研究成果の刊行に関する一覧表

書籍

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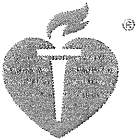

**Molecular Genetic and Functional Characterization Implicate Muscle-Restricted Coiled-Coil Gene ( *MURC*) as a Causal Gene for Familial Dilated Cardiomyopathy**

Gabriela Rodriguez, Tomomi Ueyama, Takehiro Ogata, Grazyna Czernuszewicz, Yanli Tan, Gerald W. Dorn II, Roberta Bogaev, Katsuya Amano, Hidemasa Oh, Hiroaki Matsubara, James T. Willerson and Ali J. Marian

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# Molecular Genetic and Functional Characterization Implicate Muscle-Restricted Coiled-Coil Gene (*MURC*) as a Causal Gene for Familial Dilated Cardiomyopathy

Gabriela Rodriguez, MD*; Tomomi Ueyama, MD, PhD*; Takehiro Ogata, MD, PhD*; Grazyna Czernuszewicz, MS; Yanli Tan, RN; Gerald W. Dorn II, MD; Roberta Bogaev, MD; Katsuya Amano, MD, PhD; Hidemasa Oh, MD, PhD; Hiroaki Matsubara, MD, PhD; James T. Willerson, MD; Ali J. Marian, MD

***Background***—Dilated cardiomyopathy (DCM) and hypertrophic cardiomyopathy (HCM) are classic forms of systolic and diastolic heart failure, respectively. Mutations in genes encoding sarcomere and cytoskeletal proteins are major causes of HCM and DCM. *MURC*, encoding muscle-restricted coiled-coil, a Z-line protein, regulates cardiac function in mice. We investigated potential causal role of *MURC* in human cardiomyopathies.

***Methods and Results***—We sequenced *MURC* in 1199 individuals, including 383 probands with DCM, 307 with HCM, and 509 healthy control subjects. We found 6 heterozygous DCM-specific missense variants (p.N128K, p.R140W, p.L153P, p.S307T, p.P324L, and p.S364L) in 8 unrelated probands. Variants p.N128K and p.S307T segregated with inheritance of DCM in small families ($\chi^2$=8.5, $P$=0.003). Variants p.N128K, p.R140W, p.L153P, and p.S364L were considered probably or possibly damaging. Variant p.P324L recurred in 3 independent probands, including 1 proband with a *TPM1* mutation (p.M245T). A deletion variant (p.L232-R238del) was present in 3 unrelated HCM probands, but it did not segregate with HCM in a family who also had a *MYH7* mutation (p.L907V). The phenotype in mutation carriers was notable for progressive heart failure leading to heart transplantation in 4 patients, conduction defects, and atrial arrhythmias. Expression of mutant MURC proteins in neonatal rat cardiac myocytes transduced with recombinant adenoviruses was associated with reduced RhoA activity, lower mRNA levels of hypertrophic markers and smaller myocyte size as compared with wild-type MURC.

***Conclusions***—*MURC* mutations impart loss-of-function effects on MURC functions and probably are causal variants in human DCM. The causal role of a deletion mutation in HCM is uncertain. (*Circ Cardiovasc Genet*. **2011;4:349-358.**)

**Key Words:** heart failure ■ genetics ■ cardiomyopathy ■ mutation ■ RhoA

Heart failure is a major cause of mortality and morbidity.[1] It is associated with >270 000 deaths in the United States alone.[1] Primary dilated cardiomyopathy (DCM) and hypertrophic cardiomyopathy (HCM) are prototypic genetic forms of systolic and diastolic heart failure, respectively. Molecular genetic studies have led to partial elucidation of causal genes and identification of several hundred mutations in families and cases with cardiomyopathies.[2–5] Accordingly, mutations in genes coding for sarcomere and cytoskeletal proteins have emerged as important causes of primary cardiomyopathies.[2–5] Genetic studies also have highlighted the genetic heterogeneity of cardiomyopathies, particularly for DCM.[3,4] Accordingly, the most common known gene for DCM account for ≈5% of all primary HCM cases.[4] Collectively, the known causal genes are responsible for approximately two-thirds of HCM families and a much smaller fraction of DCM families.[4,5] Thus, the causal genes for a significant number of HCM and DCM cases and families remain to be identified.

**Clinical Perspective on p 358**

The conventional approach for identification of the causal genes for single-gene disorders is genetic linkage analysis. However, the approach does not provide sufficient resolution

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*Drs Rodriguez, Ueyama, and Ogata are co-first authors.

**The online-only Data Supplement is available at http://circgenetics.ahajournals.org/cgi/content/full/CIRCGENETICS.111.959866/DC1.**

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to identify the causal mutations in small families or sporadic cases. Direct DNA sequencing of the candidate genes, particularly in view of the recent advances in DNA sequencing technologies, is an alternative approach that is being used increasingly to identify the causative mutations/genes. Among the candidate genes for cardiomyopathies are genes encoding the Z-disc proteins.[6,7] We recently identified and characterized Muscle-Restricted Coiled-Coil (*MURC*), which encodes a Z-line component protein.[8,9] We showed that MURC activates the RhoA/ROCK pathway and expression of atrial natriuretic peptide (ANP) and regulates myofiber organization.[8] MURC is also a member of the cavin complex, associated with sarcolemmal caveaolae of muscle cells.[10] Subcellular distribution of MURC is altered in myopathic fibers.[10] Collectively, the experimental data implicate MURC as a biologically plausible gene for human cardiomyopathies.[10] To delineate the causal role of *MURC* in cardiomyopathies, we sequenced the coding regions and the splice junctions of *MURC* in 690 cases with cardiomyopathies and 509 control subjects, and, whenever available, we extended the genetic analysis to family members. We complemented the genetic studies with in vitro functional studies in cardiac myocytes. The findings, collectively, implicate *MURC* as a novel gene for DCM.

## Methods

### Study Population

The study protocol was approved by the institutional review board and was in accord with the Human Subjects Committee guidelines. The participating individuals signed informed consent. The main study population comprised 383 cases with DCM, 307 cases with HCM, and 277 normal individuals.[7] We obtained phenotypic data, including 12-lead ECGs and echocardiograms in all participants. Cardiomyopathies were diagnosed according to the conventional criteria.[11] Given the relatively small number of the African Americans in the main control group, we included a second group of 232 African Americans who had normal ECGs and echocardiograms.[12] On identification of a putative mutation, defined as insertion/deletion or frame-shift, nonsynonymous, or splice sequence variants, we recruited and phenotyped additional family members whenever available.

### DNA Sequencing

We sequenced all exons and exon-intron boundaries of *MURC* in 1199 participants by the Sanger method in sense and antisense directions (primer sequence is provided in online-only Data Supplement Table I), using the Big Dye Terminator Reactions in an Applied Biosystems 3730xl Genetic Analyzer (Applied Biosystems, Inc, Foster City, CA). We analyzed the output using Variant Reporter software (Applied Biosystems, Inc). In addition, 2 investigators analyzed every sequence printout to detect the variants. We compared the sequence with the published GenBank sequence for *MURC* (GRCh37 reference genome assembly, chromosome 9, region 103340336.103350180). To reduce the possibility of sequencing errors, we repeated the sequencing reactions in all samples that contained a sequence variant. Only variants that were detected in sense and antisense directions and confirmed in independent sequencing reactions were considered as real variants.

### Mutation Calling

To ascertain a causal role, we analyzed segregation of the variants with the phenotype in the families whenever available and analyzed evolutionary conservation of the involved amino acid using PRRN (http://align.genome.jp/prrn/), charge change, and hydropathy index. We used PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/index.shtml) to predict functional effects of the variants.[13] Nonsynonymous variants that were present only in the cases with cardiomyopathies but absent in the normal control subjects were considered likely disease-causing mutations and selected for in vitro functional studies.

### Sequencing of Known Common Causal Genes for Cardiomyopathies

We sequenced the coding regions and exon-intron boundaries of *LMNA*, encoding Lamin A/C, a known gene for DCM in 103 proband with a compound phenotype of DCM and conduction defect and/or atrial fibrillation.[4] Likewise, we had previously sequenced all exons and exon-intron boundaries of *MYH7*, *MYBPC3*, *ACTC1*, *TNNT2*, *TNNI3*, *TPM1*, and *MYOZ2* in 81 probands with familial HCM. Finally, to detect compound mutations in those who carried a mutation in *MURC*, we sequenced *MYH7*, *MYBPC3*, *TNNT2*, *TNNI3*, *TPM1*, *ACTC1*, and *LMNA*, known as relatively common genes for HCM and DCM in all *MURC* mutation carriers.[5,14,15]

### Genotyping for Short Tandem Repeat Markers

To determine whether the recurring mutations occurred de novo or shared a common ancestral allele, we genotyped the individuals for 3 short tandem repeat (STR) markers (D9S180, D9S910, and D9S176) that spanned an approximately 2.5-Mbp genomic region on 9q31.1 locus. Genotyping was performed by polymerase chain reaction (PCR), using fluorescent-labeled primers and capillary electrophoresis on an ABI 3730xl Genetic Analyzer and analyzed using the GeneMapper v4.1 (Applied Biosystems).

### Plasmid Constructs

We cloned human FLAG-tagged MURC cDNA into a pcDNA3.1 vector (pcDNA3.1-hMURC). We introduced the p.N128K, p.R140W, p.L153P, p.S307T, p.P324L, and p.S364L mutations by site directed mutagenesis.[8] Sequences of mutant-specific oligonucleotide primers are shown in online-only Data Supplement Table I.

### Isolation and Culture of Neonatal Rat Cardiac Myocytes

We isolated and prepared neonatal rat cardiac myocytes (NRCM) from 1-day-old Sprague-Dawley rats as described.[8] Briefly, we digested ventricular tissues enzymatically and separated cardiac myocytes over a Percoll gradient. We changed the culture medium to serum-free medium after 24 hours and maintained the cells under serum-free conditions before experiments.

### Replication-Deficient Recombinant Adenoviruses and Gene Transfer

We generated recombinant adenoviruses expressing FLAG-tagged human wild-type (Ad-hMURC-WT), each of the mutant MURC protein or $\beta$-galactosidase (Ad-LacZ), as described previously.[8] The Ad-LacZ and Ad-hMURC-WT served as controls. We infected the NRCM with the recombinant adenoviruses at a multiplicity of infection of 10. After incubation at 37°C for 1 hour, the viral suspension was removed, and cells were cultured with serum-depleted culture media for 48 hours.

### Immunoblotting

To detect expression of the WT or mutant MURC, we electrophoresed the cell lysates in 10% SDS-polyacrylamide gels and transferred the proteins to polyvinylidene difluoride membranes (Millipore, Billerica, MA). We incubated the membranes with primary antibodies against FLAG (SIGMA, St Louis, MO) and GAPDH to detect FLAG-tagged MURC and GAPDH proteins, respectively. The secondary antibody was horseradish peroxidase–conjugated anti-mouse IgG (GE Healthcare, Waukesha, WI).

### RhoA Activation Assay

We determined RhoA activity in the protein extracts from NRCM transduced with the recombinant adenoviruses using an absorbance-

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**Table 1. Baseline Characteristics**

| n=967 (1199*) | Control* (n=277) (509*) | DCM (n=383) | HCM (n=307) | *P* NA |
|---|---|---|---|---|
| Demographics | | | | |
| Male/female | 129 (46)/148 (54) | 214 (56)/169 (44) | 167 (54)/140 (46) | 0.046 |
| Caucasian/African American | 193 (70)/84 (30) | 204 (53)/179 (47) | 258 (84)/49 (16) | <0.001 |
| Age, y | 44.24±17.50 | 51.43±13.92 | 52.40±16.23 | <0.0001 |
| BMI, kg/m$^2$ | 28.04±7.22 | 30.15±7.48 | 30.19±7.21 | 0.0003 |
| BSA, m$^2$ | 1.96±0.32 | 2.07±0.33 | 2.05±0.29 | <0.0001 |
| DM, % | 26 (10) | 90 (23) | 35 (12) | <0.001 |
| Smoker, % | 26 (10) | 57 (15) | 49 (17) | <0.001 |
| Heart rate, bpm | 71.3±12.4 | 80.1±16.2 | 70.4±12.0 | <0.0001 |
| Systolic BP, mm Hg | 123.5±15.7 | 116.8±16.8 | 128.5±17.5 | <0.0001 |
| Diastolic BP, mm Hg | 73.3±9.9 | 73.4±12.4 | 74.3±13.4 | 0.5315 |
| Echocardiographic data | | | | |
| ST, cm | 0.96±0.18 | 1.01±0.22 | 1.89±0.48 | <0.0001 |
| PWT, cm | 0.97±0.2 | 1.08±0.23 | 1.34±0.32 | <0.0001 |
| LVEDD, cm | 4.51±0.53 | 6.28±0.97 | 4.15±0.67 | <0.0001 |
| LVESD, cm | 2.90±0.53 | 5.39±1.12 | 2.45±0.70 | <0.0001 |
| LVM, g | 184.26±65.97 | 350.83±142.24 | 355.89±128.24 | <0.0001 |
| LVMI, g/m$^2$ | 94.02±25.12 | 169.50±63.22 | 173.30±63.22 | <0.0001 |
| LA size, cm | 3.46±0.76 | 4,43±1.00 | 4.25±0.79 | <0.0001 |
| LVEF | 61.47±5.37 | 24.42±8.04 | 67.03±5.15 | <0.0001 |

HCM indicates hypertrophic cardiomyopathy; DCM, dilated cardiomyopathy; NA, not applicable; BMI, body mass index; BSA, body surface area; DM, diabetes mellitus; BP, blood pressure; ST, septal thickness; PWT, posterior wall thickness; LVEDD, left ventricular end-diastolic diameter; LVESD, left ventricular systolic diameter; LV mass, left ventricular mass; LVMI, left ventricular mass indexed to body surface area; LVEF, left ventricular ejection fraction; and LA, left atrium.

*Denotes the total number of the control group with data that are included in the table. We added a second control group of African American individuals (n=232) who had normal ECGs and echocardiograms but did not have the detailed data.

based G-LISA RhoA activation assay biochemistry kit (Cytoskeleton, Denver, CO), as described.[8] We isolated total protein 2 days after transduction of NRCM with the recombinant adenoviruses per instruction of the manufacturer. We equalized the total protein concentrations among the samples at 1 mg/mL for the assay and measured the signals at an absorbance of 490 nm, using a microplate spectrometer.

## RNA Extraction and Quantitative Reverse Transcriptase–PCR

We isolated total cellular RNA from NRCM using an RNeasy Mini Kit (QIAGEN, Inc). We performed quantitative reverse transcriptase–PCR (qPCR) to quantify mRNA expression levels of rat ANP (Nppa), B-type natriuretic peptide (BNP or Nppb), and rat skeletal α-actin (SkA or Acta1) in NRCM transduced with WT or mutant MURC constructs, as described.[8] Sequences of oligonucleotide primers are shown in online-only Data Supplement Table I.

## Cardiac Myocyte Size

Cardiac myocytes were infected with the recombinant adenoviruses and cultured under serum-free conditions for 48 hours. Cells were then fixed with 4% paraformaldehyde and stained with fluorescein isothiocyanate–conjugated phalloidin (SIGMA) for the detection of actin filaments. DNA was stained with 4′,6-diamidino-2-phenylindole. We measured myocyte surface area using Image software (National Institutes of Health, Bethesda, MD) in at least 100 cells per group.

## Statistics

Statistical analyses were similar to those published recently.[7,16] We expressed the continuous variables that followed normality distribution as mean±SD. We compared differences among the continuous variables that satisfied the normality distribution by 1-way ANOVA and applied the Bonferroni correction to multiple-comparison tests. Variables that were not normally distributed were compared by Kruskal-Wallis test. We analyzed differences in the categorical variables by $\chi^2$ test.

All in vitro experiments were performed at least 3 times, and the mean values of the 3 measurements were used. Data are expressed as mean±standard error and analyzed by 1-way ANOVA with Scheffe post hoc analysis. A value of $P<0.05$ was considered significant. All statistical analyses were performed using STATA v 10.1.

# Results

## Characteristics of the Study Population

Phenotypic characteristics of the study population are shown in Table 1 and are partly published.[7,12]

## *MURC* Sequence Variants

We found 6 DCM-specific heterozygous missense variants (p.N128K, p.R140W, p.L153P, p.S307T, p.P324L, and p.S364L) in 8 probands (Table 2). These variants were absent in 509 normal individuals. The p.N128K variant cosegregated with DCM in 2 affected family members and was absent in 5 unaffected family members (Figure 1). The p.S307T was present in 4 adult family members, of whom 3 had severe DCM (Figure 1). One young adult (31 years old) and 1 child also had the p.S307T variant, whereas 3 phenotypically

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**Table 2. Nonsynonymous Variants Detected in Probands With Dilated Cardiomyopathy**

| | | Control Subjects (n=509) | | DCM (n=383) | | HCM (n=307) | |
|---|---|---|---|---|---|---|---|
| Amino Acid | Nucleotide | A 316 | C 193 | A 179 | C 204 | A 49 | C 258 |
| p.N128K | g.474C>G | 0 | 0 | 1 | 0 | 0 | 0 |
| p.R140W | g.7721C>T | 0 | 0 | 0 | 1 | 0 | 0 |
| p.L153P | g.7761T>C | 0 | 0 | 1 | 0 | 0 | 0 |
| p.S307T | g.8223G>C | 0 | 0 | 0 | 1 | 0 | 0 |
| p.P324L | g.8274C>T | 0 | 0 | 3 | 0 | 0 | 0 |
| p.S364L | g.8394C>T | 0 | 0 | 1 | 0 | 0 | 0 |
| Total No. of nonsynonymous variants per group and ethnic background | | 0 | 0 | 6 | 2 | 0 | 0 |
| Total No. of nonsynonymous variants per group | | 0 (0 %) | | 8 (2.1%) | | 0 (0%) | |

DCM indicates dilated cardiomyopathy; HCM, hypertrophic cardiomyopathy; A, African American; and C, Caucasian.

normal adults had the WT genotype. Segregation analysis that includes healthy individuals in cardiomyopathies is subject to age-dependent and incomplete penetrance. The absence of a clinical phenotype in young mutation carriers in this family may reflect partial and age-dependent penetrance of the variant. Collectively, the p.N128K and p.S307T variants were present in 5 affected family members and absent in 8 of 9 clinically normal members ($\chi^2$=8.5, $P$=0.003). The small size of these 2 families and the incomplete penetrance of the p.S307T variant impeded the power to calculate the logarithm of odds score, as a measure of linkage to strengthen the causal role of these variants. The p.P324L was a recurring variant, as it was detected in 3 probands. To determine whether the probands shared a common ancestral allele at the *MURC* locus, we genotyped the 3 families for 3 locus-specific STR markers. The 3 probands did not share the genotypes for the *MURC* locus, indicating independent origins of the mutation in these individuals (online-only Data Supplement Figure I).

We also detected a deletion variant (p.L232-R238del), which excluded 7–amino acid–long motif (ERLRQSG, Swiss-Prot ID: Q5BKX8.2) in 3 probands with HCM, 1 African American, and 2 white subjects. The deletion was absent in >500 control individuals. Two affected members of a HCM family had the deletion variant. However, it was absent in an affected member of another family with the deletion variant (online-only Data Supplement Figure II). The 2 affected family members in the latter family had a p.L907V mutation in MHY7, a known gene for cardiomyopathies.[5] The p.L907V mutation was absent in >500 normal individuals, and affects a highly conserved domain in MYH7 protein. It is predicted to be probably damaging by PolyPhen-2 analysis (score, 0.954; sensitivity, 0.74; specificity, 0.93).

In contrast to 7 phenotype-specific variants, we detected only one nonsynonymous variant (p.R272H) that was present only in the control group (Table 2). The p.R272H was predicted to be a benign variant by PolyPhen-2 analysis. To test whether *MURC* was a disease-causing gene, we performed a gene-centric analysis by coalescing the nonsynonymous variants in each group and comparing the prevalence of *MURC* variants in cases with DCM and control subjects. In support of a causal role of *MURC* variants in DCM, nonsynonymous variants were overrepresented by 25-fold in the cases (Pearson $\chi^2$=7.84, $P$=0.005).

As would be expected, we identified several polymorphisms that were common to the cases and the control subjects, including 6 nonsynonymous variants, 14 synonymous, and 8 noncoding variants (online-only Data Supplement Tables II and III). The frequencies of the nonsynonymous or synonymous polymorphisms were not significantly different between the cases and control subjects. Likewise, the age distributions of the control individuals and the DCM cases with the nonsynonymous variants were similar. Overall, the minor allele frequencies of the nonsynonymous polymorphisms were <1% in the study population. The most common variant was the p.S78L, which had a minor allele frequency of 0.009 in the entire population and 0.033 in the African American subpopulation. The p.N81K was detected in 3 African Americans, 1 with HCM, 1 with DCM, and 1 normal individual. In each case, it cosegregated with the p.S78L variant.

## Mutations in Known Genes for Cardiomyopathies

We sequenced all coding regions and exon-intron boundaries of *MYH7*, *MYBPC3*, *TNNT2*, *TNNI3*, *TPM1*, *ACTC1*, and *LMNA*, known relatively common genes for cardiomyopathies,[4,5] in all *MURC* mutation carriers. An African American patient with DCM had a compound mutation: the p.P324L variant in *MURC* and a novel nonsynonymous variant p.M245T in the *TPM1* gene. Likewise, 1 family member who had the p.L232-R238del variant in *MURC* also had a p.L907V mutation in the *MYH7* gene (probably compound mutation).

We identified 3 nonsynonymous variants in *LMNA* on screening of 103 probands with DCM associated with conduction defect and/or atrial fibrillation.[4] None had a *MURC*

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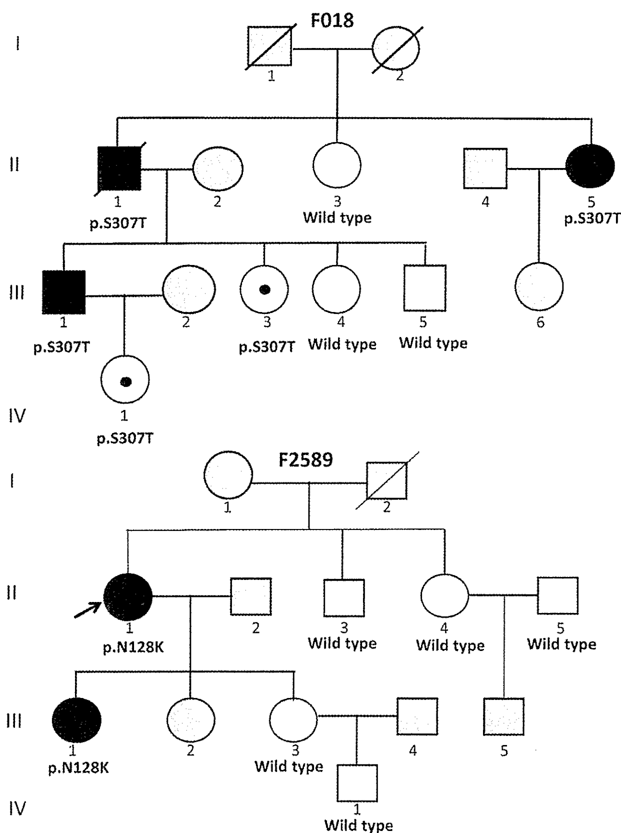


**Figure 1.** Pedigree of families with Muscle-Restricted Coiled-Coil *(MURC)* mutations: Squares and circles represent male and female members. Full and open circles and squares indicate affected and unaffected (normal) individuals, respectively. Gray squares and circles indicate individuals that were not studied. The mutation and wild-type codons are listed under those members that were studied. Individual III-3, who is 31 years old, is a mutation carrier but is phenotypically normal (nonpenetrance). Individual IV-1 in Pedigree F018 is 5 years old and noncontributory to cosegregation analysis.

mutation. Likewise, we identified 32 different mutations in *MYH7* (14 mutations), *MYBPC3* (11 mutations), *TNNT2* (2 mutations), *TNNI3* (1 mutation), *TPM1* (2 mutations), and *MYOZ2* (2 mutations) in screening of 81 probands with HCM. None had a mutation in *MURC* gene.

## Phenotypic Expression of *MURC* Mutation Carriers

The phenotype in the mutation carriers was notable for heart failure in the middle age (mean age, 45.0±6.5 years), leading to severely depressed left ventricular systolic function, which led to cardiac transplantation in 4 affected members. Cardiac tissues were not available to analyze histological correlates of MURC variants. Several affected members exhibited supraventricular arrhythmias and progressive conduction defects, requiring implantation of a permanent pacemaker and internal cardioverter/defibrillator. Detailed phenotypic data are shown in Table 3.

## Predicted Pathogenicity of the Variants

Conservation of the involved codon and domain across paralogous proteins from zebrafish to humans, a potential indicator of the biological significance of the codon, is shown in Figure 2. Likewise, the predicted effects of the nonsynonymous variants on MURC protein are shown in Table 4. Codon N128K affected a totally conserved amino acid from zebrafish to human and had a very high PolyPhen-2 prediction score (0.980; sensitivity, 0.69; specificity, 0.94), placing it in the probably damaging category (highest category).[13] The L153P also affected a highly conserved amino acid from rat *(Rattus norvegicus)* to human and was categorized as probably damaging (score, 0.975; sensitivity, 0.70; specificity, 0.94). The p.R140W and p.S364L involved conserved amino acids and were predicted to be possibly damaging (score for p.R140W, 0.840; sensitivity, 0.80; specificity, 0.90 and score for p.S364L, 0.438; sensitivity, 0.438; specificity, 0.86). In contrast, p.S307T and p.P324L involved fewer or nonconserved amino acids and were predicted to be benign.

## Functional Characterization of *MURC* Mutations

We have previously shown that MURC activates the RhoA/ROCK pathway and its downstream target, ANP.[8] Therefore, we transduced NRCM with recombinant viruses expressing either a WT or a mutant MURC protein and measured RhoA activity. Expression of the WT MURC increased RhoA activity by ≈80%, a finding that is in accord with the previous data.[8] In contrast, RhoA activity was significantly less in myocytes expressing the mutant MURC proteins as compared with the WT MURC group (Figure 3).

Likewise, expression of the WT MURC significantly increased mRNA levels of ANP (Nppa) and SkA (Acat1) by >5-fold and that of BNP (Nppb) by ≈2.6-fold (Figure 3). In contrast, mRNA levels of the Nppa, Nppb, and Acta1 in NRCM transduced with the mutant MURC recombinant viruses were attenuated significantly, as compared with WT MURC. The findings indicate that the mutations impart a loss-of-function effect on the activation of RhoA and expression of the hypertrophic markers by MURC.

To further characterize the phenotypic consequence of the MURC mutations in vitro and complement the data on RhoA activity and expression of the molecular markers of hypertrophy, we measured the effects of expression of WT and mutant MURC proteins on cardiac myocyte size. Expression of the WT MURC increased cardiac myocyte surface area by ≈3.5-fold. However, the mutant MURC had attenuated hypertrophic effects, as myocyte surface area was significantly less in the mutant groups as compared with the WT MURC group (Figure 4).

# Discussion

We have identified 6 DCM-specific heterozygous missense variants, including 4 that were predicted to be probably or possibly pathogenic. The mutations were associated with progressive heart failure, conduction defects, and cardiac arrhythmias. In vitro functional studies indicate loss-of-function effects of the mutant MURC proteins on activation of RhoA, expression of hypertrophic markers, and myocyte hypertrophy. Collectively, human molecular genetic and in vitro functional data implicate *MURC* as a causal gene for human DCM.

Several lines of evidence support the causal role of *MURC* mutations in DCM. All 6 nonsynonymous variants were

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**Table 3. Demographic, Echocardiographic, and ECG Phenotypes in DCM Patients With *MURC* Mutations**

| | AA Change | Ethnicity | Age at Diagnosis, y | Sex | BMI, kg/m$^2$ | Clinical Phenotype | ST, cm | PWT, cm | LVEDD, cm | LVEF, % | ECG |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1* | N128K | A | 54 | F | 31.0 | Heart failure, recurrent nonsustained V. Tach, ICD implantation, heart transplant | 0.9 | 0.8 | 6.8 | <10 | SR, 1st-degree AV block, LAE, LVH with ST/T changes |
| 2* | | A | 41 | F | 26.8 | Heart failure, hypotension, nonsustained VT, SVT | 0.8 | 0.9 | 6.3 | 28 | SR, LVH with ST/T changes |
| 3 | R140W | C | 41 | M | 25.9 | SCA, ICD implantation, recurrent V. Fib, heart transplantation | 1.2 | 1 | 6.6 | 26.5 | A. Fib, atrial paced rhythm, LAE, LBBB, ST/T changes |
| 4 | L153P | A | 48 | M | 23.0 | Heart failure, NYHA functional class, cardiac thrombus | 0.5 | 0.5 | 9.1 | <10 | SR, 1st-degree AV block, LAE, IVCD, V. Tach |
| 5* | S307T | C | 48 | M | 34 | Heart failure, syncope, PPI, heart transplantation | 0.8 | 0.9 | 6.9 | <10 | Ventricular paced rhythm |
| 6* | | C | 36 | M | 47.3 | Heart failure, palpitations, class III NYHA functional class on therapy | 1.2 | 1.3 | 6.7 | 32 | SR, IVCD |
| 7* | | C | 33 | F | 24.8 | Heart failure, syncope and V. Tach, ICD implantation, heart transplantation | 1.3 | 1.3 | 7.4 | <20 | SR, LAE, LBBB |
| 8 | P324L (*TPM1:* M245T) | A | 52 | F | 25.9 | Heart failure, hypotension and low cardiac output requiring inotropic support, and LV assist device. Embolic stroke, PPI, and ICD | 1.3 (0.9) | 1.4 (1) | 5 (5.5) | 22 | Dual-chamber paced rhythm, intermittent atypical AF, LVH with ST/T changes |
| 9 | P324L | A | 49 | F | 47.2 | Heart failure, class II NYHA functional class on medical therapy | 0.8 | 1 | 5.4 | 32 | SR, LVH voltage |
| 10 | P324L | A | 46 | M | 27.4 | Heart failure and palpitations, class IV NYHA functional class, evaluation for heart transplantation | 0.8 | 1 | 5.4 | 32 | SB, LVH with ST/T changes |
| 11 | S364L | A | 47 | M | 49.7 | Heart failure, palpitations, biventricular pacing (cardiac resynchronization therapy), ventricular support system, ICD | 0.8 | 1 | 6.4 | 22 | SR, LVH with ST/T, V. Tach |

DCM indicates dilated cardiomyopathy; AA, amino acid; BMI, body mass index; ST, septal thickness; PWT, posterior wall thickness; LVEDD, left ventricular end-diastolic diameter; LVEF, left ventricular ejection fraction; SCA, sudden cardiac arrest; SR, sinus rhythm; LAE, left atrial enlargement; LVH, left ventricular hypertrophy; IVCD, intraventricular conduction delay; A. Fib, atrial fibrillation; LBBB, left bundle-branch block; V. Tach, ventricular tachycardia; V. Fib, ventricular fibrillation; SB, sinus bradycardia; AF, atrial flutter; NYHA, New York Heart Association; ICD, implantable cardioverter-defibrillator; and SVT, supraventricular tachycardia.

*Individuals 1 and 2 and individuals 5, 6, and 7 are family members. Echocardiographic data are findings on presentation. Data in parenthesis indicate findings on a follow-up echocardiogram. Individual 8 had increased left ventricular wall thickness with decreased LVEF at the time of first evaluation. She had subsequent development of a DCM phenotype.

absent in >500 control individuals who had no clinical, ECG, or echocardiographic evidence of cardiomyopathies. The variants were also absent in the dbSNP (build 132). A gene-centric analysis showed that the missense variants were overrepresented in patients with DCM by 25-fold. Likewise, 2 mutations cosegregated with the phenotype, albeit in small families. Although the de novo recurring mutations in cases only imply a causal role in DCM, the p.P324L recurring

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p.N128K                              ↓
     Homo sapiens           EEIMKENKFRVVI 134
     Pan troglodytes        EEIMKKNKFRVVI 134
     Macaca mulatta         EEIMKENKFRVVI 132
     Canis familiaris       EEIMKKNKFRVVI 134
     Equus caballus         EELLKKNKFRVVI 134
     Bos taurus             EEIMKKNKFRVVI 74
     Mus musculus           EEIMKKNKFRVVI 134
     Rattus norvegicus      EEIMKKNKFRVVI 134
     Gallus gallus          EEMLRKNKFRVVI 135
     Danio rerio            VELLNKNKFRVVI 120

p.L153P                              ↓
     Homo sapiens           VVKDRNLTENQEE 159
     Pan troglodytes        VVKDRNLTENQEE 159
     Macaca mulatta         VVKDRNLTENQEE 157
     Canis familiaris       IVKDRSLTENQEV 159
     Equus caballus         IVKDRTLTEDQEV 159
     Bos taurus             VVKDRSLTESPEE 99
     Mus musculus           VVKDRSLPENQEE 159
     Rattus norvegicus      VVKDRSLPENEEE 159
     Gallus gallus          VIKEHTAGETRED 160
     Danio rerio            PKASPKGAMGGDA 145

p.P324L                              ↓
     Homo sapiens           EHEAARPVYPPHE 330
     Pan troglodytes        EHEAARSVYPPHE 330
     Macaca mulatta         EHEAARPVYPAHE 328
     Canis familiaris       DHEEARAAYPPRE 329
     Equus caballus         DREEARAGGPPRE 329
     Bos taurus             DPEEASATHPPQE 269
     Mus musculus           EKEVTKGGYSPQE 328
     Rattus norvegicus      EKEVTKVGYIPQE 328
     Gallus gallus          KKDKNSTPRGAAQ 320
     Danio rerio            SPEVAYTEPTQKQ 296

p.R140W                              ↓
     Homo sapiens           IFQEKFRCPTSLS 146
     Pan troglodytes        IFQEKFRCPTSLS 146
     Macaca mulatta         IFQEKFRCPTSLS 144
     Canis familiaris       IFQEEIRCPTSLS 146
     Equus caballus         IFQEKLRCPTSLS 146
     Bos taurus             IFQEEVQCPTSLS 86
     Mus musculus           IFQEDIPCPASLS 146
     Rattus norvegicus      IFQEDVPCPASLS 146
     Gallus gallus          IYQEETECPSSLS 147
     Danio rerio            IYQGDNEVPAVAG 132

p.S307T                              ↓
     Homo sapiens           ESLGPISELYS-- 311
     Pan troglodytes        ESLGPISELYS-- 311
     Macaca mulatta         ESLGPISELYS-- 309
     Canis familiaris       ESLGPISEIYT-- 310
     Equus caballus         EALGPISELYS-- 310
     Bos taurus             EALGPISELYP-- 250
     Mus musculus           LALGPIHEFHS-- 309
     Rattus norvegicus      LALGPIHEFHS-- 309
     Gallus gallus          TATEPFTEEISYT 301
     Danio rerio            EGTAPVPPPKG-- 280

p.S364L                                  ↓
     Homo sapiens           -DESLLLDLKHSS 364
     Pan troglodytes        -DESLLLDLKHSS 364
     Macaca mulatta         -DESLLLDLKHSS 362
     Canis familiaris       -DESLLLDLKQ-- 361
     Equus caballus         -DESLLLDLKQSS 363
     Bos taurus             -DESLLLDLKQ-- 301
     Mus musculus           -DESLLLELKQSS 362
     Rattus norvegicus      -DESLLLELKQSS 362
     Gallus gallus          GDDVPLLDLKQSL 357
     Danio rerio            -SEVPMYDMKQLS 318
```

**Figure 2.** Multiple sequence alignment (MSA) of Muscle-Restricted Coiled-Coil (MURC) variants across species: MSA is performed using software package PRRN ((http://align.genome.jp/prrn/) to infer evolutionary conservation of the amino acids affected by the MURC nonsynonymous variants identified in probands with dilated cardiomyopathy. All available sequences from different species are included.

variant affected a nonconserved amino acid and was predicted to be benign by PolyPhen-2 analysis. Functional data, assessed at multiple levels, showed concordant results and indicated loss-of-function effects of the *MURC* mutations. Furthermore, 4 variants either involved largely conserved paralogous codons and/or were predicted to affect structure and biological function of the encoded protein. The findings are also in accord with the in vitro and in vivo biological functions of MURC protein and its potential involvement in myopathic conditions.[8–10]

MURC is a biologically plausible candidate gene for cardiomyopathies. MURC is a Z-line component protein that is predominantly expressed in the heart.[8–10] Mutations in genes encoding sarcomere and cytoskeletal proteins are major causes of cardiomyopathies.[4,5] MURC is involved in muscle protein homeostasis through regulating RhoA/ROCK and association with a multiprotein complex at the caveolae.[8–10] MURC interacts with extracellular signal-related kinases, RhoA/ROCK, and serum response factor pathways, which are also implicated in heart failure.[8,9,17] RhoA appears to play a delicate role in regulating cardiac function because its overexpression or its inhibition results in conduction defects and cardiac dysfunction.[18,19] Likewise, cardiac-restricted overexpression of MURC in mice leads to heart failure, conduction defect, and atrial arrhythmias.[8] Moreover, MURC is a component of a multiprotein complex in the caveolae that regulates cardiac function.[10] Its subcellular distribution is perturbed in human muscle disease associated with Caveolin-3 dysfunction.[10] Notably mutations in *CAV3*, encoding caveolin 3, are also known to cause cardiac and skeletal myopathy.[20,21]

The causal role of the *MURC* deletion mutation in HCM is less clear. Evidence in support of a potential causal role includes recurrence in 2 different ethnic populations and hence, occurring independently, its absence in >500 normal individuals and the increasing recognition of prevalence of

**Table 4. Evolutionary Conservation and Biophysical Effects of the Putative Causal Variants**

| Amino Acid Change | Evolutionary Conservation | Charge Change | Change in Hydropathy | PolyPhen-2 Prediction |
|---|---|---|---|---|
| p.N128K | Conserved in all known proteins | Neutral to positive | − 3.5 to − 3.9 | Probably damaging |
| p.R140W | Partially conserved | Positive to Neutral | − 4.5 to − 0.9 | Possibly damaging |
| p.L153P | Highly conserved | Neutral | 3.8 to − 1.6 | Probably damaging |
| p.S307T | Partially conserved | Neutral | − 0.8 to − 0.7 | Benign |
| p.P324L | Nonconserved | Neutral | −1.6 to 3.8 | Benign |
| p.S364L | Highly conserved | Neutral | −0.8 to 3.8 | Possibly damaging |

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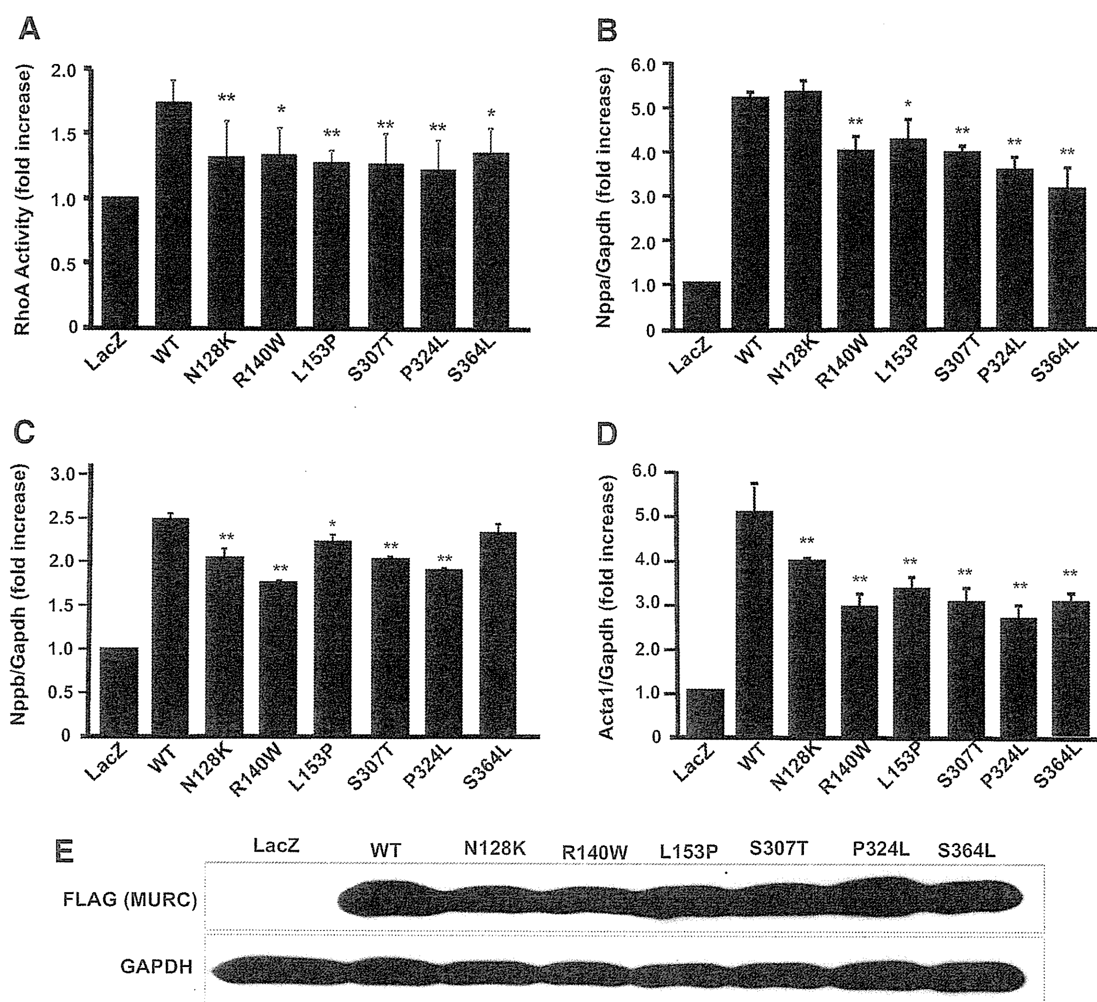


**Figure 3.** Effects of Muscle-Restricted Coiled-Coil (MURC) mutations on RhoA activation and expression of molecular markers of cardiac hypertrophy. **A**, Bar graph representing RhoA activity in neonatal rat cardiac myocytes (NRCM) infected with recombinant adenoviruses at a multiplicity of infection (MOI) of 10. The groups included Ad-LacZ (expressing β-galactosidase, as a control), wild-type human MURC (Ad-hMURC-WT), and each of the specific mutant MURCs (Ad-N128K, Ad-R140W, Ad-L153P, Ad-S307T, Ad-P324L, or Ad-S364L) that were identified in patients with dilated cardiomyopathy (DCM) (n=3 independent experiments per group. **B**, **C**, and **D**, MicroRNA expression levels of atrial natriuretic peptide (ANP; gene, *Nppa*), B-type or brain natriuretic peptide (BNP; gene name, *Nppb*), and skeletal α-actin (SkA; gene name, *Acta1*) in cardiac myocytes transduced at an MOI of 10 with control recombinant adenoviruses or viruses expressing WT or each of the 6 mutant MURC proteins. Cells were harvested 48 hours after the infection for extraction of RNA and quantitative reverse transcriptase–polymerase chain reaction. **E**, Immunoblot of myocytes proteins extracted 48 hours after transduction with the recombinant viruses and probed with an anti-FLAG antibody to detect expression of the WT or mutant MURC proteins (**upper blot**). Immunoblotting was performed to assess equal expression level of the WT and mutant MURC proteins among the groups. The **lower blot** shows expression of GAPDH, which was used as a control for loading conditions. *$P<0.05$ and **$P<0.01$ as compared with WT MURC.

compound mutations in cardiomyopathies.[22–25] However, the deletion variant was not present in an affected family member with the phenotype and hence, conventionally cannot be considered a causal variant in this family. The 2 affected members in this small family also had a missense mutation (p.L907V) in *MYH7*, which is predicted to be probably damaging. It is possible that the deletion variant codependently contributes to phenotypic expression of HCM in this family. These findings highlight the challenge of establishing the causal role of the DNA sequence variants in sporadic cases and small families, as opposed to large families, wherein cosegregation of the phenotype with inheritance of the variants (genetic linkage) could support the causality. While the actual LOD score is determined by the family structure and information content of the locus markers, typically >7 affected family members are necessary to achieve a significant LOD score in a linkage analysis of a familial disease with an autosomal dominant mode of inheritance, assuming 100% penetrance. Unfortunately, neither the number of the affected individuals in our families is adequate nor the penetrance is complete to perform a meaningful linkage analysis. The challenge is expected to have considerable clinical implications, as whole-exome or whole-genome sequencing gains clinical applications. The task is daunting because each diploid human genome contains >10 000 nonsynonymous variants, the majority of which are not associated with any discernible clinically phenotype (discussed in Reference 26).[26] Accordingly, several lines of genetic and functional data must be incorporated in discerning clinical significance of the DNA sequence variants in

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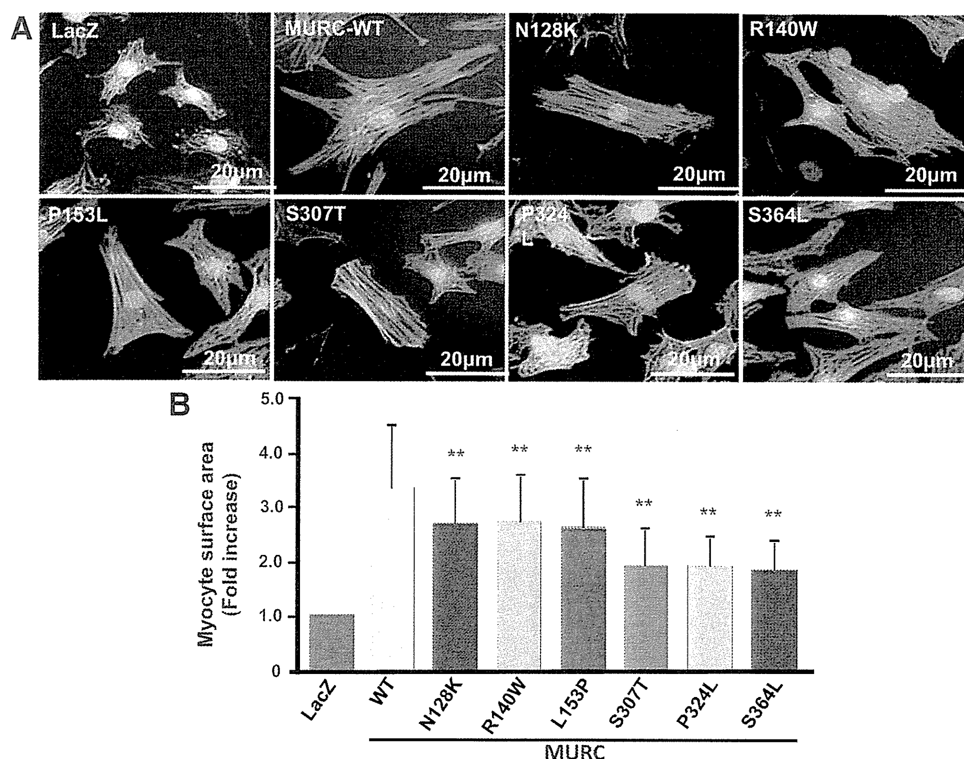


**Figure 4.** Induction of cardiac myocyte hypertrophy. **A**, Representative immunofluorescence images of neonatal rat cardiac myocytes (NRCM) transduced with a control virus or recombinant viruses, at a multiplicity of infection of 10, expressing wild-type (WT) mutant Muscle-Restricted Coiled-Coil (MURC) (Ad-LacZ, Ad-hMURC-WT, Ad-N128K, Ad-R140W, Ad-L153P, Ad-S307T, Ad-P324L, or Ad-S364L). Yellow bar indicates 20 μm scale. **B**, Bar graph depicting the mean and standard error of myocyte surface area in each experimental group. NRCM were infected with the recombinant viruses and stained with fluorescein isothiocyanate–conjugated phalloidin 48 hours after the transduction. Myocyte surface area was measured in at least 100 cells per group. *$P<0.05$ and **$P<0.01$ as compared with WT MURC.

sporadic cases or small families. Although our data strongly support the causal role of the MURC variants in DCM, one must consider the possibility that the *MURC* variants may not be true disease-causing variants but are susceptibility alleles that require a second mutation or injury to cause the clinical phenotype. There is also a possibility that these variants are functional variants that do not play significant roles in susceptibility to cardiomyopathies. We submit that the strengths of the genetic and functional data favor the causal role of *MURC* variants in the pathogenesis of human cardiomyopathies.

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## Disclosures

None.

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## CLINICAL PERSPECTIVE

Heart failure is a major cause of mortality and morbidity. Genetic mutations are important causes of susceptibility to heart failure. Familial dilated cardiomyopathy (DCM) is a classic form of systolic heart failure caused by genetic mutations. The known causal genes for DCM typically code for sarcomere and cytoskeletal proteins. Prompted by the experimental data, which implicate Muscle-Restricted Coiled-Coil (MURC) in regulating cardiac function, we investigated the causal role of the *MURC* gene in DCM. We sequenced the protein coding regions of *MURC* in 383 probands with DCM, 307 with hypertrophic cardiomyopathy, and 509 healthy control subjects. We found 6 missense mutations that were present only in DCM patients and were absent in control subjects. Two of these mutations segregated with inheritance of DCM in small families. One mutation recurred in 3 independent probands, including 1 proband who also had another mutation in the $\alpha$-tropomyosin gene. We also found 1 deletion mutation in 3 unrelated probands with hypertrophic cardiomyopathy that might be a causative or susceptibility variant. The clinical features associated with *MURC* mutations included progressive heart failure that often led to heart transplantation, conduction defects, and atrial arrhythmias. Studies in cultured cardiac myocytes showed that the mutations reduced RhoA activity, an important signal transducer in the heart and impaired myocyte hypertrophic response. We conclude that *MURC* probably is a causal gene for DCM. We suggest elucidation of the molecular genetic and pathogenesis of heart failure is an essential step in the ultimate treatment of this potentially deadly disease.

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**SUPPLEMENTAL MATERIAL**

**CIRCCVG/2011/959866**

# Molecular Genetic and Functional Characterization Implicate Muscle-Restricted Coiled-Coil Gene (*MURC*) As a Causal Gene For Familial Dilated Cardiomyopathy

Gabriela Rodriguez, MD, et al.



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