was located almost exclusively at the Z-band region of sarcomeres indicating normal structure of cardiac myocytes from the proband, normal heart and IDCM controls. Similarly, the myopalladin labelling was restricted to the Z-band region for normal control and IDCM controls. Labelling of the functionally normal right ventricle of the patient with the R1088H mutation was indistinguishable from controls indicating good quality of the heart sample preparation. In contrast, in the dilated left ventricle of the R1088H carrier, the myopalladin labelling indicated a reduced localization to the Z-band region (Figure 2). The mean percentage of the myopalladin signal that was localized to the Z-band (see Supplementary material online, Figure S1) was consistently decreased (53 ± 9.4%, n = 18) compared with control normal heart (78 ± 7.3%, n = 9) and four independent control IDCM explanted hearts (82 ± 9.3%, n = 5; 87.1 ± 5.4%, n = 4; 89.4 ± 6.7%, n = 6; 85.2 ± 5%, n = 4). Of note, the right ventricle of the patient also shows normal MYPN localization at Z-band region (86.2 ± 5%, n = 4). This result indicated a mislocalization of almost half of the myopalladin outside of the actn2 labelled Z-band, which is specifically measured in the diseased tissue from the R1088H heterozygous mutation carrier.

We used the RNC cellular model to monitor the GFP-tagged myopalladin proteins after transient cell transfection with the WT or mutated MYPN cDNA cloned in pEGFP (WT vector was kindly provided by Dr S Labeit7). Results are presented in Figure 3 and are representative of at least 85% of the GFP positive cells for each transfection. The transfection efficiency was obtained by GFP positive cell counting and was estimated to 7% for each expression vector with very little variation from one construct to another. As expected from previously published results,7 a striated pattern for WT myopalladin (WT-GFP-MYPN) expression was detected after specific immunolabelling directed against GFP. Labelling was observed at the sarcomeric I-Z-I structure, localizing with the resident protein actn2 (Figure 3A). The observed actn2 labelling was highly similar in cells transfected with pEGFP alone indicating that WT-GFP-MYPN overexpression did not alter the observed cellular phenotypes (not shown). Similarly, the R1088H-GFP-MYPN expressing RNC were phenotypically indistinguishable from the WT-GFP-MYPN (compare Figure 3A and B), suggesting that the in vitro experimental condition used were unable to reveal the cellular alteration associated with mutation R1088H.

In contrast, the myocytes expressing the mutant proteins P1112L-GFP-MYPN and V1195M-GFP-MYPN (Figure 3C and D) showed a sarcomeric apparatus disorganization with actn2 striated labelling fainting rapidly from day 1 to day 2 after transfection. Importantly, after 24 h, we noticed the absence of GFP-MYPN mutated protein at the I-Z-I structure. At 48 h, cells were not striated, either with GFP or actn2 labelling. Moreover, in all this cases, the cells were consistently small and round shaped suggesting cytoskeletal disruption.

Finally, P1112L- and V1195M-GFP-MYPN transfected cell survival was drastically reduced over time (Figure 4). GFP expressing cell counting, 48 h after transfection, indicated a 60% reduction in living cell number compared with WT-GFP-MYPN expressing cells (MANOVA; P < 10⁻⁵). Note that only a few of the remaining attached cells were TUNEL positive. However it does not exclude that detached cells underwent an apoptosis related death.

A well-described mechanism for heterozygous early nonsense mutation effect on morbidity is haploinsufficiency arising from non-sense mediated mRNA decay (NMD).24 In order to evaluate the relative amount of mRNA expression originating from the truncation allele (I83fsX105), we measured the ectopic mRNA level expressed in lymphoblastoid cells from heterozygous patient II-1 (Pedigree A). Reverse transcription of total mRNA was followed by PCR amplification and allele specific endonuclease digestion. The upper band originating from mutated allele was almost undetectable, indicating an important reduction of the mutated mRNA level in the patient’s cells (Figure 5). This was confirmed by RT–PCR cloning in pGEM T-easy vector and subsequent insert sequencing of 20 randomly picked clones. This showed that WT allele sequence was present in 85% of the clones (17/20) (not shown). These results suggested haploinsufficiency as the disease causing mechanism for I83fsX105 mutation.

4. Discussion

Despite the identification of numerous disease genes responsible for DCM, none appears to be of major prevalence and mutations are identified in less than 30% of monogenic cases. These observations suggest that numerous DCM genes remain to be identified. Here, we have used a sequencing-based mutation screening to search for mutation in the MYPN, a gene encoding a largely unknown structural protein of the sarcomeric I-Z-I region. From 114 independent DCM cases screened, we have identified four independent mutations in MYPN. Our genetic results indicated strong genetic evidences for a role of the newly identified
mutations in DCM. In addition, we performed a functional analysis of the mutated proteins by expression in the RNC model. Clearly, two out of three expressed missense mutations were associated with a major disorganization of the cell structure and early mortality compared to cells expressing WT myopalladin, confirming that we did identify functional mutations rather than rare neutral polymorphisms. For the R1088H mutation, despite the absence of measurable phenotypic changes in the heterologous RNC transfected cellular model, we had access to the explanted left and right ventricles of one heterozygous carrier allowing to demonstrate a marked left ventricular specific effect of the mutation on myopalladin localization. The mutation was strongly associated with myopalladin mislocalization outside the I-Z-I region only in the diseased left ventricle slices from the patient. Moreover, compared to WT, the marked and highly reproducible phenotypes induced by MYPN-L1112 and MYPN-M1195 expression indicated a clear effect of at least two of the missense mutations identified in this assay. The absence of observed difference between transfected cells expressing WT or R1088H myopalladin remain unclear. Several mechanisms could lead to DCM in response to mutation encoding replacement of amino acid at different position in the myopalladin protein. The discrepancy between \textit{in vivo} and \textit{in vitro} effects of R1088H mutation on sarcomere organization suggests that several factors, including mechanical overload, contribute to DCM. This hypothesis is also supported by the absence of myopalladin mislocalization in the right ventricular tissue from R1088H carrier. The needs for a trigger to reveal R1088H mutation effect in the left ventricle is consistent with the absence of mislocalization and observed phenotype for this specific mutation when expressed in unstressed RNC model. In addition to specific localization abnormalities

\textbf{Figure 2} Immunohistochemical labelling of heart tissue sample from R1088H mutation carrier and controls. Heart cryosection were labelled with antibodies specifically directed against myopalladin (green) or alpha-actinin 2 (red). Confocal microscopy picture are representative of the labelling obtained from (A) a normal heart left ventricle, (B) control IDCM left ventricle, (C) R1088H mutation carrier IV-1 in pedigree B explanted right ventricle, and (D) left ventricle. Note the specific decreased colocalization of the myopalladin with alpha-actinin positive Z-band region in diseased left ventricle (D, merged). Scale bar: 10 µm.

\textbf{Figure 3} Immunofluorescence based localization of WT vs. mutated myopalladin in transfected rat neonate cardiomyocytes. Cells are fixed and labelled with anti-GFP (green) or anti actn2 (red) antibodies at indicated times after transfection (12, 24, 48 h) with vectors expressing (A) GFP-MYPN WT or the GFP-MYPN mutants, (B) R1088H, (C) P1112L, and (D) V1195M. The sarcomeric structure of cells is rapidly disrupted when mutant P1112L and V1195M are expressed. Scale bar: 5 µm.
revealed by tissue labelling, the disease causing status of this familial mutation is highly supported by genetic evidences, as R1088H is absent from 800 control chromosomes, affects amino acid conserved among species and displays strong positive linkage corresponding to the maximum Lod score calculated for the family.

The molecular mechanism linking mutations to DCM remains hypothetical. On one hand a haploinsufficiency mechanism appears associated with the I83fsX105 heterozygous mutation. This mechanism is in accordance with the apparently milder phenotype of patient carrying the truncation mutation (I83fsX105) as described for numerous diseases causing heterozygous premature termination codon (PTC) mutation. In the absence of cardiac tissue samples from patients with mutation I83fsX105, we have taken advantage from the illegitimate transcription of the MYPD gene in lymphoblastoid cell lines derived from one patient. This approach has previously been used to study other genes that are also expressed in tissues with low availability.

Heart section labelling from heterozygous R1088H carrier indicated mislocalization of near half of the myopalladin signal. This observation would be consistent with the hypothesis of mislocalization of the mutated isoform originating from the mutated allele in the tissue and would fit either with a haploinsufficiency mechanism, as only half of

Figure 4 Survival of transfected rat neonate cardiomyocytes expressing WT or mutated myopalladin. Cells were transfected with indicated vectors expressing GFP alone (as a control for 100% survival, not drawn) or fused to WT-MYPN (squares) or R1088H-GFP-MYPN (triangles), P1112L-GFP-MYPN (plain circles) and V1195M-GFP-MYPN (empty circles). Mean ± SD GFP-positive cell counting from four independent transfection experiments clearly shows a very low and similar time-dependent survival rate for cells expressing P1112L and V1195M as compared to control or WT-GFP-MYPN and R1088H-GFP-MYPN expressing cells. Groups were compared statistically by analysis of variance for repeated measures.

Figure 5 I83fsX105 heterozygous mutation effects and allele specific mRNA expression measurement. (A) Schematic representation of MYPN gene region carrying the I83fsX105 mutation, electrophoresis sequence profile and partial translated protein sequence. The gene region encompassing exon 2 and the Ins/del T735 heterozygote mutation is transcribed into both mutated and WT mRNA. RT-PCR amplification with primers (pF and pR) leads to an 898 bp DNA fragment mixture of both WT and mutated (grey dot) cDNA. The heterozygote frame-shift mutation effect on protein translation with an aberrant 22 amino acid peptide C-terminal (italic) sequence translated from the T735 deletion allele. (B) Restriction enzyme scheme and EtBr-agarose gel electrophoresis of RT-PCR products from I83fsX105 heterozygous carrier mRNA showing absence of expression of the PTC transcript. Total mRNA from lymphoblastoid cell line from patient II-1 (family A) was reverse transcribed and amplified with cDNA specific primers (see A). Allele specific endonuclease digestion by BccI of PCR product cleaves only WT allele (lane 4) generating a 390 bp DNA fragment (and 128 bp, not in picture) from undigested mutant specific allele (518 bp). The control signal at 518 and 390 originating from each allele was obtained by BccI restriction analysis of amplification from pcDNA-GFP-MYPN-WT (lane 1), pcDNA-GFP-I83fsX105 (lane 2) or an equimolar mixture of both plasmids (lane 3).
functional protein may be correctly located, or from a poison peptide based mechanism if the mutated protein impairs the cellular physiology. Supporting this second option, the left ventricle mislocalization of myopalladin is similar to what is observed in the transfected RNC model expressing missense mutations P1112L and V1195M for which the most likely mechanism is a dominant negative effect. However, our expression experiments in RNC indicated a more drastic functional effect with a lack of correct localization of mutated myopalladin associated with rapid sarcomeric structure dislocation and high cellular death rate. Cellularity pattern after mutant transfection is consistent with that observed when the N-terminal domain of myopalladin is overexpressed in chicken cardiomyocytes.7 N-terminal domain of myopalladin is interacting with CARP (cardiac ankyrin repeat protein), a transcription repressor shown to be overexpressed in cardiac remodelling associated with numerous cardiac conditions such as left ventricular hypertrophy10,11 and dilated cardiomyopathy.13

An interesting hypothesis to explain the link between the sarcomere breakdown and cell death observed in our transfection experiments is that mutant myopalladin could alter CARP signalling leading to cardiac dilation and dysfunction. Such hypothesis would have to be confirmed in animal models in order to better understand the myopalladin related pathophysiology of DCM.

Supplementary material

Supplementary material is available at Cardiovascular Research online.

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We would like to thank the patients and their families for their participation; C. Pouzet, C.M. Bachelet and A. Daphin for microscopy analysis expertise; E. Deroubaix for her helpful participation in rat experiments is that mutant myopalladin could alter CARP signalling leading to cardiac dilation and dysfunction. Such hypothesis would have to be confirmed in animal models in order to better understand the myopalladin related pathophysiology of DCM.

Finding

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References


