Genetic basis of hypertrophic cardiomyopathy

Bos, J.M.

Publication date
2010

Citation for published version (APA):

General rights
It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations
If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: https://uba.uva.nl/en/contact, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.
Chapter 3

Cardiac Ankyrin Repeat Protein Gene (ANKRD1)
Mutations in Hypertrophic Cardiomyopathy

Takuro Arimura*, J. Martijn Bos*, Akinori Sato, Toru Kubo, Hiroshi Okamoto, Hirofumi Nishi, Haruhiyo Harada, Yoshinori Koga, Mousumi Moulik, Yoshinori L. Doi, Jeffrey A. Towbin, Michael J. Ackerman, Akinori Kimura
* These authors equally contributed to the work


Editorial comment by:
L. Mestroni. Phenotypic Heterogeneity of Sarcomeric Gene Mutations: a Matter of Gain and Loss?
Abstract

Objectives: The purpose of this study was to explore a novel disease gene for hypertrophic cardiomyopathy (HCM) and evaluate functional alteration(s) caused by mutations.

Background: Mutations in genes encoding myofilaments or Z-disc proteins of the cardiac sarcomere cause HCM, but the disease-causing mutations can be found in half of the patients, indicating that novel HCM-susceptibility genes await discovery. We studied a candidate gene ANKRD1 encoding for cardiac ankyrin repeat protein (CARP) that is a Z-disc component interacting with N2A domain of titin/connectin and N-terminal domain of myopalladin.

Methods: We analyzed 384 HCM patients for mutations in ANKRD1 and in the N2A domain of titin/connectin gene (TTN). Interaction of CARP with titin/connectin or myopalladin was investigated using co-immunoprecipitation assay to demonstrate the functional alteration caused by ANKRD1 or TTN mutations. Functional abnormalities caused by the ANKRD1 mutations were also examined at the cellular level in neonatal rat cardiomyocytes.

Results: Three ANKRD1 missense mutations, Pro52Ala, Thr123Met and Ile280Val, were found in 3 patients. All mutations increased binding of CARP to both titin/connectin and myopalladin. In addition, TTN mutations, Arg8500His and Arg8604Gln, in the N2A domain were found in 2 patients and these mutations increased binding of titin/connectin to CARP. Myc-tagged CARP showed that the mutations resulted in abnormal localization of CARP in cardiomyocytes.

Conclusion: CARP abnormalities may be involved in the pathogenesis of HCM.

Keywords

Hypertrophic cardiomyopathy, mutation, Z-disc, CARP, titin/connectin
Abbreviations

Ab  Antibody
ANKRD1  Ankyrin repeat domain 1
CARP  Cardiac ankyrin repeat protein
cDNA  Complementary deoxyribonucleic acid
Co-IP  Co-immunoprecipitation
DAPI  4′,6-diamidino-2-phenylindole
DCM  Dilated cardiomyopathy
HCM  Hypertrophic cardiomyopathy
PCR  Polymerase chain reaction
WT  Wild-type
Introduction

Cardiomyopathy is a primary heart muscle disorder caused by functional abnormalities of cardiomyocytes. There are several clinical subtypes of cardiomyopathy and the most prevalent subtype is hypertrophic cardiomyopathy (HCM)[1,2]. HCM is characterized by hypertrophy and diastolic dysfunction of cardiac ventricles accompanied by cardiomyocyte hypertrophy, fibrosis and myofibrillar disarray[1]. Although the etiology of HCM has not been fully elucidated, 50-70% of the patients with HCM have apparent family histories consistent with autosomal dominant genetic trait[2], and recent genetic analyses have revealed that a significant percentage of HCM is caused by mutations in the genes encoding for myofilaments and Z-disc proteins of the cardiac sarcomere with the majority of mutations identified in MYH7-encoded beta myosin heavy chain and MYBPC3-encoded myosin binding protein C[2].

ANKRD1 (ankyrin repeat domain 1)-encoded “cardiac adriamycin responsive protein” [3] or “cardiac ankyrin repeat protein”(CARP)[4], is a transcription co-factor and an early differentiation marker of cardiac myogenesis, expressed in the heart during embryonic and fetal development. CARP expression is up-regulated in the adult hearts at end-stage heart failure [5]. In addition, increased CARP expression was found in hypertrophied hearts from experimental murine models [6, 7]. These observations suggest a pivotal role of CARP in cardiac muscle function in both physiological and pathological conditions. Although CARP is known to be involved in the regulation of gene expression in the heart, Bang et al. demonstrated that CARP located to both the sarcoplasm and nucleus, suggesting a shuttling of CARP in cellular components [8]. Within the I-band region of sarcomere, CARP bound to both N2A domain of titin/connectin encoded by TTN and the N-terminal domain of myopalladin encoded by MYPN. Hence, titin/connectin and myopalladin function in part as anchoring proteins of "sarcomeric CARP” [8, 9].
Titin/connectin is the most giant protein expressed in the striated muscles, which is involved in sarcomere assembly, force transmission at the Z-disc, and maintenance of resting tension in the I-band region[10, 11]. In cardiac muscle, there are two titin isoforms, N2B and N2BA. The N2B isoform contains a cardiac specific N2B domain, and the N2BA isoform contains both N2B and N2A domains. Both N2A and N2B domains, within the extensible I-band region, function as a molecular spring that develops passive tension; the expression of N2B isoform results in a higher passive stiffness than that of N2AB isoform. We previously reported an HCM-associated mutation localizing to the N2B domain[12], and Gerull et al.[13] reported other TTN mutations in the Z/I transition domain. These observations suggest that the I-band region of titin/connectin contains elastic components extending with stretch to generate passive force, which plays an important role in the maintenance of cardiac function.

Another protein that anchors CARP at the Z/I band is myopalladin, a cytoskeletal protein containing 3 proline-rich motifs and 5 Ig domains. The proline-rich motifs in the central part is required for binding to nebulin/nebulette, and the Ig domains at the N-terminus and C-terminus are involved in the binding to CARP and sarcomeric α-actinin, respectively[8]. It was suggested that myopalladin played key roles in sarcomere/Z-disc assembly, myofibrillogenesis, recruitment of the other Z/I-band elements, and signaling in the Z/I-band[8].

In this study, we analyzed unrelated patients with heretofore genotype-negative HCM for mutations in ANKRD1 and found 3 mutations that showed abnormal binding to myopalladin and titin/connectin. In addition, we searched for mutations in the reciprocal CARP-binding N2A domain of titin/connectin and identified 2 HCM-associated mutations in TTN causing abnormal binding to CARP. We report here that abnormal CARP assembly in the cardiac muscles may be involved in the pathogenesis of HCM.
Methods

Subjects
A total of 384 unrelated patients with HCM were included in this study. The patients were diagnosed based on medical history, physical examination, 12-lead electrocardiogram, echocardiography, and other special tests if necessary. The diagnostic criteria for HCM included LV wall thickness >13mm on echocardiography, in the absence of coronary artery disease, myocarditis, and hypertension. The patients had been analyzed previously for mutations in previously published myofilament- and Z-disc associated genes and no mutation was found in any of the known HCM-susceptibility genes (15-18). Ethnically-matched healthy individuals (400 and 300 from Japan and USA, respectively) were used as controls. Blood samples were obtained from the subjects after given informed consent. The protocol for research was approved by the Ethics Reviewing Committee of Medical Research Institute, Tokyo Medical and Dental University (Japan) and by the Mayo Foundation Institutional Review Board (US).

Mutational analysis
Using intronic primers, each translated ANKRD1 exon was amplified by polymerase chain reaction (PCR) from genomic DNA samples. TTN exons 99 to 104 corresponding to the N2A domain including binding domains to CARP and p94/calpain were amplified by PCR in exon-by-exon manner. Sequence of primers and PCR conditions used in this study are available upon request. PCR products were analyzed by direct sequencing or by denaturing high performance liquid chromatography (DHPLC) followed by sequencing analysis. Sequencing was performed using Big Dye Terminator chemistry (version 3.1) and ABI3100 DNA Analyzer (Applied Biosystems, CA, USA).
**Co-immunoprecipitation (co-IP) assay**

We obtained cDNA fragments of human ANKRD1 and TTN by RT-PCR from adult heart mRNA. A wild-type (WT) full-length CARP cDNA fragment spanned from bp249 to bp1208 of GenBank Accession No. NM_014391 (corresponding to aa1-aa319). Three equivalent mutant cDNA fragments containing C to G (Pro52Ala mutation), C to T (Thr123Met mutation) or A to G (Ile280Val mutation) substitutions were obtained by primer-directed mutagenesis method. A WT TTN cDNA fragment encoding N2A domains (from bp25535 to bp26465 of NM_133378 corresponding aa8437-aa8747) was obtained and 3 TTN mutants carrying T to C (non disease-associated ile8474Thr polymorphism), G to A (HCM-associated Arg8500His mutation) or G to A (HCM-associated Arg8604Gln mutation) substitutions were created by the primer-mediated mutagenesis method. The cDNA fragments of ANKRD1 were cloned into myc-tagged pCMV-Tag3 (Stratagene, CA, USA), while TTN and MYPN cDNA fragments were cloned into pEGFP-C1 (Clontech, CA, USA). These constructs were sequenced to ensure that no errors were introduced.

Cellular transfection and protein extractions were performed as described previously [14], and co-IP assays were performed using the Catch and Release v2.0 Reversible Immunoprecipitation System according to the manufacturer's instructions (Millipore, Billerica, MA). Immunoprecipitates were separated on SDS-PAGE gels and transferred to a nitrocellulose membrane. After a pre-incubation with 3% skim milk in PBS, the membrane was incubated with primary rabbit anti-myc polyclonal or mouse anti-GFP monoclonal Ab (1:100, Santa Cruz Biotechnology, CA, USA), and with secondary goat anti-rabbit (for polyclonal Ab) or rabbit anti-mouse (for monoclonal Ab) IgG HRP-conjugated Ab (1:2000, Dako A/S, Grostrup, Denmark). Signals were visualized by Immobilon Western Chemiluminescent HRP Substrate (Millipore, MA, USA) and Luminescent Image Analyzer LAS-3000mini (Fujifilm, Tokyo, Japan), and their densities were quantified by using Multi Gauge ver3.0 (Fujifilm, Tokyo, Japan).

Numerical data were expressed as means ± SEM. Statistical differences were analyzed using one-way ANOVA and Student’s t test for paired values. Means were compared by independent samples t-tests without correction for multiple comparisons. A p-value<0.05 was considered to be statistically significant.
Indirect immunofluorescence microscopy

All care and treatment of animals were in accordance with "Guidelines for the Care and Use of Laboratory Animals" published by the National Institute of Health (NIH Publication 85-23, revised 1985) and subjected to prior approval by the local animal protection authority. Neonatal rat cardiomyocytes were prepared as described previously[14]. Eighteen and 48 hours after the transfection, cardiomyocytes were washed with PBS, fixed for 15 min in 100% ethanol at -20°C. Transfected cells were incubated in blocking solution, and stained by primary rabbit anti-myc polyclonal Ab (1:100, Santa Cruz Biotechnology) and mouse anti-α-actinin monoclonal Ab (1:800, Sigma-Aldrich), followed by secondary sheep anti-rabbit IgG FITC-conjugated Ab (1:500, Chemicon, Victoria, Australia) and Alexa fluor 568 goat anti-mouse IgG (1:500, Molecular Probes, OR, USA). All cells were mounted on cover-glass using Mowiol 4-88 Reagent (Calbiochem, Darmstadt, Germany) with 4',6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich) and images from at least 200 transfected cells were analyzed with an LSM510 laser-scanning microscope (Carl Zeiss Microscopy, Jena, Germany).
Results

Identification of ANKRD1 (CARP) and TTN mutations in HCM

Eleven distinct sequence variations in ANKRD1 were identified among the 384 patients with HCM (Figure 1A). Four intronic variants, 2 non-synonymous substitutions and 1 synonymous variation were polymorphisms, because they were also found in the controls. A nonsense mutation (c.423C>T in exon 2 yielding Gln59ter) was found in 2 patients with familial HCM and was absent in the controls, but was not co-segregated with the disease in both families, suggesting that they were not associated with HCM. In contrast, 3 missense mutations, Pro52Ala (c.402C>G in exon 2), Thr123Met (c.616C>T in exon 4) and Ile280Val (c.1086A>G in exon 8), identified in three unrelated HCM patients, were not found in the controls.

Sequence variations in TTN at the N2A domain containing binding region to CARP and p94/calpain were searched for in the patients and 8 variations were identified (Figure 1B). An intronic variation and 3 synonymous variations were polymorphisms observed in the controls. Two non-synonymous variations, Ile8474Thr (c.25645T>C in exon 99) and Asp8672Val (c.26239A>T in exon 102), were not associated with HCM, because Ile8474Thr was found in the controls and Asp8672Val did not co-segregate with the disease in a multiplex family. On the other hand, 2 missense mutations, Arg8500His (c.25723G>A in exon 99) and Arg8604Gln (c.26035G>A in exon 100), identified in familial HCM patients, were not found in the controls.

Clinical phenotypes

Clinical findings of the patients carrying the ANKRD1 or TTN mutations are summarized in Table 1. All patients manifested with HCM except CM1288 II-2 who had mild cardiac hypertrophy. Her father had died suddenly of unknown etiology at the age of 30. Two unaffected brothers of the patient did not harbor the mutation (Figure 1C). The proband patient with the TTN Arg8606Gln mutation (CM1480, Table 1) showed asymmetric septum hypertrophy. A family study revealed that his father had unexplained sudden cardiac death. His son (CM1481, Table 1) was affected and carried the same mutation (Figure 1D).
Figure 1: Mutational analyses of ANKRD1 and TTN in HCM. (A) Sequence variations found in ANKRD1. Single letter code was used to indicate the amino acid residue. Solid boxes represent protein coding region corresponding to exons 1-9. Dotted boxes indicate ankyrin repeat domains encoded by exons 5-8. (B) Sequence variations found in TTN. Solid boxes represent Ig domains corresponding to exons 98, 99 and 102-104. Dotted boxes indicate tyrosine-rich motif encoded by exons 99-101. (C and D) Pedigrees of HCM families with the ANKRD1 T123M (C, CM 1288 family) and the TTN R8604Q (D, CM 1480 family). Filled square and filled circle indicate affected male and female, respectively. Open square and open circle represent unaffected or unexamined male and female, respectively. An arrow indicates the proband patient. Presence (+) or absence (-) of the mutations is noted.
Table 1: Clinical characteristics of individuals carrying ANKRD1 or TTN mutations

<table>
<thead>
<tr>
<th>ID</th>
<th>Mutation</th>
<th>Age, gender</th>
<th>Age at onset</th>
<th>Clinical Dx</th>
<th>FH of HCM</th>
<th>NYHA</th>
<th>LVDd (mm)</th>
<th>LVDs (mm)</th>
<th>IVS (mm)</th>
<th>PW (mm)</th>
<th>%FS</th>
<th>%EF</th>
<th>Other remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mayo I</td>
<td>ANKRD1</td>
<td>44, male</td>
<td>30</td>
<td>HCM</td>
<td>No</td>
<td>II</td>
<td>-</td>
<td>-</td>
<td>22</td>
<td>-</td>
<td>-</td>
<td>70</td>
<td>LVH on ECG; provokable gradient 100mmHg, but asymptomatic</td>
</tr>
<tr>
<td>Mayo II</td>
<td>ANKRD1</td>
<td>65, male</td>
<td>41</td>
<td>HCM</td>
<td>No</td>
<td>III</td>
<td>38</td>
<td>16</td>
<td>14</td>
<td>14</td>
<td>-</td>
<td>84</td>
<td>Mid-ventricular-apical hypertrophy with midventricular wall thickness up to 35mm</td>
</tr>
<tr>
<td>CM1288</td>
<td>ANKRD1</td>
<td>62, female</td>
<td>40</td>
<td>HCM</td>
<td>No</td>
<td>I</td>
<td>41</td>
<td>22</td>
<td>13</td>
<td>13</td>
<td>46</td>
<td>78</td>
<td>Lateral LV hypertrophy (15mm), LAD=37 mm, ECG; abnormal Q-wave in II, III, aVF, V4-6</td>
</tr>
<tr>
<td>Mayo III</td>
<td>ANKRD1</td>
<td>82, female</td>
<td>61</td>
<td>HCM</td>
<td>No</td>
<td>III</td>
<td>52</td>
<td>30</td>
<td>20</td>
<td>14</td>
<td>-</td>
<td>70</td>
<td>Septal ablation (relieved obstruction 73mmHg, -&gt; 22mmHg)</td>
</tr>
<tr>
<td>CM89</td>
<td>TTN</td>
<td>59, male</td>
<td>53</td>
<td>HCM</td>
<td>No</td>
<td>I</td>
<td>42</td>
<td>25</td>
<td>28</td>
<td>8</td>
<td>40</td>
<td>79</td>
<td>LVH (ASH)</td>
</tr>
<tr>
<td>CM1480</td>
<td>TTN</td>
<td>52, male</td>
<td>43</td>
<td>HCM</td>
<td>Yes</td>
<td>I</td>
<td>41</td>
<td>24</td>
<td>18</td>
<td>10</td>
<td>41</td>
<td>80</td>
<td>LVH (ASH), Atrial fibrillation ECG; Inverted T-wave in V4-V6</td>
</tr>
<tr>
<td>CM1481</td>
<td>TTN</td>
<td>25, male</td>
<td>16</td>
<td>HCM</td>
<td>Yes</td>
<td>I</td>
<td>45</td>
<td>27</td>
<td>22</td>
<td>9</td>
<td>40</td>
<td>66</td>
<td>LVH (ASH), ECG; Inverted T-wave in V1-V3</td>
</tr>
</tbody>
</table>

Dx, diagnosis; EF, ejection fraction; FH, family history; LAD, left atrial dimension; LVH, left ventricular hypertrophy; LVDd, left ventricular dimension diastole, LVDs, left ventricular dimension systole, IVS, intraventricular septum; FS, fractional shortening; NYHA, New York Heart Association.
**Altered interaction between titin/connectin and CARP caused by the TTN or CARP mutations**

To investigate the functional alterations caused by the CARP mutations in the binding to titin/connectin N2A domain, WT-, Pro52Ala-, Thr123Met-, or Ile280Val-CARP construct was co-transfected with the WT TTN-N2A construct into COS-7 cells. Western blot analyses of immunoprecipitates from the transfected cells demonstrated that HCM-associated CARP mutations significantly increased binding to TTN-N2A (2.22±0.76 AU, p<0.05, 1.98±0.52 AU, p<0.01 or 2.16±0.64 AU, p<0.05, respectively) (Figure 2A and B). Reciprocally, the effect of titin/connectin mutations in binding to CARP was assessed. TTN-N2A constructs, WT-, HCM-associated mutants (Arg8500His- and Arg8604Gln-TTN), or non-disease-related variant (Ile8474Thr) TTN-N2A were co-transfected with WT CARP. Western blot analyses showed that Arg8500His and Arg8604Gln significantly increased the binding to CARP (2.78±0.40 or 3.16±0.40 AU, respectively, p<0.001 in each case) (Figure 2A and B), while the non-disease related variant (Ile8474Thr) did not alter the binding (1.18±0.11 AU), despite equal expression of proteins.

**Altered interaction between myopalladin and CARP caused by the CARP mutations**

Because CARP bound also to myopalladin, we investigated the effects of CARP mutations in binding to myopalladin. WT or mutant CARP construct was co-transfected with a MYPN construct. Western blot analysis revealed that binding of mutant CARPs, Pro52Ala, Thr123Met or Ile280Val, to myopalladin was significantly increased (3.60±0.67 AU, p<0.001, 1.87±0.47 AU, p<0.01 or 2.48±0.45 AU, p<0.001, respectively) (Fig. 2C and D).
Figure 2: Binding of CARP to titin/connectin and myopalladin. Binding of CARP to titin/connectin (TTN) or myopalladin (MYPN) was analyzed by co-IP assays. (A) Myc-tagged CARPs co-precipitated with GFP-tagged TTN-N2A domain were shown (top panel). Expressions of GFP-tagged TTN-N2A (middle panel) and myc-tagged CARP (lower panel) were confirmed by immunoblotting of whole cell supernatants. Binding pairs were WT CARP in combination with WT, I8474T, R8500H or R8604Q mutant TTN-N2A, or WT TTN-N2A with WT, P52A, T123M or I280V mutant CARP. Dashes indicate no GFP- or myc-tagged proteins (transfected only with pEGFP-C1 or pCMV-Tag3 vectors, respectively). (B) Densitometric data obtained in the co-IP assay. Data for WT CARP with WT TTN-N2A were arbitrarily defined as 1.00 arbitrary unit (AU). Data are represented as mean ± SEM. (n= 6 for each case). *** p<0.001 vs WT; ** p<0.01 vs WT; * p<0.05 vs WT.
Figure 2 cont’d. (C): Myc-tagged CARP co-precipitated with GFP-tagged full-length MYPN was detected by immunobloting using anti-myc antibody (top panel). Expressed amounts of GFP-tagged MYPN (middle panel) and myc-tagged CARP (lower panel) were confirmed as in (A). Binding pairs were full-length WT-MYPN with WT, P52A, T123M or I280V mutant CARP. (D) Densitometric analysis of myc-blotting data in (C). Data were arbitrarily represented as intensities and that for WT CARP with full length or N-terminal half WT MYPN was defined as 1.00 AU. Data are expressed as mean± SEM. (n = 9 for each case). *** p<0.001 vs WT; ** p<0.01 vs WT.
Altered localization of CARP caused by the mutations
To further investigate the functional consequence of the CARP mutations, we examined cellular distribution of the mutant CARP proteins expressed in neonatal rat primary cardiomyocytes. Cells were transfected with myc-tagged WT or mutant CARP constructs, co-immunostained for myc (a marker for CARP) and α-actinin (a marker for Z-disc). WT and mutant myc-CARP proteins were expressed at a similar level in the transfected cells as assessed by Western-blot analyses, suggesting that the mutations did not affect the expression level and stability of CARP proteins (data not shown). Control cells expressing myc-tag alone showed negative staining for myc-tag with striated staining pattern of sarcomeric α-actinin at the Z-disc (data not shown). In premature cardiomyocytes containing Z-bodies (Z-disc precursors), myc-tagged WT CARP was mainly targeted to nucleus and colocalization of CARP with α-actinin, which formed patchy dense bodies in the cytoplasm, was observed (Figure 3A-C). No apparent changes in localization of mutant CARP proteins were observed in the nascent and immature cardiomyocytes (Figure 3D-F, G-I and J-L).

In the mature cardiomyocytes where Z-discs were well organized, myc-tagged WT CARP was assembled in the striated pattern at the Z-I bands and co-localized with α-actinin (Figure 4A-C). It was found that most (≈90%) of mature cardiomyocytes did not contain nuclear CARP (Figure 4A-C). On the other hand, higher intensity of CARP-related fluorescence at the Z-I bands and diffused localization in the cytoplasm was observed in the most (≈80%) of mature cardiomyocytes expressing myc-tagged mutant CARPs, albeit that the Z-disc assembly was not impaired (Figure 4D-F, G-I and J-L). Quite interestingly, myc-tagged mutant CARP proteins displayed localization within the nuclear and/or at nuclear membrane in ≈60% of mature cardiomyocytes (Figure 4D-F, G-I and J-L).
Discussion

CARP encoded by ANKRD1 is a nuclear transcription co-factor expressing in the embryonic hearts. Its expression progressively decreases in adult hearts [3, 4] and reappears in the hypertrophied or failing adult heart [5, 15], suggesting that CARP may be involved in the regulation of muscle gene expression. CARP also localizes in cardiac sarcomere although the roles of “sarcomeric CARP” are not fully elucidated. Several reports have demonstrated that CARP binds titin/connectin [9], myopalladin [8] and desmin [16] at the ZI-region of sarcomere. In this study, we found that the HCM-associated ANKRD1 mutations increased the binding of CARP to titin/connectin and myopalladin, and HCM-associated TTN mutations in its reciprocal CARP N2A-binding domain increased the binding of titin/connectin to CARP. These observations in association with HCM suggested that the assembly or binding of sarcomeric CARP with titin/connectin and/or myopalladin would be required for the maintenance of cardiac function.

In the nascent myofibrils, myc-tagged CARP proteins were detected within the nucleus irrespective of mutations. Because CARP is an early differentiation marker during heart development, recruitment of CARP into nuclei may be important in the embryonic gene expression. Interestingly, abnormal intra-nuclear accumulation of myc-tagged mutant CARP proteins was observed in mature myofibrils. It is well known that the embryonic and fetal gene program of cardiac cytoskeletal proteins is initiated during the cardiac remodeling [17, 18]. Hence, one could hypothesize that nuclear CARP may cause embryonic/fetal gene expression in mature myofibrils and this abnormal gene expression is a possible mechanism leading to the pathogenesis of HCM. It was reported that CARP negatively regulated expression of cardiac genes including MYL2, TNNC1 and ANP [3, 4].
Conversely, another report suggested that different expression level of CARP did not correlate with the altered expression of cardiac genes such as MYL2, MYH7, ACTC, CACTN, TPM1, ACTN2 and DES [19]. Thus, the role of CARP as a regulator of cardiac gene expression remains to be resolved. During the preparation of this paper, Cinquetti et al. [20] reported other CARP mutations, rearrangements or Thr116Met, in association with the cyanotic congenital heart anomaly known as total anomalous pulmonary venous return (TAPVR). These mutations were demonstrated to be associated with increased expression or stability of CARP. It is not clear whether the mutations associated with HCM altered expression or stability of CARP, though our data suggested that HCM-associated CARP mutations did not alter the stability. The molecular mechanisms underlying the CARP-related pathogenesis should be different between TAPVR and HCM.
Figure 3: Distribution myc-tagged CARP in immature rat cardiomyocytes. Neonatal rat cardiomyocytes transfected with myc-tagged WT (A-C) or mutant (P52A, T123M or I280V) (D-F, G-I or J-L, respectively) CARP constructs were fixed 18 h after the transfection, and stained with DAPI and anti-α-actinin antibody followed by secondary antibody (B, E, H, and K). Merged images were shown in C, F, I, and L. In the immature cardiomyocytes showing nascent myofibrils with Z bodies (Z-disc precursors), myc-tagged CARPs were preferentially localized to the nucleus and mutant CARP showed relatively low expression in the cytoplasm. Scale bars=10 μm.
Figure 4: Distribution of myc-tagged CARP in mature rat cardiomyocytes. Neonatal rat cardiomyocytes transfected with myc-tagged WT (A-C) or mutant (P52A, T123M or I280V) (D-F, G-I or J-L, respectively) CARP constructs were fixed 48 h after the transfection, and stained with DAPI and anti-β-actinin antibody followed by secondary antibody (B, E, H, and K). Merged images were shown in C, F, I, and L. In the mature cardiomyocytes showing myofibrils with Z-discs, normal localization of myc-tagged WT CARP at the Z-discs was observed (A-C). In contrast, myc-tagged mutant CARP proteins showed intense localization at the I-discs (colocalization with β-actinin) and diffused localization in the cytoplasm (D-F, G-I and J-L). In addition, myc-tagged mutant CARPs expressed at high levels around the nuclear membrane (white arrow) and/or in the nucleus (white arrowhead).
Conclusions

We identified 3 missense CARP mutations in < 1% of unrelated patients with HCM, which not only increased the binding of sarcomeric CARP to I-band components but also resulted in the mis-localization of CARP to the nucleus. Although the molecular mechanisms of HCM due to the CARP mutations remain to be elucidated, our findings imply that HCM may be associated with the abnormal recruitment of CARP in cardiomyocytes leading to pathological hypertrophy.

Acknowledgements

We thank Drs. H. Toshima, C. Kawai, K. Kawamura, M. Nagano, T. Sugimoto, S. Ogawa, A. Matsumori, S. Sasayama, R. Nagai, and Y. Yazaki for their contributions in clinical evaluation and blood sampling from patients with cardiomyopathy, and Ms. M. Yanokura, M. Emura and A. Nishimura for their technical assistance.
References


