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Tcap Gene Mutations in Hypertrophic Cardiomyopathy and Dilated Cardiomyopathy

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OBJECTIVES We sought to explore the relationship between a Tcap gene *(TCAP)* abnormality and cardiomyopathy.

BACKGROUND Hypertrophic cardiomyopathy (HCM) and dilated cardiomyopathy (DCM) cause severe heart failure and sudden death. Recent genetic investigations have revealed that mutations of genes encoding Z-disc components, including titin and muscle LIM protein (MLP), are the primary cause of both HCM and DCM. The Z-disc plays a role in establishing the mechanical coupling of sarcomeric contraction and stretching, with the titin/Tcap/MLP complex serving as a mechanical stretch sensor. Tcap interacts with the calsarcin, which tethers the calcineurin to the Z-disc.

METHODS The *TCAP* was analyzed in 346 patients with HCM (236 familial and 110 sporadic cases) and 136 patients with DCM (34 familial and 102 sporadic cases). Two different in vitro qualitative assays—yeast two-hybrid and glutathion S-transferase pull-down competition—were performed in order to investigate functional changes in Tcap's interaction with MLP, titin, and calsarcin-1 caused by the identified mutations and a reported DCM-associated mutation, R87Q.

RESULTS Two *TCAP* mutations, T137I and R153H, were found in patients with HCM, and another *TCAP* mutation, E132Q, was identified in a patient with DCM. It was demonstrated by the qualitative assays that the HCM-associated mutations augment the ability of Tcap to interact with titin and calsarcin-1, whereas the DCM-associated mutations impair the interaction of Tcap with MLP, titin, and calsarcin-1.

CONCLUSIONS These observations suggest that the difference in clinical phenotype (HCM or DCM) may be correlated with the property of altered binding among the Z-disc components. (J Am Coll Cardiol 2004;44:2192–201) © 2004 by the American College of Cardiology Foundation

Cardiomyopathy is one of the major causes of sudden death and/or progressive heart failure, and cases of cardiomyopathy are mainly classified into hypertrophic cardiomyopathy (HCM) and dilated cardiomyopathy (DCM). Hypertrophic cardiomyopathy is characterized by left ventricular (LV) hypertrophy accompanied by myofibrillar disarray and manifests as diastolic dysfunction of the cardiac ventricles, whereas DCM is characterized by a dilated ventricular cavity with systolic dysfunction (1). Recent genetic investigations have revealed that HCM is caused by mutations in the genes for sarcomeric proteins, such as *MYH7* (cardiac beta-myosin heavy chain), *TNNT2* (cardiac troponin T), and *ACTC* (cardiac alpha-actin) (2). Mutations in the sarcomere genes also cause DCM (3,4). In addition, mutations in the genes for Z-disc or costameric proteins, such as *DMD* (dystrophin), *DES* (desmin), and *SGCD* (δ-sarcoglycan), cause DCM (5–7).

The Z-disc plays various roles in sarcomeric organization, force transduction, and force transmission (8). Because the Z-disc is important in establishing the mechanical coupling of the sarcomere, defects in the Z-disc proteins may lead to either HCM or DCM. Titin, also known as connectin, is a component of the sarcomere and contains distinct motifs that sequentially unfold as the muscle is stretched (9). Titin forms a complex with alpha-actinin and alpha-actin at the

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Abbreviations and Acronyms

CS-1 = calsarcin-1
DCM = dilated cardiomyopathy
GST = glutathion S-transferase
HCM = hypertrophic cardiomyopathy
LV = left ventricle/ventricular
MLP = muscle LIM protein
PCR = polymerase chain reaction
RT = reverse transcription
SSCP = single-strand conformation polymorphism
Thio = thioredoxin
WT = wild-type
Y2H = yeast two-hybrid

Z-disc (Fig. 1) (10,11). We previously reported on two *TTN* (titin) mutations at the actinin-interaction domain, which increases and decreases the ability of titin to interact with alpha-actinin: an HCM-associated mutation that increases the ability of titin to interact with alpha-actinin and a DCM-associated mutation that decreases it (12,13). Titin also interacts with another Z-disc component, titin-cap (Tcap/telethonin) (14,15), and we found another DCM-associated *TTN* mutation at the Tcap-interaction domain, which impaired the interaction with Tcap (13). In addition, other DCM-associated *TTN* mutations not located in the Z-disc region have been reported (13,16), although the functional changes caused by these mutations remain unknown.

Tcap is a 19-kd protein required for sarcomerogenesis in striated muscles (14). It is phosphorylated by titin kinase at Ser157 and is suggested to play a key role in sarcomere assembly (17). Tcap interacts with calsarcin and minK at the N-terminal half and the C-terminal regions, respectively (18,19). Tcap also interacts with muscle LIM protein (MLP), and a DCM-associated *MLP* mutation abolishes the interaction (20). In addition, another DCM-associated *MLP* mutation was reported to impair the interaction of Tcap with alpha-actinin-2, whereas a DCM-associated alpha-actinin-2 mutation impaired its interaction with MLP (21). On the other hand, four *MLP* mutations were reported in HCM, one of which was shown to decrease the binding to alpha-actinin (22).

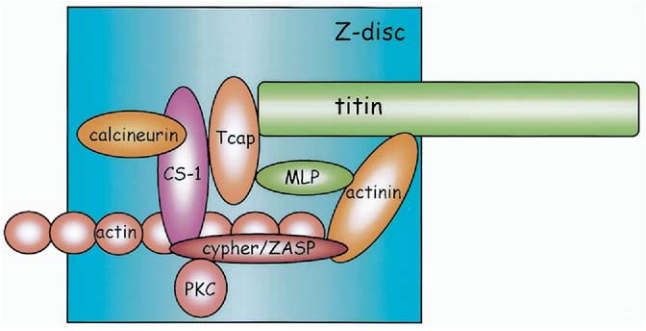

Figure 1. Schematic representation of Z-disc proteins. The Z-disc proteins mentioned in the text and their interactions are schematically shown. CS-1 = calsarcin-1; MLP = muscle LIM protein; PKC = protein kinase C; ZASP = Z-band alternatively spliced PDZ-motif protein.

To further explore the relationship between an abnormality in Z-disc proteins and cardiomyopathy, we searched for mutations in the Tcap gene *(TCAP)* in a cohort of patients with HCM and DCM. Two HCM-associated *TCAP* mutations and a novel DCM-associated *TCAP* mutation were identified in this study. We investigated the interaction of Z-disc components titin, MLP, and calsarcin-1 (CS-1) with Tcap in the presence of the *TCAP* mutations. We report here that the HCM-associated and DCM-associated mutations showed different changes in Tcap's interaction properties with these Z-disc components.

METHODS

Subjects. A total of 346 and 136 genetically unrelated patients with HCM and DCM, respectively, were chosen as subjects. Among them, 332 HCM and 135 DCM patients were Japanese, and 14 HCM patients and 1 DCM patient were Korean. A family history of the disease (i.e., at least one blood relative with the same disease and/or unexplained sudden cardiac death <45 years of age) was observed in 236 patients with HCM and 34 with DCM. The patients with HCM were diagnosed based on medical history, physical examination, electrocardiogram, and echocardiogram (LV wall thickness >14 mm), whereas patients with DCM were diagnosed based on a medical history, physical examination, electrocardiogram, and echocardiogram (impaired LV function and fractional shortening <25%), in the absence of coronary artery disease, as evaluated by coronary angiography (12,13,23). When a patient was found to have a *TCAP* mutation, blood relatives were examined for the mutation, if available. Control subjects consisting of 240 Japanese and 70 Korean healthy individuals were tested for the mutations identified in this study. Blood samples were obtained from each subject after obtaining informed consent. The study protocol was approved by the Ethics Reviewing Committee of Medical Research Institute, Tokyo Medical and Dental University, and Institutional Reviewing Board of the Samsung Medical Center.

Analysis of known disease genes for HCM and DCM. Patients with HCM were screened for mutations in *MYH7, TNNT2, MYBPC3* (cardiac myosin-binding protein C), *TNNI3* (cardiac troponin I), *MYL2* (myosin regulatory light chain), *MYL3* (myosin essential light chain), *TPM1* (alpha-tropomyosin), *TTN, MLP,* and *ACTC,* whereas patients with DCM were analyzed for mutations in *ACTC, DES, DMD, LMNA* (lamin A/C), *TTN, MYH7, TNNT2, MYBPC3,* and *TNNI3,* by using the polymerase chain reaction (PCR) single-strand conformation polymorphism (SSCP) method (24–26), and the mutations were confirmed by sequencing the PCR products. The SSCP method we employed usually detects a point mutation with an efficacy of about 90% (25,26). The conditions of the PCR and primers used in the analyses are available on

Table 1. Nucleotide Sequence of Primers Used in This Study

Name	Sequence (5′ to 3′)
TCAP-1F	GCTATTTAAAGGGCCTGGGA
TCAP-1R	CAGGGACTCTAGCAGACC
TCAP-2	AGAGAGCAACAGCTCCCAG
TCAP-2R	GCGGGCACAGCGGAGCC
MYBPC3-Ex6F	TAAGGGTGCGGAGCCTTGT
MYBPC3-Ex6R	GGGAGCCCGAGCCCAGGACA
TCAP-EcoF	GAATTCATGGCTACCTCAGAGCTG
TCAP-BamR	GGATCCTCAGCCTCTCTGTGCTTCCTG
TCAP-87MR	CGGCAGTACCTGCTGGCAGGG
TCAP-87MF	CAGCTGCCCTACCAGCAGGTA
TCAP-132MF	ACCGCCAGCAGGTGGCTG
TCAP-132MR	CAGCCACCTGCTGGCGGT
TCAP-137MF	GTGGCTGAGATCATAAAGCAG
TCAP-137MR	GGGCAGCTGCTTTATGATCTC
TCAP-153MR	GGACAGGGAGCGATGAAGGGC
TCAP-153MF	CCCGGTGCCCTTCATCGCTCC
MLP-EcoF	GAATTCATGCCAAACTGGGGCGGAGGCGCAA
MLP-BamR	GGATCCTCATTCTTTCTTTTCCACTTGTTGTGTAAGGCC
CS-1-EcoF	GAATTCATGCTATCACATAATACTATG
CS-1-BamR	GGATCCATCTGGGGTGTTGGGAGGAGTCAA
TTN-NcoIF	CCATGGTGACAACTCAAGCACCGAC
TTN-BamR	GGATCCAGGTACTTCTTCTTCACCTTGA
Tcap-XhoT7R	ACCCATTTGCTGTCCACCAGTCATGCTAGCCATCTCGAGGCCTCTCTGTGCTTC
Tcap-120XhoR	CTCGAGCTCCAGCGCCAGCAGCTCCTG
MLP-Xho1R	CTCGAGTTCTTTCTTTTCCACTTGTTGTGTAAGGCC

request. The screening analysis revealed 36 *MYH7*, 24 *MYBPC3*, 20 *TTNT2*, 6 *TNNI3*, 2 *MYL2*, 1 *TPM1*, 1 *TTN*, and 2 *MYL3* mutations in 91 familial HCM cases, and 5 *MYBPC3*, 3 *TNNT2*, 3 *TNNI3*, 1 *MYL3*, 1 *TTN*, and 2 *MYH7* mutations in 15 sporadic HCM cases. In addition, we found 1 *DES* and 3 *TTN* mutations in four familial DCM cases and 1 *TTN* and 3 *DMD* mutations in four sporadic DCM cases (details will be reported elsewhere).

Mutational analysis of *TCAP*. The primers used in this study are listed in Table 1. Both TCAP-1F and -1R for exon 1 and TCAP-2F and -2R for exon 2 were used in the analysis of *TCAP*. The PCR products from the patients were sequenced from the primers on both strands. Controls were analyzed only for exon 2.

Analysis of the cardiac myosin-binding protein C gene *(MYBPC3)* in a Korean family. Exon 6 of *MYBPC3* was amplified by PCR with MYBPC3-Ex6F and -Ex6R. The PCR products showed an abnormal SSCP pattern in patients with HCM, and the sequencing analysis demonstrated that this was due to an A-to-G change in codon 236. This change destroyed an AluI site. In order to detect the mutation in the blood relatives, the PCR products were digested by AluI and electrophoresed in 2.0% agarose gel.

Yeast two-hybrid (Y2H) assay. A human *TCAP* complementary deoxyribonucleic acid (cDNA) fragment (nucleotides 11–514; GenBank AJ000491, corresponding to amino acids 1–167) was amplified from cDNA generated from total cardiac RNA by the reverse transcription (RT)-PCR method using primers TCAP-EcoF and -BamR. Cardiomyopathy-associated mutations were introduced into the cDNA fragment by the primer-mediated mutagenesis method, as described previously (13). The following primers were used: R87Q: TCAP-EcoF, -87MR, -87MF, and -BamR; E132Q: TCAP-EcoF, -132MF, -132MR, and -BamR; T137I: TCAP-EcoF, -137MF, -137MR, and -Bam; R153H: TCAP-EcoF, -153MR, -153MF, and -Bam. Both S157A and S157D were constructed from cDNA clones obtained from Dr. S. Labeit (15). The PCR fragments were cloned into pCRII (Invitrogen, Carlsbad, California) for sequence confirmation. In order to obtain bait plasmids, normal and mutant *TCAP* cDNA fragments were cleaved by digestion with EcoRI and BamHI, gel-purified, and inserted into pGBKT7 (Clontech, Palo Alto, California). A human *MLP* cDNA fragment (nucleotides 58–642; GenBank U49837, corresponding amino acids 1–194) and a CS-1 cDNA fragment (nucleotides 86–424; GenBank BC005195, corresponding amino acids 1–113) were also obtained by the RT-PCR method with primers MLP-EcoF and -BamR, and CS-1-EcoF and -BamR, respectively. A human titin Z1-Z2 cDNA fragment (nucleotides 123–731; GenBank NM-003319, corresponding amino acids 1–203) was obtained by the RT-PCR method with primers TTN-NcoIF and -BamR. The MLP, the Z1-Z2 region of titin, and CS-1 cDNA fragments were cloned into pGADT7 (Clontech), and the nucleotide sequences were confirmed. The *TCAP* constructs and either MLP, titin, or CS-1 constructs were used for co-transformation of *Saccharomyces cerevisae* (strain *Y187*) on agar plates lacking leucine and tryptophan. Several independent transformants (n = 8 to 10) were tested for beta-galactosidase activity, as described previously (12,13).

Transformants carrying either bait plasmids or activator plasmids did not show any significant beta-galactosidase activity, demonstrating no self-activation. The data are presented as the mean value ± SD. The Student *t* test was used to estimate statistical significance.

Glutathion S-transferase (GST) pull-down competition assay. Each cDNA fragment used in the GST pull-down competition assay was generated by the PCR from each Y2H construct as a template, using combinations of the following primers: full Tcap fragment: Tcap-EcoF and -XhoT7R; short Tcap fragment: Tcap-EcoF and -120XhoR; titin Z1-Z2 fragment: TTN-NcoIF and -BamR; MLP: MLP-EcoF and -Xho1R; CS-1: CS-1-EcoF and -BamR. The *TCAP* fragments were cloned into pBAD/Thio-TOPO (Invitrogen) encoding the C-terminal polyhistidine tag to obtain a thioredoxin (Thio), T7, and His-tag fusion constructs. The titin, MLP, and CS-1 fragments were cloned into pGEX5X-1 (Amersham, Piscataway, New Jersey) to obtain GST-fusion constructs. All constructs were sequenced to ensure that no PCR errors were introduced. The GST-fusion constructs were used for the transformation of BL21-Gold (Stratagene, La Jolla, California). The transformants were collected in PBS with 1% Triton X-100 and sonicated. The GST-fusion proteins were purified and fixed on glutathione-sepharose beads as per the manufacturer's instructions. Thio-fusion *TCAP* constructs were introduced into TOP10 (Invitrogen). Thio-fusion proteins were prepared under the denaturing condition (6-M urea) and purified by binding with Ni^{2+}-charged resi, as per the manufacturer's instructions. A GST-only construct was used as a negative control. Binding assays were performed as described in the literature (14). Briefly, 5 μl each of Thio-fusion full Tcap protein and short Tcap protein (each 0.5 μg/μl) were added to 5 μl of either GST-fusion titin, MLP, or CS-1 (each 0.5 μg/μl) in 100 μl of binding buffer (20 mmol/l Tris, 50 mmol/l NaCl, pH 8.0). The ratio of Thio-fusion proteins to each GST-fusion protein used in the assay was determined in preliminary experiments, in which fixed amounts of Thio-fusion proteins were mixed with various amounts of GST-fusion proteins. The mixture was incubated for 1 h at 4°C, washed three times with 400 μl of binding buffer, suspended in sodium dodecyl sulfate (SDS) sample buffer, separated by SDS-polyacrylamide gel electrophoresis (PAGE) in 10% polyacrylamide gels, and transferred to duplicated nitrocellulose membranes. The membranes were blocked with 3% nonfat dry milk, incubated overnight at 4°C with anti-T7 tag monoclonal antibody (Novagen, San Diego, California) (1:7500) or goat anti-GST antibody (Amersham) (1:1000) as the first antibody. After washing with Tris-buffered saline (50 mmol/l Tris, 150 mmol/l NaCl, pH 7.5) containing 0.05% Tween 20, the membranes were incubated with anti-mouse immunoglobulin G conjugated with alkaline phosphatase (AP) (Promega, Madison, Wisconsin) (1:7500) or anti-goat immunoglobulin G conjugated with AP (Chemicon, Temecula, California) (1:7500), respectively, as the second antibody for 1 h at room temperature, and the immunocomplex was stained by using an Alkaline Phosphatase Conjugate Substrate Kit (Bio-Rad, Hercules, California). To confirm the specificity of the assay, we used affinity-purified rabbit polyclonal anti-Tcap antibody (kindly provided by Dr. S. Labeit) as the first antibody, substituting for anti-T7 antibody, and obtained similar results. The experiments were repeated at least five times, and each blot was analyzed by National Institutes of Health imaging in order to measure the contents of wild-type (WT) or mutant full Tcap protein in relation to that of WT short Tcap. The data are represented as the mean value ± SD. The Student *t* test was used to estimate statistical significance.

RESULTS

Identification of *TCAP* mutations in HCM. *TCAP* was analyzed by direct sequencing in a cohort of patients with HCM. There were three variations not accompanied by amino acid replacement: a CAC-to-CAT change in codon 52, a GGC-to-GGA change in codon 75, and a GCC-to-GCA change in codon 151. They were found in both patients and healthy controls at a similar frequency (variant allele frequencies in the patients and controls were 0.004 and 0.003, 0.003 and 0.003, and 0.432 and 0.440, respectively), indicating that these variations are polymorphisms not associated with the disease. In contrast, two missense mutations—a C-to-T transition in codon 137 replacing ACA (Thr) with ATA (Ile) (T137I) and a G-to-A transition in codon 153 replacing CGT (Arg) with CAT (His) (R153H)—were found in Japanese patients. The clinical characteristics of the patients and their blood relatives carrying these mutations are shown in Table 2.

A T137I mutation was found in a female patient (JH945 II-2) (Figs. 2a and 2b) and her son, both affected with HCM (JH945 III-1) (Table 2). On the other hand, an R153H mutation was found in a male patient (JH14 II-1) (Figs. 2c and 2d) and his sister (JH14 II-3) (Fig. 2c), both with HCM; this mutation was inherited by his sons, who did not suffer from HCM at the time of the examination (JH14 III-1 and III-2, respectively) (Table 2). These missense mutations were not found in unrelated controls. In addition, the patient cohort was screened for mutations in the known disease genes for HCM, and the patients carrying the *TCAP* mutations did not show any mutations in the known disease genes.

Identification of a novel *TCAP* mutation in DCM. While analyzing a cohort of DCM patients, we found a novel *TCAP* mutation in a Korean patient with DCM (SM728 II-5) (Fig. 3a). The patient had suffered from heart failure at the age of 34 years (Table 2) and undergone heart transplantation at the age of 35 years. The analysis demonstrated a missense mutation in codon 132, a replacement of GAG (Glu) with CAG (Gln) (E132Q) (Fig. 3b). This mutation was not found in the controls. A family study revealed that his mother (SM728 I-3) and maternal uncle (SM728 I-4) were affected by HCM, but no *TCAP* muta-

Table 2. Clinical Characteristics of Individuals Carrying the *TCAP* or *MYBPC3* Mutation

ID	Mutation	Age (yrs), Gender	NYHA Class	LVEDD (mm)	LVESD (mm)	IVS_{max} (mm)	PWT_{max} (mm)	FS (%)	ECG Findings
JH945 II-2	*TCAP* T1371	62, F	I	44	27	17	11	39	WPW syndrome, LVH
JH945 III-1	*TCAP* T1371	29, M	I	43	28	16	8	35	LVH, abnormal Q-wave
JH14 II-1	*TCAP* R153H	48, M	I	42	30	23	12	29	Abnormal Q-wave
JH14 II-2	*TCAP* R153H	67, F	I	38	28	17	13	26	LVH
JH14 III-1	*TCAP* R153H	21, M	I	46	27	10	9	41	Normal
JH14 III-2	*TCAP* R153H	19, M	I	46	28	10	10	39	Normal
SM728 I-2	*TCAP* E132Q	63, M	I	53	30	11	11	43	Normal
SM728 II-5	*TCAP* E132Q	34, M	III	55	50	10	9	9	RVH, I-AV block
SM728 III-4	*TCAP* E132Q	5, F	I	32	21	4	6	34	Normal
SM728 I-3	*MYBPC3* S256G	64, F	I	55	27	21	9	51	LVH, T-wave abnormality
SM728 I-4	*MYBPC3* S256G	60, M	I	55	31	27	11	44	Inverted T-wave in V_5–V_6
SM728 II-3	*MYBPC3* S256G	39, M	I	52	29	12	10	44	Normal
SM728 III-1	*MYBPC3* S256G	12, F	I	37	23	9	7	38	Normal
SM728 III-2	*MYBPC3* S256G	10, M	I	37	23	7	7	38	Normal

For ID, see Figures 1 and 2. Age represents age at examination.
ECG = electrocardiographic; F = female; FS = fractional shortening; I-AV = first-degree atrioventricular block; IVS_{max} = maximum intraventricular septal wall thickness; LVEDD = left ventricular end-diastolic diameter; LVESD = left ventricular end-systolic diameter; LVH = left ventricular hypertrophy; M = male; NYHA = New York Heart Association; PWT_{max} = maximum posterior wall thickness; RVH = right ventricular hypertrophy.

tion was found in either of them (Fig. 3a). Instead, the *TCAP* mutation was transmitted from the father (SM728 I-2) to his son (SM728 III-4), who did not manifest a DCM phenotype at the time of the examination (Fig. 3a) (Table 2).

We searched for mutations in the known HCM-causing genes in his mother, and a missense mutation of *MYBPC3, S236G*, which is a replacement in codon 236 of AGC (Ser) with GGC (Gly), was identified (Fig. 3c). This mutation was also found in the affected uncle (Fig. 3a) and in another unrelated Japanese patient (data not shown), but not in the controls, suggesting that it may be a disease-causing mutation. As shown in Figure 3a, the *MYBPC3* mutation was not inherited by the proband, but was found in his brother (SM728 II-3) and his brother's offspring (SM728 III-1 and III-2), none of whom had HCM at the time of the examination (Table 2). Because both mother and uncle developed HCM after the age of 50 years, it is possible that the *MYBPC3* mutation is associated with late-onset HCM (2). These data demonstrate that this family has two different gene mutations: one is the *TCAP* mutation associated with DCM and the other is the *MYBPC3* mutation associated with HCM. In addition, the DCM cohort was screened for mutations in the known disease genes for DCM, and the patient carrying the *TCAP* mutation did not have any mutations in the known disease genes thus far tested.

As shown in Figure 4, the *TCAP* mutations identified in this study were found in the evolutionary conserved residues (except for E132Q, but this position is occupied by an acidic residue in the rat and mouse) at or near the binding domains to titin (14), MLP (20), and calsarcin-1 (19).

Alterations in the interaction of Tcap with MLP, titin and CS-1 caused by *TCAP* mutations, as found in the Y2H assay. In order to investigate the functional consequences of *TCAP* mutations for Tcap's interaction with MLP, titin, and CS-1, we performed Y2H assays. Wild-type and mutant Tcap's were constructed in an activator plasmid and co-transformed with a bait plasmid containing cDNA for MLP, titin (Z1-Z2 domains), or CS-1. Because the R153H mutation was found near the phosphorylation site, Ser157, and because this site is important in Tcap's interaction with minK (18), we investigated two other constructs, S157A (a model of the unphosphorylated form) and S157D (a model of the phosphorylated form).

We measured beta-galactosidase activity in several independent transformants in order to investigate qualitatively the strength of protein-protein interaction (Table 3). It was

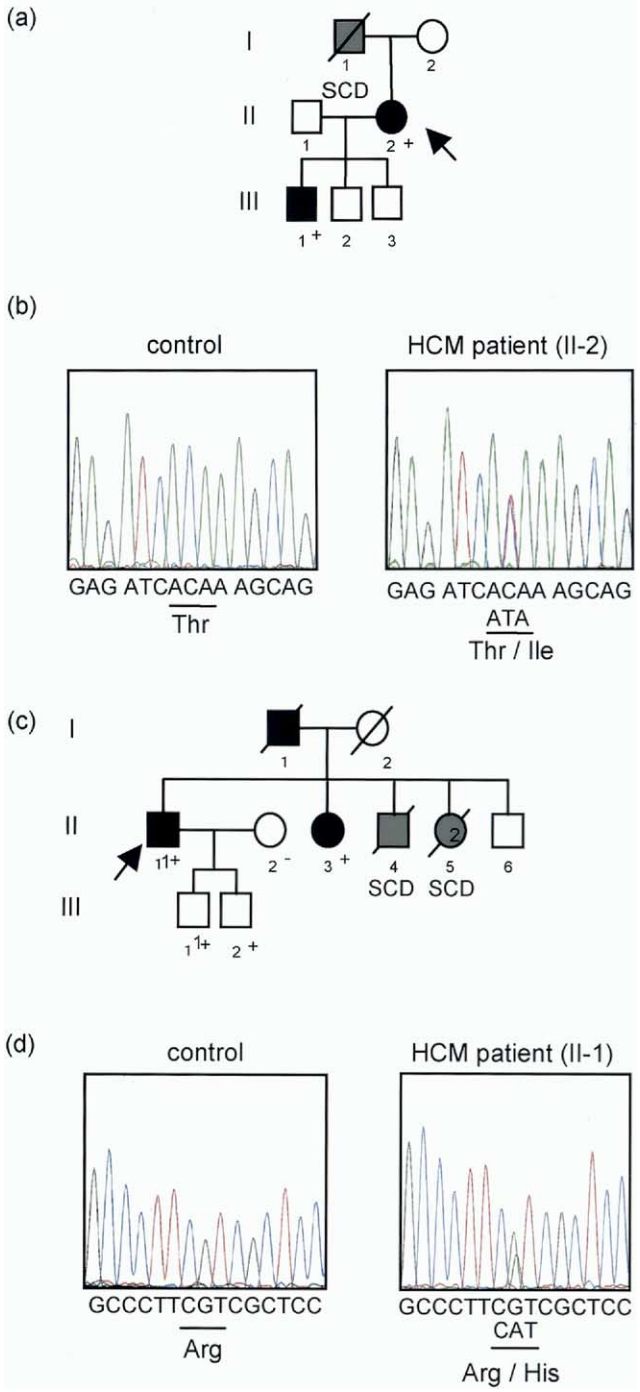

Figure 2. Mutational analysis of *TCAP* in hypertrophic cardiomyopathy (HCM). **Filled and open symbols** indicate affected and unaffected individuals, respectively. The presence (+) or absence (−) of a *TCAP* mutation is noted. SCD = sudden cardiac death (JH14: II-4 and II-5 died after exercise at age 44 and 33 years, respectively; JH945: I-1 died after exercise at age 34 years). **(a)** Pedigree of a Japanese HCM family. The **arrow** indicates the proband (JH945 II-2). A *TCAP* mutation was found in II-2 and III-1. **(b)** Direct sequencing data of *TCAP* exon 2 from a control **(left)** and the proband **(right)**. Codon 137 of the control was ACA (Thr), whereas the patient was heterozygous for an ATA mutation (Ile). **(c)** Pedigree of another Japanese HCM family. A *TCAP* mutation was found in II-1, II-3, III-1, and III-2. **(d)** Direct sequencing data of *TCAP* exon 2 from a control and the proband (JH14 II-1). Codon 153 of the control was CGT (Arg), whereas the patient was heterozygous for a CAT mutation (His).

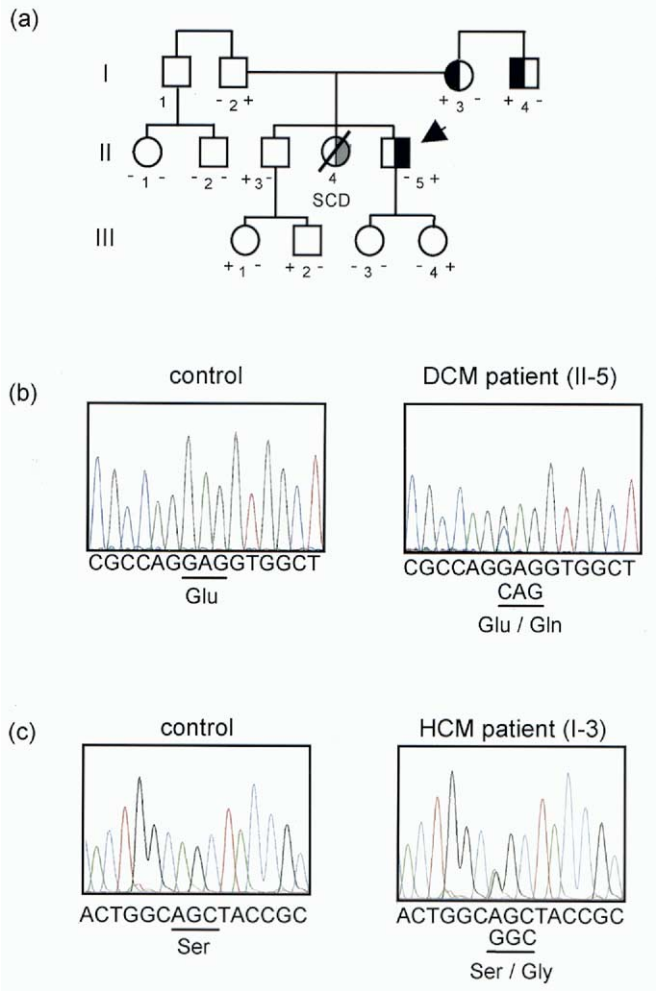

Figure 3. Mutational analyses of *TCAP* and *MYBPC3* in a Korean family. **(a)** Pedigree of a Korean family with dilated cardiomyopathy (DCM) and hypertrophic cardiomyopathy (HCM). **Right-half-filled and left-half-filled symbols** indicate DCM and HCM, respectively. Plus symbols to the right and left of individual IDs represent *TCAP* (E132Q) and *MYBPC3* (S236G) mutations, respectively. A *TCAP* mutation was found in I-2, II-5, and III-4, whereas an *MYBPC3* mutation was found in I-3, I-4, II-3, III-1, and III-2. **Open symbols** represent individuals without cardiac disease phenotypes. SCD = sudden cardiac death (II-4: suffered from heart failure and died suddenly at the age of 26 years). **(b)** Direct sequencing data of *TCAP* exon 2 from a control and the proband (SM728 II-5). Codon 132 of the control was GAG (Glu), whereas that of SM728 was heterozygous for a CAG mutation (Gln). **(c)** Direct sequencing data of *MYBPC3* exon 6 from the mother of the proband (SM728 I-3). Codon 236 of the control was AGC (Ser), whereas that of the mother was heterozygous for a GGC mutation (Gly).

demonstrated that the DCM-associated mutations, R87Q and E132Q, significantly impaired the interaction (both were $p < 0.01$). On the other hand, the HCM-associated mutations, T137I and R153H, showed no statistical difference ($p = 0.29$ and 0.54, respectively). The unphosphorylated Tcap model (S157A) and the phosphorylated model (S157D) showed virtually no change ($p = 0.49$ and 0.70, respectively).

We next investigated Tcap's interaction with titin. The DCM-associated Tcap mutations showed statistically significant impairment (both were $p < 0.001$). In clear

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                121         132  137              153 ▼ 160
Human           TALGGQCVDR QEVAEITKQL PPVVPVSKPG ALRRSLSRSM
HCM(JH945)      ---------- ------I--- ---------- ----------
HCM(JH14)       ---------- ---------- ---------- --H-------
DCM(SM728)      ---------- -Q-------- ---------- ----------
Bovine          ---------- -D-------- ---------- T---------
Rat             --------E- -D-------- ---------- P---T-----
Mouse           --------E- -D-------- ---------- P---T-----
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Figure 4. Alignment of Tcap amino acid sequences. The amino acid sequence of human Tcap was aligned with that of bovine, rat, and mouse Tcaps. The mutations found in patients with hypertrophic cardiomyopathy (HCM) and dilated cardiomyopathy (DCM) are represented along with the phosphorylation site, Ser157, indicated by an **arrowhead**. **Dashes** indicate identity to human Tcap.

contrast, the HCM-associated mutations significantly augmented the interaction (both were $p < 0.001$). On the other hand, the unphosphorylated model and phosphorylated model showed no change in the interaction ($p = 0.48$ and $p = 0.15$, respectively).

Because calsarcin-3, a skeletal muscle-specific calsarcin, was reported to interact with Tcap, and cardiac muscle expresses another calsarcin, CS-1 (19), we examined the interaction between Tcap and CS-1. Co-transformants of Tcap and CS-1 constructs showed that the beta-galactosidase activity of WT Tcap colonies was 1.04 ± 0.03 units, whereas no beta-galactosidase activity was observed in colonies carrying either Tcap or CS-1. These data suggest that CS-1 could interact with Tcap. The DCM-associated mutations significantly impaired the interaction (both were $p < 0.001$). The HCM-associated mutations, in clear contrast, augmented the interaction (both were $p < 0.001$). Unphosphorylated or phosphorylated Tcap models showed no change ($p = 0.58$ and $p = 0.19$, respectively).

These observations demonstrate that the DCM-associated mutations impair the interaction of Tcap not only with MLP but also with titin and CS-1. On the other hand, the HCM-associated mutations augment Tcap's interaction with titin and CS-1 without significant changes in the interaction with MLP. In addition, the phosphorylation status of Tcap does not affect its interaction with these Z-disc components.

Table 3. Relative Binding of Wild-Type and Mutant Tcap to MLP, Titin, and CS-1, as Assessed by the Y2H Assays

	MLP	Titin	CS-1
WT	1.0*	1.0*	1.0*
R87Q	0.35 ± 0.07†	0.31 ± 0.04‡	0.76 ± 0.09‡
E132Q	0.67 ± 0.11†	0.41 ± 0.03‡	0.63 ± 0.04‡
T137I	1.11 ± 0.17	1.57 ± 0.12‡	1.27 ± 0.11‡
R153H	1.11 ± 0.26	1.28 ± 0.12‡	1.25 ± 0.04‡
S157A	0.94 ± 0.25	0.98 ± 0.07	0.97 ± 0.18
S157D	1.10 ± 0.34	0.94 ± 0.11	0.96 ± 0.08

*Relative beta-galactosidase activity in wild-type (WT) Tcap colonies was defined as 1.0. The beta-galactosidase activities (arbitrary unit) for WT Tcap with MLP, titin, and CS-1 were 0.42 ± 0.08, 8.88 ± 0.35, and 1.04 ± 0.03, respectively. †$p < 0.01$. ‡$p < 0.001$. Data are presented as the mean value ± SD.
CS-1 = calsarcin-1; MLP = muscle LIM protein.

GST pull-down competition assay of the interaction between Tcap and the other Z-disc components, MLP, titin, and CS-1. In order to investigate the altered interaction between Tcap and the other Z-disc components by an independent method, we performed GST pull-down competition assays. The MLP, titin, and CS-1 proteins were expressed as GST-fusion proteins. The WT and mutant Tcap proteins were expressed as Thio-fusion proteins. These recombinant Tcap proteins were designated as test-Tcap-WT or test-Tcap-mutant (e.g., test-Tcap-R87Q). A short form of Tcap (amino acids 1–120) protein was also constructed as a Thio-fusion protein, to be used as a competitor (designated as comp-Tcap). Equal amounts of test-Tcap and comp-Tcap (Fig. 5a) were complexed with each GST-fusion protein. After incubation and washing, the Tcap proteins were eluted from the complex, separated by SDS-PAGE, and immunoblotted with anti-GST antibody and anti-T7 antibody so that the amounts of GST-fusion proteins complexed with the test-Tcap in the presence of comp-Tcap could be measured. Test-Tcap-WT did not form a complex with GST-only proteins (data not shown).

As shown in Figure 5b, the interaction between GST-MLP and test-Tcap-R87Q was weak, compared with that between GST-MLP and test-Tcap-WT. Similarly, the interaction between GST-MLP and test-Tcap-E132Q was weak. In contrast, the interactions between the GST-MLP and HCM-associated mutant Tcap, test-Tcap-T137I or -R153H, were comparable with that with test-Tcap-WT. Neither S157A nor S157D showed remarkable changes. The results obtained with GST-titin (Fig. 5c) and GST-CS1 (Fig. 5d) demonstrated that the DCM-associated mutations impaired the interaction with titin and CS-1, respectively. On the other hand, the HCM-associated mutations showed an augmented interaction with titin and CS1 (Figs. 5c and 5d, respectively).

The assays were repeated and each blot was analyzed by National Institutes of Health imaging to measure semi-quantitatively the intensity of each test-Tcap in comparison with that of comp-Tcap. The altered interaction between Tcap and the Z-disc components, as assessed by the relative intensity of the bands, is shown in Table 4. Regarding the interaction with MLP, the DCM-associated mutations showed significantly lower values (both were $p < 0.001$), whereas the HCM-associated mutations showed no change ($p = 0.78$ and 0.30, respectively). As for the interaction with titin, the DCM-associated mutations showed lower values (both were $p < 0.001$). On the other hand, the HCM-associated mutations clearly showed higher values (both were $p < 0.001$). The interaction with CS-1 was at a comparable level. The DCM-associated mutations gave lower values ($p < 0.001$), whereas the HCM-associated mutations gave higher values ($p < 0.001$). Both S157A and S157D showed virtually no change in the interaction with MLP, titin, and CS-1.

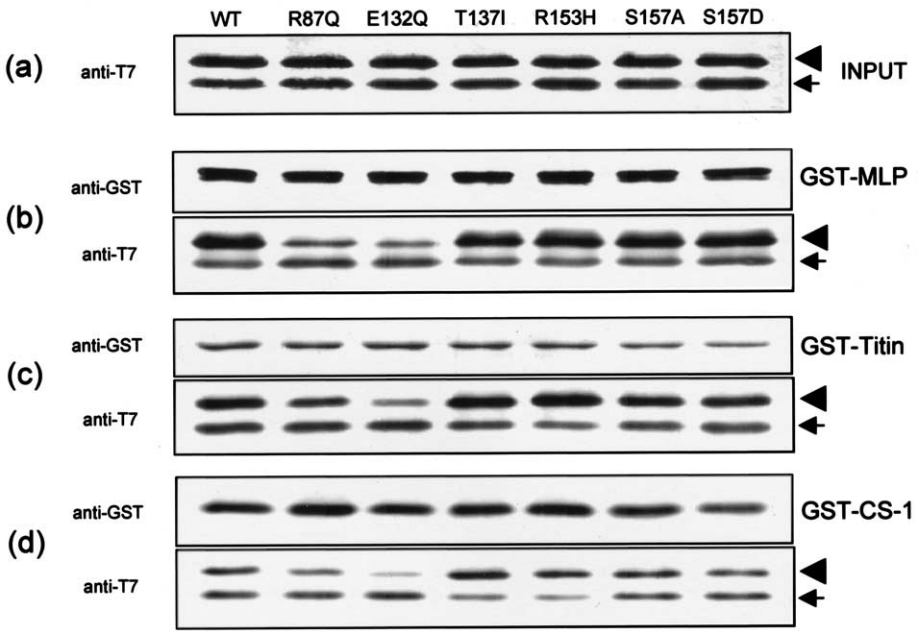

Figure 5. The glutathion S-transferase (GST) pull-down competition assays between Tcap and the other Z-disc components. Wild-type (WT) and mutant (R87Q, E132Q, T137I, R153H, S157A, and S157D) Tcap (test-Tcap) and a short Tcap (amino acids 1–120, comp-Tcap) were fused to Thio and T7-tag at the N- and C-terminus, respectively. MLP, titin (Z1-Z2 domain), and calsarcin-1 (CS-1) were fused to GST. The molecular weights of GST-titin, GST-MLP, GST-CS-1, test-Tcap, and comp-Tcap are 50, 47, 39, 35, and 30 kd, respectively. Equivalent amounts of test-Tcap (indicated by **arrowheads**) and comp-Tcap (indicated by **arrows**) were mixed with each GST-fusion protein. After incubation and washing, the complex was resolved in SDS-PAGE and immunoblotted with anti-GST antibody and anti-T7 antibody. **(a)** Input amounts of test-Tcap and comp-Tcap; **(b)** GST-MLP protein and Tcap proteins in complex; **(c)** GST-titin protein and Tcap proteins in complex; and **(d)** GST-CS-1 protein and Tcap proteins in complex.

DISCUSSION

In this study, we identified for the first time, *TCAP* mutations associated with HCM. The HCM-associated mutations were found in the evolutionary conserved residues and were absent in healthy controls. In addition, we identified another DCM-associated mutation. The penetrance of the *TCAP* mutations may not be high, as there were several unaffected blood relatives carrying the mutations. In this cohort study, 2 of 236 familial HCM patients and 1 of 34 familial DCM patients were found to carry the *TCAP* mutations, suggesting that *TCAP* mutations may be a rare cause of cardiomyopathy.

The Z-disc of *MLP* knock-out mice showed widened, dispersed, and irregular structures, and the cardiomyocytes of *MLP* knock-out mice did not exhibit a hypertrophic response to mechanical stress. On the other hand, as a completely preserved Z-disc structure was observed in *MLP* and *PLB* (phospholamban gene) double knock-out mice, it is presumed that the promotion of calcium cycling can prevent Z-disc defects by enhancing cardiac relaxation and reducing wall stress (20). These observations suggest an important role of the Z-disc complex, titin/Tcap/MLP, in response to cardiac muscle stretching. Through the Y2H and GST pull-down competition assays, we demonstrated the functional alterations caused by the *TCAP* mutations. Due to the impaired interaction of Tcap with MLP, titin, and CS-1 caused by DCM-associated mutations, mutation-prone cardiomyocytes are more easily extended than the normal cardiomyocytes in the presence of mechanical stretch. The Z-disc with mutant Tcap could affect the stretch response and the calcium signals via CS-1 and the calcineurin complex.

Table 4. Relative Binding of Wild-Type and Mutant Tcap to MLP, Titin, and CS-1, as Measured by the GST Pull-Down Competition Assays

	MLP	Titin	CS-1
WT	1.0*	1.0*	1.0*
R87Q	0.22 ± 0.04†	0.65 ± 0.07†	0.51 ± 0.15†
E132Q	0.23 ± 0.02†	0.53 ± 0.10†	0.27 ± 0.10†
T137I	0.99 ± 0.09	1.31 ± 0.04†	1.98 ± 0.46†
R153H	0.97 ± 0.06	1.43 ± 0.08†	1.91 ± 0.39†
S157A	0.96 ± 0.05	1.04 ± 0.10	1.09 ± 0.10
S157D	0.95 ± 0.06	0.94 ± 0.07	0.93 ± 0.10

*Relative ratio of intensity from wild-type (WT) test Tcap to that of comp Tcap was defined as 1.0. †$p < 0.001$. Data are presented as the mean value ± SD.

GST = glutathion S-transferase; other abbreviations as in Table 3.

In clear contrast, due to the augmented interaction of Tcap with titin and CS-1 caused by HCM-associated mutations, the Z-disc with mutant Tcap might exert an increased passive tension. If the length of the muscle fiber is constant, the increased passive tension leads to increased calcium sensitivity in muscle contraction (27). Therefore, it is presumed that *TCAP* mutations in HCM increase calcium sensitivity. The HCM-associated mutations in *TPM1*, *MYL2*, *TNNT2*, *MYH7*, and *TNNI3* are reported to increase calcium sensitivity (28–32), and the increased calcium sensitivity may be a common fundamental change caused by HCM-associated sarcomere mutations. It may be

worth noting that Mohapatra et al. (21), by using the immunoprecipitation method, found a DCM-associated *MLP* mutation leading to decreased interaction with alpha-actinin, whereas Geier et al. (22), by means of an Y2H assay, revealed that an HCM-associated *MLP* mutation decreases the binding to alpha-actinin. It is not clear why DCM-associated and HCM-associated mutations exhibited similar functional changes, but it is difficult to compare the results because different assay systems were used in these studies; in particular, the Y2H assay might not be adequate for the protein-protein interaction in mammalian cells. In our study, we used the pull-down assay in addition to the Y2H assay in order to demonstrate the functional changes.

The Z-disc has a functional and mechanical coupling to the T-tubule system, a site of initial Ca^{2+}-induced Ca^{2+} release that links excitation and contraction. Calsarcin-1 binds calcineurin, a well-known mediator of hypertrophy. Overexpression of calsarcin can inhibit calcineurin activity (19), and CS-1 interacts with Cypher/ZASP, which binds protein kinase C (PKC) (Fig. 1) (19,33). Of interest is that we recently identified in a DCM family a Cypher/ZASP mutation that increases the binding to protein kinase C (33), and other Cypher/ZASP mutations were reported in several DCM families (34). These observations suggest that Z-disc proteins may play a role in monitoring local Ca^{2+} signals at the Z-disc and regulating the activation of compensatory pathways (35). Our results suggest that the titin/Tcap/MLP/alpha-actinin complex plays an important role as a stretch sensor controlling cardiac hypertrophy, as well as cardiac failure. The modulation of the mechanical links between the stretch sensor and the Ca^{2+} signals could become a therapeutic target in the future.

Scope of this study. We investigated the interaction of Tcap with titin, MLP, and CS-1, using two different methods. Because both methods are in vitro qualitative assays and recombinant proteins were used, the alterations in the interaction caused by the *TCAP* mutations might be different in vivo. A cohort of HCM and DCM patients was analyzed for mutations in the known disease genes by the SSCP method, which detects a point mutation with an efficacy of 90% or less (26). In addition, some disease genes, such as *MYH6* (cardiac alpha-myosin heavy chain) and *MYLK2* (myosin light chain kinase) for HCM and *EMD* (emerin) and *G4.5* (tafazzin) for DCM, were not analyzed in the patients carrying *TCAP* mutations. As such, the possibility remains that these patients might have additional disease-related mutations.

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