**Dilated Cardiomyopathy-Associated FHOD3 Variant Impairs the Ability to Induce Activation of Transcription Factor Serum Response Factor**

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**Background:** Dilated cardiomyopathy (DCM) is characterized by a dilated left ventricular cavity with systolic dysfunction manifested by heart failure. It has been revealed that mutations in genes for cytoskeleton or sarcomere proteins cause DCM. However, the disease-causing mutations can be found only in far less than half of patients with a family history, indicating that there should be other disease genes for DCM. Formin homology 2 domain containing 3 (FHOD3) is a sarcomeric protein expressed in the heart that plays an essential role in sarcomere organization during myofibrillogenesis. The purpose of this study was to explore a possible novel disease gene for DCM.

**Methods and Results:** We analyzed 48 Japanese familial DCM patients for mutations in FHOD3, and a missense variant, Tyr1249Asn, which was predicted to modify the 3D structure and damage protein function, was found in a case with adult-onset DCM. Functional studies revealed that the DCM-associated mutation significantly reduced the ability to induce actin dynamics-dependent activation of serum response factor, although no remarkable change in the cellular localization was induced in neonatal rat cardiomyocytes transfected with a mutant construct of FHOD3.

**Conclusions:** The DCM-associated FHOD3 variant may cause DCM by interfering with actin filament assembly. *(Circ J 2013; 77: 2990–2996)*

**Key Words:** Actin assembly; Dilated cardiomyopathy; FHOD3; Mutation

**Dilated cardiomyopathy (DCM) is a primary cardiac disorder caused by functional abnormalities in cardiomyocytes and is characterized by ventricular chamber dilation with decreased contractility. DCM is a major cause of chronic heart failure and the most common indication for cardiac transplantation. Various etiologies include genetic abnormalities, viral infections, alcohol, mitochondrial dysfunction and metabolic disorders. Most of the genetic causes are mutations in genes for sarcomeric proteins, including contractile elements, sarcolemma elements, Z-disc elements, and Z-I region components, which play key roles in the generation and transmission of contractile force.*

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**Editorial p2879**

Fromin homology 2 domain containing 3 (FHOD3) is a member of the formin family. We have previously reported that FHOD3 is a sarcomeric protein that is predominantly expressed in the heart and plays an essential role in the regulation of actin assembly and sarcomere organization during myofibrillogenesis. FHOD3 contains multiple domains, including GTPase-binding and diaphanous inhibitory domains at the N-terminus, formin homologous 1 (FH1), formin homologous 2 (FH2) and diaphanous autoregulation (DA) domains at the C-terminus. The FH2 domain mediates actin filament nucleation and polymerization, which are accelerated by FH1-mediated
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straight actin filaments, between the FH1 and FH2 domains regulates the formation of

jects was used to amplify the C-terminal functional domains, 

Genomic DNA extracted from peripheral blood cells of sub-

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written informed consent. The research protocol was approved

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We have demonstrated that FHod3 knockout mice display abnormal maturation of muscle fibers in the heart, resulting in embryonic lethality with ventricular hypokinesia. In addition, transgenic mice expressing a mutant FHOD3 protein with Ile1127Ala (I1127A) substitution in the FH2 domain, which is defective in actin-assembly activity, exhibits phenotypic changes similar to DCM. Moreover, it has recently been reported that knockdown of flhos, the fly homolog of mammalian FHOD, in Drosophila develops heart failure resembling to DCM. However, there is no report on FHOD3 mutation in patients with DCM.

We report here a heterogeneous sequence variation of FHOD3 identified in a Japanese patient with familial DCM. The FHOD3 variant was found at the conserved residue in the FH2 domain and impaired the ability to induce actin dynamics-dependent activation of a transcription factor, serum response factor (SRF). This is the first report demonstrating a functional abnormality of a DCM-associated FHOD3 variant.

Methods

Subjects

A total of 48 genetically unrelated Japanese patients with DCM were the subjects of this study. Each patient had an apparent family history (ie, at least 1 patient with DCM, heart failure or sudden cardiac death among the first-degree family relatives) and was diagnosed based on medical history, physical examination, 12-lead ECG, echocardiography, and other special tests if necessary. The diagnostic criteria for DCM have been described previously. The patients had been analyzed for mutations in the known cardiomyopathy-associated genes, and none was found in any of them, although the largest gene in humans, the titin gene (TTN) consisting or more that 360 exons, was analyzed for approximately 30% of the gene region. As controls, we included 400 healthy Japanese individuals.

Blood samples were obtained from each subject after giving written informed consent. The research protocol was approved by the Ethics Review Committee of the Medical Research Institute, Tokyo Medical and Dental University, Tokyo, Japan.

Mutational Analysis

Genomic DNA extracted from peripheral blood cells of sub-

including the FH1/FH2 domains and DA domain, encoded by exons from 16 to 25 of human FHOD3 (GenBank Accession No. NM_025135.2), and an alternative T(D/E)5XE exon between exons 22 and 23, by polymerase chain reaction (PCR) in an exon-by-exon manner using specific primer pairs listed in Table 1. The PCR products were analyzed by direct sequencing on both strands, from the same primers used in the PCR, using Big Dye Terminator chemistry (version 3.1) and ABI3100 DNA Analyzer (Applied Biosystems, Foster City, CA, USA).

Comparison of Amino Acid Sequence of FHOD3 From Various Species

Amino acid sequences of human FHOD3 predicted from NM_025135 were aligned with those of chimpanzee, rhesus monkey, mouse, rat, rabbit, cattle, cat, dog, opossum, platypus, tetraodon and zebrafish.

Molecular Modeling

To predict possible effects of an amino acid substitution on the structure and function of human FHOD3, we used the SWISS-MODEL program (http://swissmodel.expasy.org/) and PolyPhen-2 program (http://genetics.bwh.harvard.edu/pph2/).

Plasmids for Expression of FHOD3

Complementary DNA (cDNA) for mouse FHod3 was obtained by reverse transcriptase-PCR from total mRNA of the heart. The cDNA contains 28 exons of 1,586 amino acids, with the T(D/E)5XE region-encoding exon between exons 24 and 25 in the clone NM_175276.3. The mutant cDNA fragment carrying a T to A mutation, leading to the Tyr1388Asn substitution, which was equivalent to the Tyr1249Asn substitution in human FHOD3, was prepared by PCR-mediated site-directed mutagenesis. The cDNA fragments were cloned into a pEF-BOS vector to be expressed as Flag-tagged proteins in mammalian cells. The constructs were sequenced for confirmation of their identities.

SRF Reporter Gene Activity

SRF activation by FHOD3 was estimated using a serum response element (SRE)-dependent reporter as described previously, with minor modifications. Briefly, mouse NIH3T3 fibroblasts (2×10⁴) were plated onto a 24-well plate and transfected using LipofectAMINE PLUS (Invitrogen) with 260 ng of pEF-BOS–FHOD3, 40 ng of pRL-TK encoding for Renilla Luciferase (Promega) and 100 ng of pSRE-Luc reporter plasmid (Clontech). After 24 h of transfection, the culture medium was changed to decrease the concentration of fetal calf

Table 1. Primers Used For PCR Amplification and Sequencing of FHOD3

<table>
<thead>
<tr>
<th>Exon</th>
<th>Forward primer sequence (5’&gt;3’)</th>
<th>Reverse primer sequence (5’&gt;3’)</th>
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<tr>
<td>16a (5’ side)</td>
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<td>AGGTCCACACATCTAGGACATC</td>
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<tr>
<td>16b (3’ side)</td>
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<td>CAGAACGAGACCTGTGAG</td>
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</tr>
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<tr>
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<td>25</td>
<td>CTGTCGCTTCGCTGCTCATTAC</td>
<td>TCCATCCAGCAAGCTGTTG</td>
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Figure 1. Mutational analysis of *FHOD3* in dilated cardiomyopathy (DCM). (A) Sequence variations found in this study. DCM-associated mutation and polymorphisms are indicated above and below, respectively, the schematic representation of the *FHOD3* gene. Known polymorphisms are indicated with reference single nucleotide polymorphism (rs) number in parentheses. Single-letter code is used to indicate the amino acid residue. (Solid boxes) Coding exons for FH1, FH2 and DA domains. Position of T(D/E)<XE exon is indicated. (B) Direct sequencing data of *FHOD3* exon 22 from a control (Left) and the DCM patient CM753 (Right). Codon 1249 of the control was TAT (Tyr), whereas the patient was heterozygous for an AAT (Asn) mutation. (C) Pedigree of DCM family carrying the Y1249N mutation (CM 753 family) is shown. (Filled square) males, (filled circles) females. (Open squares) unaffected males, (open circles) unaffected females. Proband patient CM753 (arrow). Presence (+) or absence (−) of the mutation is noted for the analyzed individuals. SCD, sudden cardiac death. HF, heart failure. (D) Alignment of amino acid sequences of *FHOD3* from various species around the DCM-associated mutation, Y1249N. Protein sequence of human FHOD3 predicted from the nucleotide sequences aligned with those of chimpanzee, rhesus monkey, mouse, rat, rabbit, cattle, cat, dog, opossum, platypus, tetraodon and zebrafish. (E) 3D models of FH2 domain encoded by exons 21 and 22 in the presence of Tyr1249 (Left) or Asn1249 (Right), which are indicated by arrows.
serum from 10% to 0.5%, with or without latrunculin A (Invitrogen), latrunculin B (Invitrogen), or cytochalasin D (Sigma). After culture for another 24h, luciferase activity was determined using the dual luciferase reporter assay system kit (Promega) with a luminometer (Auto Lumat LB953; EG&G Berthold). SRE firefly luciferase counts were normalized to the activity of the internal control, Renilla luciferase, and calculated as fold transactivation in comparison with the counts from cells transfected with the empty pEF-BOS vector.

Indirect Immunofluorescence Microscopy
All care and treatment of animals were in accordance with the 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 (NRCs) from 1-day-old Sprague-Dawley rats were prepared as described previously. NRCs (1 × 10^5  cells) were plated onto the Collagen Type I Cellware 8-Well Culture Slide (BD Biosciences) in low-glucose DMEM supplemented with 0.01 mg/ml insulin (Sigma-Aldrich, St. Louis, MO, USA), 10% fetal bovine serum, and 1% penicillin/streptomycin at 37°C with 5% CO_2 for 24 h. Each Flag-tagged FHOD construct (300 ng) was transfected into cells with TransFectin Lipid Reagent (Bio-Rad) according to the manufacturer’s instructions. At 48h after transfection, the NRCs were washed with PBS and fixed for 15 min in 100% ethanol at −20°C. Transfected cells were incubated in blocking solution and stained by primary mouse anti-α-actinin (1:400, Sigma-Aldrich) and rabbit anti-flag (1:100, Sigma-Aldrich) monoclonal antibodies, followed by secondary Alexa fluor 568 goat anti-mouse IgG 1 (1:400, Molecular Probes) and FITC-conjugated sheep anti-rabbit IgG (1:100, Chemicon). The cells were mounted on a cover-glass using Mowiol 4-88 Reagent (Calbiochem, Darmstadt, Germany) with 4’6-diamidino-2-phenylindole (Sigma-Aldrich), and images from at least 200 transfected cells were analyzed using an LSM510 laser-scanning microscope (Carl Zeiss Microscopy, Jena, Germany).

Results
Identification of FHOD3 Mutations in DCM
Sequence variations of FHOD3 in the regions corresponding to the FH1/FH2 and DA domains were searched for in 48 proband patients with familial DCM and in total, 5 variations were identified (Figure 1A). Among them, 2 synonymous substitutions, p.Asp709Asp (D709D; c.2127T>C in exon 16, rs3809993) and p.Asn984Asn (N984N; c.2952C>T in exon 17, rs3744903), and 1 non-synonymous variation, p.Val1151Ile (V1151I; c.3451G>A in exon 20, rs2303510), were known polymorphisms found in the dbSNP database (http://www.ncbi.nlm.nih.gov/SNP/snps_ref.cgi?locusId=80206). A novel synonymous variation, p.Arg912Arg (R912R; c.2736T>C in exon 16), found in 1 patient was considered to be a polymorphism, because no effect on FHOD3 function could be predicted.

On the other hand, a missense variation, p.Tyr1249Asn

Table 2. Clinical Characteristics of the Patient Carrying the FHOD3 Mutation

<table>
<thead>
<tr>
<th>ID</th>
<th>Mutation</th>
<th>Age at exam (years) and sex</th>
<th>Age at onset (years)</th>
<th>Clinical diagnosis</th>
<th>NYHA</th>
<th>LVDd (mm)</th>
<th>LVDs (mm)</th>
<th>IVST (mm)</th>
<th>LAD (mm)</th>
<th>%FS</th>
<th>%EF</th>
<th>Other remarks</th>
</tr>
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<tr>
<td>CM753</td>
<td>Y1249N</td>
<td>48, female</td>
<td>48</td>
<td>DCM</td>
<td>I</td>
<td>55</td>
<td>43</td>
<td>13</td>
<td>13</td>
<td>49</td>
<td>23</td>
<td>44</td>
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</table>

%EF, percent ejection fraction; %FS, percent fractional shortening; CK, creatine kinase; IVST, interventricular septum thickness; LAD, left atrial dimension; LVDd, left ventricular end-diastolic dimension; LVDs, left ventricular end-systolic dimension; PWT, posterior wall thickness.

Figure 2. Serum response factor reporter gene activity of FHOD3. (A) NIH3T3 cells cotransfected with pSRE-Luc, pHRL-TK, and pEF-BOS encoding wild-type (WT) Fhod3; and then treated with 200 nmol/L latrunculin A, 500 nmol/L latrunculin B or 5 μmol/L cytochalasin D. (B) NIH3T3 cells cotransfected with pEF-BOS encoding WT or mutant Fhod3; I1127A substitution (IA) or Y1388N mutation (YN). (C) pEF-BOS for WT and mutant Fhod3 cotransfected at a 1:1 ratio for mimicking the heterozygous state. Luciferase activity was normalized for transfection efficiency with Renilla luciferase activity. Fold activation was calculated as compared with normalized luciferase activity in cells transfected with empty pEF-BOS vector, which was arbitrarily defined as 1.00. Each graph represents the mean±SD of data from 5 independent transfection experiments. *P<0.005.
and performed a reporter assay using a luciferase reporter plasmid under the control of SRF (pSRF-Luc). It is established that activation of SRF-dependent transcription correlates well with actin polymerization status: the SRF coactivator MAL/MRTF-A associates with G-actin in the resting state, but dissociates during actin polymerization to directly interact with SRF, resulting in transcriptional activation. Indeed, actin polymerization induced by formin family proteins leads to the activation of SRF-dependent transcription. This assay system also appears to reflect actin dynamics, because reporter activity was enhanced by treatment of cells with cytochalasin D (Figure 2A), an agent that disrupts the MAL/MRTF-A interaction with G-actin and thus activates SRF-dependent transcription. Under these conditions, FHOD3 exhibited the ability to induce SRF-dependent transcriptional activation (Figure 2A). FHOD3 likely activated SRF via actin dynamics, because the response was effectively blocked by latrunculin A and latrunculin B (Figure 2A), both of which are known to inhibit actin polymerization and thus increase the level of G-actin, leading to impairment of SRF activity. In contrast to FHOD3-WT, a mutant mouse Fhod3 construct carrying the I1127A mutation, which has been demonstrated to be defective in in-vivo actin-assembling activity, failed to induce the activation of SRF-dependent transcription (Figure 2B). Therefore, FHOD3 appeared to activate SRF in a manner dependent on actin-assembling activity.

To quantitatively estimate the effect of the Y1249N variant, we constructed a mutant mouse Fhod3 carrying an equivalent substitution, Y1388N, and transfected NIH 3T3 cells and performed a reporter assay using a luciferase reporter plasmid under the control of SRF (pSRF-Luc). It is established that activation of SRF-dependent transcription correlates well with actin polymerization status: the SRF coactivator MAL/MRTF-A associates with G-actin in the resting state, but dissociates during actin polymerization to directly interact with SRF, resulting in transcriptional activation. Indeed, actin polymerization induced by formin family proteins leads to the activation of SRF-dependent transcription. This assay system also appears to reflect actin dynamics, because reporter activity was enhanced by treatment of cells with cytochalasin D (Figure 2A), an agent that disrupts the MAL/MRTF-A interaction with G-actin and thus activates SRF-dependent transcription. Under these conditions, FHOD3 exhibited the ability to induce SRF-dependent transcriptional activation (Figure 2A). FHOD3 likely activated SRF via actin dynamics, because the response was effectively blocked by latrunculin A and latrunculin B (Figure 2A), both of which are known to inhibit actin polymerization and thus increase the level of G-actin, leading to impairment of SRF activity. In contrast to FHOD3-WT, a mutant mouse Fhod3 construct carrying the I1127A mutation, which has been demonstrated to be defective in in-vivo actin-assembling activity, failed to induce the activation of SRF-dependent transcription (Figure 2B). Therefore, FHOD3 appeared to activate SRF in a manner dependent on actin-assembling activity.

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Figure 3. Distribution of cardiac α-actinin and transiently expressed Flag chimeras of FHOD3 in neonatal rat cardiomyocytes (NRCs). NRCs transfected with Flag-tagged Fhod3 constructs for WT (A–C) or Y1388N mutant (D–F) were fixed 48 h after transfection, and stained with Flag (A, D) and anti-α-actinin (B, E) antibodies followed by secondary antibodies. Merged images are shown in (C) and (F). In the NRCs showing myofibrils with Z-discs, WT-FHOD3 (A–C) and mutant FHOD3 (D–F) were similarly observed at the A-band region.
sure the ability to induce actin dynamics-dependent activation of the SRF reporter gene. It was revealed that the DCM-associated variant led to significant impairment of SRF activation (Figure 2B). In addition, significant impairment to a similar extent was observed in cells cotransfected with equal amounts of the WT and variant Fhod3 constructs, which mimicked the heterozygous state (Figure 2C).

**Cellular Localization of Mutant Fhod3**

Because Fhod3 is known to localize at the A-band, we analyzed cellular localization of Fhod3-WT or DCM-associated Fhod3 variant. NRCs were transfected with Flag-tagged WT or variant Fhod3 construct, co-immunostained for Flag-tag and a Z-disc marker, cardiac α-actinin, and examined under confocal microscopy, which showed Flag-Fhod3-WT mainly localizing at the A-band in NRCs (Figures 3A–C) as reported previously, and no apparent change in the localization of the DCM-associated variant type Flag-Fhod3 (Figures 3D–F), suggesting that overexpression of variant Fhod3 was neither deleterious to maintaining sarcomere structure nor causative of drastic change in the cellular localization of Fhod3.

**Discussion**

In the present study, we identified a DCM-associated sequence variation in the gene for sarcomeric protein, Fhod3, which was not found in either control subjects or a public database for sequence variations including the 1,000 genome database. The Y1249N variant affected the evolutionary conserved residue, predicted to be deleterious by PolyPhen-2 and to induce sequence variations including the 1,000 genome database, which was not found in either control subjects or a public database.

**Study Limitations**

First, we could not prove co-segregation of the Fhod3 variant with DCM in the family, because both the father and elder sister of the proband patient had died more than 30 years ago and hence we could not investigate whether they had the same variant. Second, because we did not complete the analysis of TTN, which is reported to be a major disease gene for DCM, a possibility remains that the patient had a TTN mutation in a region so far not analyzed. However, it should be noted here that Golbus et al have analyzed genome data from 1,092 individuals in the 1,000-genome database and reported that approximately 9% of the general population had a TTN variation and, more specifically, 3% had a protein-truncating TTN variation. Therefore, the clinical significance of TTN variations should be discussed with caution. Third, although we demonstrated the functional alteration caused by the DCM-associated Y1249N variant in this study, definite in vivo proof of disease-
causing property was not obtained. The latter 2 issues should be addressed in future studies. Finally, we have identified only 1 case of FHOD3 variant in this study, so it was difficult to conclude if FHOD3 is a disease gene for DCM. Identification of another FHOD3 variant in another DCM patient will support a genetic association between FHOD3 and DCM.

In conclusion, we report here for the first time a heterozygous sequence variation of FHOD3 in a patient with familial DCM, which reduced the actin dynamics-dependent activity of the SRF reporter gene. Because an in vitro study has significant limitations in mimicking the DCM heart, further studies of the functional role of FHOD3 in adult cardiac muscle will help us understand the association between abnormal function of FHOD3 and DCM.

Acknowledgments
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Disclosures
None declared.

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