

Green Tea Catechin Normalizes the Enhanced Ca^{2+} Sensitivity of Myofilaments Regulated by a Hypertrophic Cardiomyopathy–Associated Mutation in Human Cardiac Troponin I (K206I)

Chad M. Warren, MSc; Chehade N. Karam, PhD; Beata M. Wolska, PhD; Tomoyoshi Kobayashi, PhD; Pieter P. de Tombe, PhD; Grace M. Arteaga, MD; J. Martijn Bos, MD, PhD; Michael J. Ackerman, MD, PhD; R. John Solaro, PhD

Background—Hypertrophic cardiomyopathy (HCM) is the most common inherited cardiovascular disease characterized by thickening of ventricular walls and decreased left ventricular chamber volume. The majority of HCM-associated mutations are found in genes encoding sarcomere proteins. Herein, we set out to functionally characterize a novel HCM-associated mutation (K206I-TNNI3) and elucidate the mechanism of dysfunction at the level of myofilament proteins.

Methods and Results—The male index case was diagnosed with HCM after an out-of-hospital cardiac arrest, which was followed by comprehensive clinical evaluation, transthoracic echocardiography, and clinical genetic testing. To determine molecular mechanism(s) of the mutant human cardiac troponin I (K206I), we tested the Ca^{2+} dependence of thin filament–activated myosin-S1–ATPase activity in a reconstituted, regulated, actomyosin system comparing wild-type human troponin complex, 50% mix of K206I/wildtype, or 100% K206I. We also exchanged native troponin detergent extracted fibers with reconstituted troponin containing either wildtype or a 65% mix of K206I/wildtype and measured force generation. The Ca^{2+} sensitivity of the myofilaments containing the K206I variant was significantly increased, and when treated with 20 $\mu\text{mol/L}$ (-)-epigallocatechin gallate (green tea) was restored back to wild-type levels in ATPase and force measurements. The K206I mutation impairs the ability of the troponin I to inhibit ATPase activity in the absence of calcium-bound human cardiac troponin C. The ability of calcium-bound human cardiac troponin C to neutralize the inhibition of K206I was greater than with wild-type TnI.

Conclusions—Compromised interactions of K206I with actin and hcTnC may lead to impaired relaxation and HCM. (*Circ Cardiovasc Genet.* 2015;8:765-773. DOI: 10.1161/CIRCGENETICS.115.001234.)

Key Words: actin ■ actomyosin ■ calcium ■ echocardiography ■ troponin

The linkage of mutations in sarcomeric proteins to hypertrophic cardiomyopathy (HCM) not only provides insights into the control of cardiac remodeling by the state of the sarcomeres but also provides insights into the molecular mechanisms controlling the contraction and relaxation of the heart.¹⁻³ Experiments reported here highlight these insights in the case of a heterozygous mutation in *TNNI3*-encoded cardiac troponin I identified as p.Lys206Ile (also p. K206I, c. 617 A>T) in a 13-year-old male, who was diagnosed with HCM after an out-of-hospital cardiac arrest in 2009. These observations stimulated us to investigate the fundamental mechanisms for these phenotypic findings in reconstituted systems with myofilaments regulated by wild-type (WT) human cardiac

troponin I (hcTnI), heterozygously expressed mutant hcTnI (K206I/WT), and mutant hcTnI (K206I).

Clinical Perspective on p 773

The site of the mutation is of particular interest in that, despite extensive investigations of structure–function relations, there are significant gaps in our understanding of the role of the mobile domain (MD) of hcTnI (containing residues 167–210) in the regulation of thin filament–thick filament interactions. There is ample evidence that the relaxed state of the myofilaments involves an inhibition of the reaction of myosin cross-bridges with the thin filament. The inhibition occurs at Ca^{2+} concentrations below the threshold for binding

Received January 14, 2014; accepted November 6, 2015.

From the Department of Physiology and Biophysics, Center for Cardiovascular Research (C.M.W., C.N.K., B.M.W., T.K., R.J.S.) and Division of Cardiology, Department of Medicine (B.M.W.), University of Illinois at Chicago; Department of Cell and Molecular Physiology, Stritch School of Medicine, Loyola University Chicago, Maywood, IL (P.P.d.T.); and Division of Pediatric Critical Care and Physiology, Department of Pediatrics (G.M.A.), Department of Molecular Pharmacology and Experimental Therapeutics, Windland Smith Rice Sudden Death Genomics Laboratory (J.M.B., M.J.A.), Division of Pediatric Cardiology, Department of Pediatrics (J.M.B., M.J.A.), and Division of Cardiovascular Diseases, Department of Medicine (M.J.A.), Mayo Clinic, Rochester, MN.

The Data Supplement is available at <http://circgenetics.ahajournals.org/lookup/suppl/doi:10.1161/CIRCGENETICS.115.001234/-DC1>.

Correspondence to R. John Solaro, PhD, Department of Physiology and Biophysics, Center for Cardiovascular Research, University of Illinois-Chicago, 835 S Wolcott Ave, RM E202 (M/C 901), Chicago, IL 60612. E-mail solarorj@uic.edu

© 2015 American Heart Association, Inc.

Circ Cardiovasc Genet is available at <http://circgenetics.ahajournals.org>

DOI: 10.1161/CIRCGENETICS.115.001234

to a regulatory site on human cardiac troponin C (hcTnC), in which actin binding hcTnI inhibitory peptides flanking a switch peptide act together with the interactions of human cardiac troponin T (hcTnT) with tropomyosin to block the actin-myosin interaction.¹ As the Ca²⁺ concentration increases, sites on the thin filament are released from inhibition by promotion of binding of a switch peptide of hcTnI to Ca²⁺-bound hcTnC (calcium-bound human cardiac troponin C).^{4,5} How the MD of hcTnI, which contains K206, participates in establishing relaxation and in switching on activation remains unclear. The location of the mutation in hcTnI is the C-terminal region that did not resolve in the crystal structure, and whose function remains unclear.⁶ A potentially significant role for the MD of hcTnI has been established in studies testing the regulatory role of hcTnI with truncations at the C-terminus.^{7–10} Our demonstration of HCM linked to a single missense mutation extends our understanding of the role of the hcTnI C-terminus in long-term regulation of cardiac remodeling.

The aim of this study is to provide evidence that the newly discovered K206I mutation in *TNNI3* is a HCM-susceptibility mutation, and to determine the effect and mechanism of the mutation on myofilament Ca²⁺ sensitivity. The *TNNI3* gene has thus far been linked to 31 HCM-associated mutations,^{2,11} however, the *TNNI3* gene is only represented in <2% of patients with genotype-positive HCM, making mutations in *TNNI3* relatively rare in this population.¹² Interpretation of novel genetic variants in genes, such as *TNNI3*, is difficult because of background noise (≈5%), and thus in vitro analysis is essential to infer pathogenicity.¹³ Our studies revealed that compared with controls, myofilaments regulated by K206I demonstrate modified Ca²⁺ responsiveness and altered interactions among thin filament proteins. The green tea catechin (-)-epigallocatechin gallate (EGCG) has previously been shown to decrease Ca²⁺ sensitivity in myofilaments,^{14,15} thus we tested whether EGCG could be a potential therapeutic for this K206I mutation in *TNNI3*. EGCG was able to restore myofilament Ca²⁺ sensitivity to near WT levels.

Methods

Detailed Methods are available in the Data Supplement.

Clinical Demographics and Echocardiography

The subject was identified during the evaluation of HCM at Mayo Clinic and was enrolled in a Mayo Clinic IRB-approved research protocol no. 811–98 and, after discussing with the provider, signed a consent form to participate. Retrospective review of the electronic medical record was performed to identify the clinical phenotype of the patient. All clinical and genetic tests were performed as standard clinical care in the evaluation and treatment of the index's case disease. Transthoracic echocardiography was performed clinically according to standard protocols, and appropriate images and tracings from all angles were obtained (GE, Vivid 7).

Genetic Testing

Two independent genetic screens were used to identify the genotype. First, a comprehensive clinical genetic test was performed on the index case before evaluation at Mayo Clinic for 18 HCM genes and catecholaminergic polymorphic ventricular tachycardia-associated genes: *RYR2*, *KCNJ2*, and *CASQ2* on the index case (GeneDx, Gaithersburg, MD). For a full list of the 18 HCM-associated genes used in the genetic test, please see link: <http://www.genedx.com/>

test-catalog/disorders/hypertrophic-cardiomyopathy-hcm/. Second, targeted in-house mutation-specific confirmatory testing for the patient-specific variant was performed for the index case and the index case's parents. Genotyping of the phenotype positive sibling was performed elsewhere.

Protein Purification, Reconstitution, and Western blotting

Details are available in the Data Supplement.

Actomyosin-S1 ATPase Assay

The micro-ATPase assay was performed as previously described with some minor modifications.¹⁶

Measurements of Ca²⁺-Dependent Activation of Force in Troponin-Exchanged Skinned Fibers

pCa-force measurements were performed with troponin-exchanged skinned papillary fiber bundles from hearts of Friend Virus B NIH mice (3–4 months) as previously described.¹⁷

Data and Statistical Analysis

pCa-ATPase and pCa-force relations were fit to a modified 4-parameter Hill equation by nonlinear least squares regression with GraphPad Prism 6.0.¹⁸ The equation is defined as $y = \text{bottom} + (\text{top} - \text{bottom}) / (1 + 10^{(pCa - pCa_{50}) \cdot \text{HillSlope}})$. Herein, bottom is the *Y* value at the bottom plateau; top is the *y* value at the top plateau; pCa₅₀ is the *x* value when the response is halfway between bottom and top; and Hill slope describes the steepness of the curve and is known as the Hill coefficient. The graphs were generated with GraphPad Prism 6.0. The pCa-ATPase and pCa-force relations were analyzed by 2-way ANOVA with Tukey's post hoc test (GraphPad Prism 6.0) with a level of significance set at *P*<0.05 in the experiments with 2 independent variables (EGCG and mutant). The pCa-ATPase experiments were analyzed by 1-way ANOVA with Tukey's post hoc test (GraphPad Prism 6.0) with a level of significance set at *P*<0.05 in experiments with 1 independent variable (mutant). All values are presented as mean±SEM. Normal Gaussian distributions of the data sets were tested with an D'Agostino-Pearson omnibus test along with Brown-Forsythe test to check constant variances among groups. Percent maximal ATPase activity was normalized to highest rate in the data set, which was set to 100%. Binary comparisons between WT and K206I at each data point in the hcTnI inhibition and hcTnC neutralization experiments were analyzed by a 2-tailed Student *t* test with a level of significance set at *P*<0.05 with Microsoft Excel 2007.

Results

Patient Characteristics and Echocardiography

The index case was a 13-year-old male, diagnosed with HCM after an out-of-hospital cardiac arrest in 2009, when he was jogging during physical education class. He was resuscitated, requiring 2 shocks with an automated external defibrillator, and was implanted with an implantable cardioverter defibrillator (ICD) shortly after the event. Over the 4 following years, he has received at least 12 discrete appropriate, ventricular fibrillation-terminating ICD shocks. The first one occurred about 5 months after placement of the ICD, and the last one was as recent as February of 2013, despite numerous changes in medications. During the most recent episode, he received multiple shocks in the setting of polymorphic ventricular tachycardia and needed 7 ICD discharges to return to normal rhythm. All events occurred in the setting of some level of aerobic activity.

After this last event, he was referred to Mayo Clinic to evaluate the possible role of left cardiac sympathetic denervation as part of his treatment plan. On referral, a follow-up transthoracic echocardiogram was performed in June 2013 showing nonobstructive HCM with severe septal hypertrophy of 25 mm, posterior wall thickness of 18 mm, and LV mass of 443 g shown in Figure 1A and 1B and Table 1. He also had grade 2/4 diastolic dysfunction (pseudonormalized E/A ratio, 1.67) with an enlarged left atrium (left atrium volume index by A-L mL/m² of 45) and E/e' (medial) of 16.7 (Table 1). However, the proband did not have very severe repolarization abnormalities on ECG.

Genetic Analysis and Family History

The index case has a positive family history of HCM (Figure 1C). On diagnosis of the index case, a screening echocardiogram revealed left ventricular hypertrophy in a younger sibling (III.2). In November of 2013, she had a cardiac arrest while running. She received 3 automated external defibrillator shocks to rescue and was implanted with an ICD shortly after the incident, which has shocked her several times. Neither parent demonstrates clinical or echocardiographic phenotype of HCM (II.1 and II.2). The mother also had a cardiac magnetic resonance imaging performed in January 2014, which showed no evidence of HCM. There was no family history of HCM or history of sudden cardiac death in the extended family.

Because of his out-of-hospital cardiac arrest and positive echocardiographic results for HCM, a comprehensive clinical genetic analysis of 18 HCM- and catecholaminergic polymorphic ventricular tachycardia-associated genes was performed before evaluation at Mayo Clinic, and a novel, heterozygous mutation was identified in *TNNI3*-encoded cardiac troponin I. This variant—p.Lys206Ile (also annotated as p.K206I, c.617 A>T)—was classified as likely disease-causing based on the nonconservative nature of the amino acid substitution,

conservation of this residue across species, the presence of a previously published, HCM-associated mutation in the same residue (K206Q),¹⁹ and the absence of this variant in over 6000 controls. Mutation-specific testing of this variant showed that the index case's phenotype-positive sister III.2 and his phenotype-negative mother were both genotype positive for *TNNI3*-K206I (Figure 1C).

Protein Purification and Reconstitution

To determine functional effects of the hcTnI-K206I mutation, recombinant proteins (troponin subunits) were purified and reconstituted into a functional complex with actin, tropomyosin, and myosin-S1. Actin, tropomyosin, and myosin-S1 were natively purified and are shown individually along with the recombinant troponin proteins separated in 15% SDS-PAGE (Figure 2). The protein complexes used in functional assays are also shown in the presence and absence of calcium, where hcTnC mobility is affected because of conformational changes induced in hcTnC by calcium binding (Figure 2). The electrophoretic mobility of hcTnC in SDS-PAGE did not differ when present in myofilaments containing either TnI K206I or WT TnI in the presence or absence of Ca²⁺, suggesting that the genetic variant did not cause major conformational changes in hcTnC (Figure 2).

Actomyosin-S1 ATPase and Therapeutics of Green Tea (EGCG)

ATPase activity assays demonstrated that the mutant K206I and 50% mutant K206I/WT caused a significant increase in the Ca²⁺ sensitivity with cardiac myosin-S1 and skeletal myosin-S1 (Figure 3; Table 2; Table I in the Data Supplement). The max ATPase activity of skeletal myosin-S1 was also significantly increased in the K206I compared with the WT (Table 2; Figure 3B). The K206I mutation had a similar functional impact on both cardiac

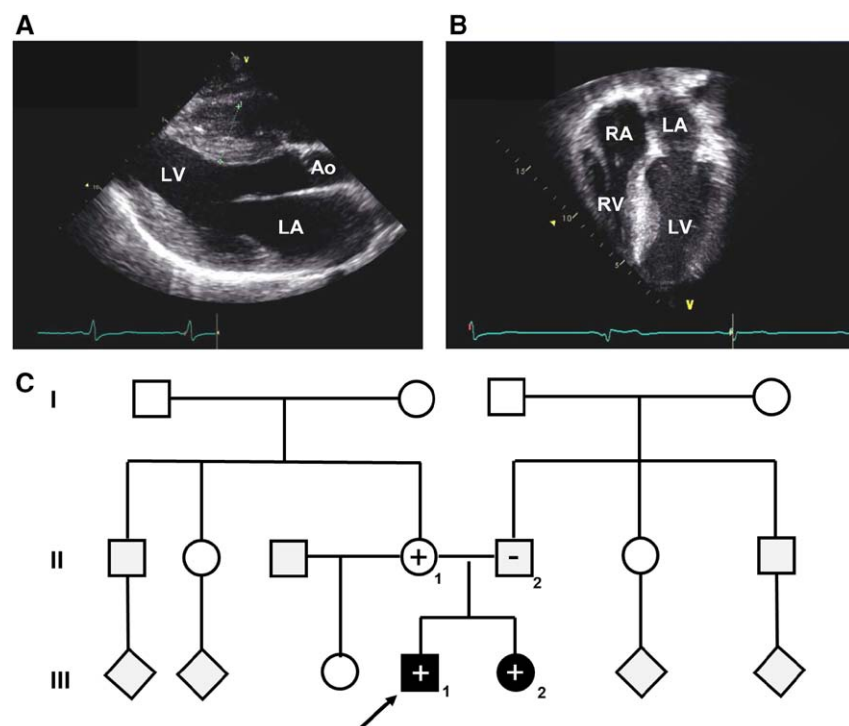


Figure 1. Echocardiogram (A and B) and pedigree (C) of index case with hypertrophic cardiomyopathy (HCM) and the K206I-TNNI3 missense mutation. Echocardiogram of index case showing severe septal hypertrophy on parasternal long axis (A) and 4-chamber views (B). The pedigree (C) shows the affected index case (III.1) and his sister (III.2), both genotype positive for TNNI3-K206I. Both parents are not demonstrating clinical or echocardiographic phenotype of HCM, but genetic analysis demonstrated the mother (II.1) to be TNNI3-K206I positive. There was no family history of HCM or sudden cardiac death in the extended family. Ao indicates aortic outflow tract; LA, left atrium; LV, left ventricle; RA, right atrium; and RV, right ventricle.

Table 1. Echo Parameters of Index Case Mayo Clinic Echocardiogram

Echo Parameters	TNNI3-K206I Patient	Mayo Clinic Reference Value
Ventricular septal thickness, mm	25*	6.7–12.5
Posterior wall thickness, mm	18*	6.6–11.4
LVEDD, mm	47	43–57
EF, %	54*	54.5–71.3
LV mass, g	443*	111–241
E/e', medial	16.7*	<12
E/A ratio	1.67	0.8–3.6
Deceleration time, ms	147	144–220
LA volume index by A-L, mL/m ²	45*	16–28

EF indicates ejection fraction; LA, left atria; LV, left ventricular; and LVEDD, LV end-diastolic dimension.

*Increased/decreased from reference value.

and skeletal myosin S1 (Figure 3A and 3B). To determine the concentration of EGCG that could decrease the Ca²⁺ sensitivity, dose response curves were generated as shown in Figure 4. WT (IC₅₀=15.80±1.0) and K206I (IC₅₀=13.40±0.4) mutant dose-response curves showed no significant differences at pCa 5.8 (Figure 4A), however, there was a significant difference in the 50% K206I/WT (IC₅₀=21.40±1.3) compared with the others. Dose-response curves were also performed at pCa 4.5 (Figure 4B), and the IC₅₀s for all 3 groups were significantly different from each other: WT, 19.40±0.01; K206I/WT, 22.00±0.01; and K206I, 31.70±0.01.

Because of the relatively low rates of ATPase activity in preparations with cardiac myosin-S1, we tested the effect of EGCG on preparations with skeletal-S1. Cardiac and skeletal-S1 demonstrate a similar impact of the K206I and 50% K206I/WT on Ca²⁺ response (Figure 3; Table 2; Table I in the Data Supplement). After treatment with 20 μmol/L EGCG, the K206I and 50% K206I/WT groups showed a similar decrease in Ca²⁺ sensitivity compared with WT (Figure 3B; Table 2). Interestingly, the effect of EGCG on the max ATPase activity of WT and to a lesser extent K206I/WT myofilament was significantly greater than K206I myofilaments in an apparent WT dose-dependent manner. This indicates that the K206I mutant impairs the normal interaction of the EGCG at saturating Ca²⁺ in this basic reconstituted system (Figure 3B).

pCa–Force Relation in Mouse Cardiac Skinned Fibers Exchanged With Human Troponin

Ca²⁺ sensitivity of mouse papillary skinned cardiac fiber bundles that were exchanged with either WT or 65% K206I/WT troponin (K206I/WT) was increased in the 65% K206I/WT group compared with the WT in a similar trend as the ATPase results (Figure 5). To determine the effects of EGCG, dose-response curves were generated at both pCa₅₀ (5.6) and high Ca²⁺ (pCa 4.5; Figure 5A and 5B). EGCG (20 μmol/L) was chosen based on the dose-response curve at pCa 5.6 to restore Ca²⁺ sensitivity to near WT levels. Treatment with 20 μmol/L EGCG reduced the Ca²⁺ sensitivity to near WT levels as with the ATPase activity (Figure 5C). Interestingly, the max force of the WT was not affected as greatly by the EGCG as the max ATPase.

Troponin I Inhibition and Ca²⁺-Dependent hcTnC–hcTnI Interactions on ATPase Activity

In the presence of actin–tropomyosin–myosin-S1, the K206I mutation impairs the ability of the hcTnI to inhibit ATPase activity compared with WT in the absence of Ca²⁺ and at all concentrations tested (Figure 6A). When tropomyosin was removed from the assay conditions, the hcTnI (1 μmol/L) inhibition was still significantly compromised (Figure 6B), suggesting that the inhibitory effect can act through both actin and tropomyosin or actin alone.

Percent maximum ATPase activity was measured at high Ca²⁺ (pCa 4.5) as a function of varying molar ratios of hcTnC:hcTnI to determine Ca²⁺-dependent interactions. Figure 6C summarizes the ability of hcTnC to neutralize the inhibition of hcTnI, which was significantly enhanced with the K206I compared with the WT (all concentrations tested except 2.0 μmol/L). These data indicate an increased interaction between hcTnI (K206I) and hcTnC or increased affinity of Ca²⁺ to hcTnC.

Discussion

This is the first study to report a discovered novel hcTnI K206I missense mutation associated with HCM that induces increased myofilament Ca²⁺ sensitivity, which was restored to WT levels with EGCG. The *TNNI3* missense mutation was discovered in a 13-year-old male after an out-of-hospital cardiac arrest with severely arrhythmogenic HCM that may be evolving toward dilated cardiomyopathy at a relatively young age. After clinical evaluation, both the index case and his phenotype-positive sister were diagnosed with HCM and found to harbor the K206I variant, whereas their mother is genotype (+) but phenotype (–).

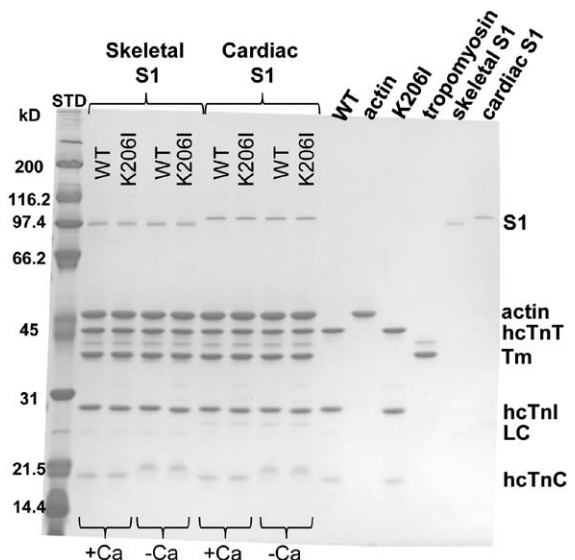


Figure 2. SDS-PAGE (15%) of purified proteins and reconstituted thin filaments with S1 used in the actomyosin-S1 ATPase reactions. +Ca indicates pCa 4.5 (high calcium); –Ca, pCa>9.0 (low calcium); hcTnI, human cardiac troponin I; hcTnC, human cardiac troponin C; hcTnT, human cardiac troponin T; K206I, cardiac troponin I with a point mutation at position K206I; LC, myosin light chain; S1, myosin-S1 fragment; STD, molecular weight standard; Tm, tropomyosin; and WT, wild-type cardiac troponin I.

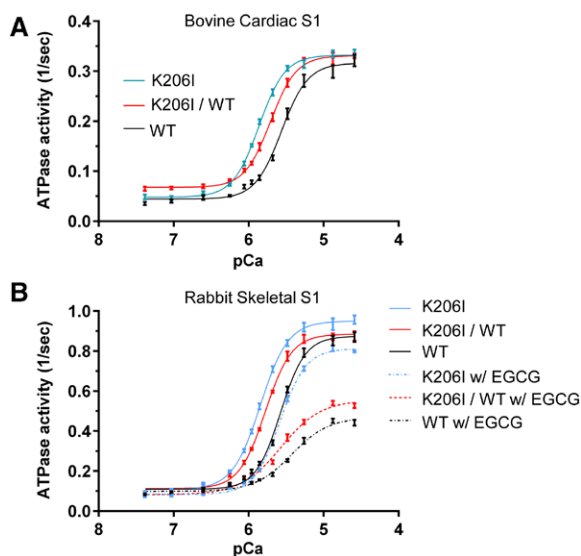


Figure 3. Ca^{2+} -dependent actomyosin-S1-ATPase activity of reconstituted thin filaments with myosin-S1 as a function of pCa. Data points are represented as mean \pm SEM (n=5). Actin and tropomyosin were both from bovine cardiac tissue. All troponin subunits were human cardiac. **A**, Myosin-S1 was from bovine cardiac tissue. **B**, Myosin-S1 was from rabbit skeletal tissue. (-)-epigallocatechin gallate (w/EGCG, 20 $\mu\text{mol/L}$) was added to the reactions. K206I/WT indicates 50% mix of K206I mutant and wild-type troponin I; K206I, 100% mutant cardiac troponin I at position K206I; and WT, wild-type cardiac troponin I.

The known variable expressivity and incomplete penetrance in HCM creates the challenge in clinical diagnosis and decision making in HCM.²⁰ This is further complicated by the fact that the background rate of rare genetic variants in healthy individuals is $\approx 5\%$ in the 9 most common sarcomeric HCM genes.¹³ The genes represent 90% to 95% of genetically identifiable HCM, making clinical interpretation difficult, especially in the minor genes, including *TNNI3*.¹³ Although the current variant K206I-*TNNI3* was classified by the genetic test company as likely disease-causing based on the nonconservative nature of the amino acid substitution, conservation of this residue across species, the presence of an HCM-associated mutation in the same residue,¹⁹ and the absence

of this variant in over 6000 controls, in vitro characterization can be extremely useful to determine pathogenicity of a rare, novel variant. A recently reported case demonstrated variable expressivity in HCM in a family that has a HCM mutation in *MYBPC3* R597Q.¹¹ The ages of phenotype manifestation were hypervariable (16, 42, and 66 years),¹¹ and all were genotype (+) suggesting the possibility that onset of a phenotype in our K206I could be variable and linked to other factors, such as the individuals epigenetics.

Further evidence for K206I-*TNNI3* being an HCM pathogenic mutation is supported by biochemical functional analysis, and another very closely related mutation, K206Q-*TNNI3*, described by Kimura et al¹⁹ in 1997. As in our study with K206I, the increase in Ca^{2+} sensitivity induced by K206Q, determined by in vitro motility assays, was independent of myosin isoform population.²¹ Myofilament ATPase activity assays also showed an increase in Ca^{2+} sensitivity with a K206Q mutant,²² similar to our measurements with the K206I variant (Figures 3 and 4; Table 2), supporting the assertion that K206I is a pathogenic mutation. To further support the pathogenicity of the K206I mutation, we also demonstrated that the mutation caused an increase in Ca^{2+} sensitivity in pCa-force measurements (Figure 5; Table 3). Our findings provide further support for the hypothesis that increased myofibrillar Ca^{2+} sensitivity is one of the final common pathways for sarcomeric HCM.^{3,23}

Mutations that affect normal molecular mechanisms of myofilament activation and relaxation are likely to involve multiple hcTnI domains. The carboxyl-terminus of hcTnI has 3 domains: the inhibitory region containing residues 130 to 150 responsible for inhibiting contraction, the switch region containing residues 151 to 166 responsible for binding to hcTnC in the presence of Ca^{2+} to trigger thin filament activation, and the MD containing residues 167 to 210 responsible for stabilizing the interaction with actin in the absence of Ca^{2+} .^{10,24,25} Our data on the effect of the K206I mutation provides insights into the role of the hcTnI MD in control of cardiac function. Current understanding of the role of the MD has come largely from truncation studies. For example, myofilaments controlled by hcTnI-1 to 193 missing 18 C-terminal amino acids demonstrate reduced tension compared with

Table 2. Summary for the Ca^{2+} -Dependent Actoskeletal-S1 ATPase Assays

	Minimum (1/s)	Maximum (1/s)	pCa50	Hill Slope
K206I	0.11 \pm 0.01	0.95 \pm 0.01*	5.84 \pm 0.01*	2.34 \pm 0.12
K206I / WT	0.11 \pm 0.01	0.88 \pm 0.01†	5.78 \pm 0.01*,†	2.48 \pm 0.12
WT	0.11 \pm 0.01	0.87 \pm 0.01	5.58 \pm 0.01	2.62 \pm 0.16
K206I w/EGCG	0.08 \pm 0.01†,‡	0.81 \pm 0.01†,§	5.58 \pm 0.01†,‡,§	2.48 \pm 0.09§
K206I / WT w/EGCG	0.08 \pm 0.01†,‡	0.56 \pm 0.01*,†,‡,§,	5.57 \pm 0.02†,‡,§	1.63 \pm 0.11*,†,‡,
WT w/EGCG	0.10 \pm 0.01	0.47 \pm 0.01*,†,‡	5.43 \pm 0.03*,†,‡	1.77 \pm 0.16*,†,‡

Data are presented as mean \pm SEM. $P < 0.05$ as indicated by the symbols (*, †, ‡, §, and ||) in the table within each parameter, and n=5. Note the Ca^{2+} sensitivity is restored back to near WT levels when treated with 20 $\mu\text{mol/L}$ EGCG (see also Figure 3B). EGCG indicates (-)-epigallocatechin gallate; and WT, wildtype.

*Significantly different vs WT.

†Significantly different vs K206I.

‡Significantly different vs K206I/WT.

§Significantly different vs WT w/EGCG.

||Significantly different vs K206I w/EGCG.

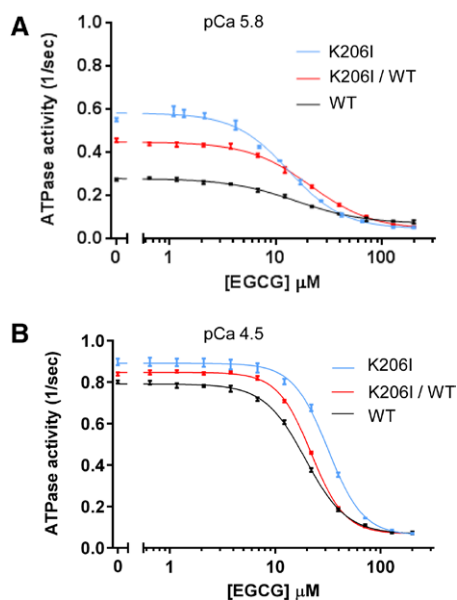


Figure 4. Dose–response curve for (–)-epigallocatechin gallate (EGCG) on actomyosin-S1–ATPase activity. Data points are represented as mean±SEM (n=3–4). **A**, Dose–response curves for pCa 5.8, which is the pCa₅₀ for the mutant. **B**, Dose–response curves for pCa 4.5, which is the max ATPase. K206I indicates cardiac troponin I at position K206I; K206I/WT, 50% mix of K206I mutant and wildtype; and WT, wild-type cardiac troponin I.

controls.^{9,26,27} These data indicate that the MD is required for homeostatic control of tension. Moreover, hcTnI-1 to 193 did not affect Ca²⁺-binding to hcTnC in the ternary troponin complex, but did alter Ca²⁺ binding in the presence of actin–tropomyosin.²⁷ This suggests that long-range interactions to regulatory domains (troponin subunits, actin, and tropomyosin) of the thin filament are under the control of the MD. Thus, it is plausible that these long-range interactions are influenced by the charge state of the MD.

Our data suggest the compromised interactions of K206I with actin and hcTnC may lead to impaired relaxation and HCM. The ability of K206I to inhibit actomyosin ATPase rate was significantly reduced compared with WT with or without tropomyosin present in the reaction. This implies that K206I impaired hcTnI intrinsic inhibitory activity (Figure 6A and 6B). The MD has been reported to have potential interactions with tropomyosin, but we found that the inhibitory effect of K206I was unaffected when tropomyosin was not included in the thin filament reconstitution. Thus, the effects of the inhibitory and switch peptide seem to dominate the influence of the mutation on response to Ca²⁺. In skinned cardiac myofibrils regulated by K206Q hcTnI, there was no change in the inhibition of hcTnI from the WT albeit with different experimental conditions.²² The 2 mutations (K206Q and K206I) differ from each other by the polar hydrophilic nature of glutamine versus the hydrophobic nature of isoleucine of the substituted amino acids. In terms of inhibitory function, the 206 codon of hcTnI seems functionally dynamic, suggesting different mechanisms resulting from a specific mutation. In addition to the K206I's compromised inhibitory activity, we also found that neutralization of the inhibitory effect of hcTnI by hcTnC is enhanced in the presence of Ca²⁺ (Figure 6C). The increased neutralization

of the inhibitory hcTnI by hcTnC suggests an enhanced interaction with hcTnC. The possibility also exists that the Ca²⁺-binding affinity to hcTnC is affected by the K206I mutation. In contrast, experiments with the similar K206Q-mutation noted no significant differences in Ca²⁺ binding.²²

EGCG modulates myofilament force and ATPase rate most likely by interacting with hcTnC. EGCG binds to the carboxyl terminus of hcTnC (ChcTnC) leading to decreased Ca²⁺ sensitivity.^{14,28,29} EGCG binding is specific for ChcTnC,²⁸ and it has been proposed to affect the interaction of hcTnC with hcTnI, thus affecting sensitivity of the thin filament for Ca²⁺.^{15,29–31} In the K206I and K206I/WT variants, Ca²⁺ sensitivity was restored close to WT levels when myofilaments were treated with 20 μmol/L EGCG. EGCG treatment had a much greater effect on actomyosin (maximum 1/s and Hill Slope) preparations regulated by the WT and 50% K206I/WT versus the K206I. The greater inhibitory effect of EGCG on actomyosin

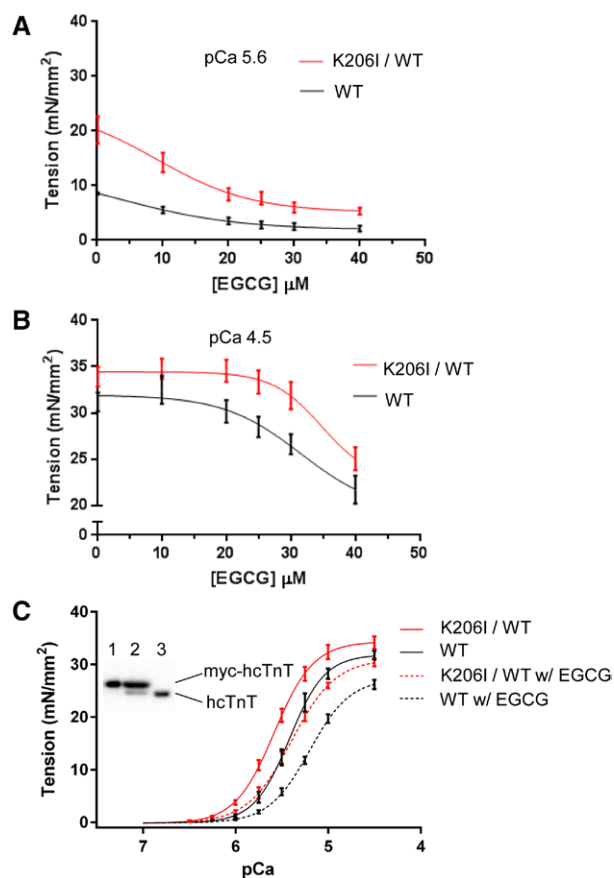


Figure 5. pCa–force relation in mouse cardiac skinned fibers exchanged with 100% WT or mixed 65% mutant K206I/35% WT. Mean±SEM, n=5 to 7 fibers. **A**, Dose–response curves for pCa 5.6, which is the pCa₅₀ for the mutant. **B**, Dose–response curves at pCa 4.5, which is the maximum tension. **C**, pCa–force relation. (–)-epigallocatechin gallate (w/EGCG, 20 μmol/L) was added to the reactions. Inset is a representative Western blot for human cardiac troponin T (hcTnT) to determine amount of troponin that was exchanged. K206I/WT indicates 65% mix of K206I mutant and wildtype; and WT, wild-type cardiac troponin I. hcTnT, endogenous troponin contained in a fiber; myc-hcTnT, exogenous troponin. Lane 1, exogenous purified troponin used for exchange; lane 2, exchanged fiber bundle; and lane 3, non-exchanged mouse heart (negative control). Troponin exchange efficiency was 86±1%, n=8.

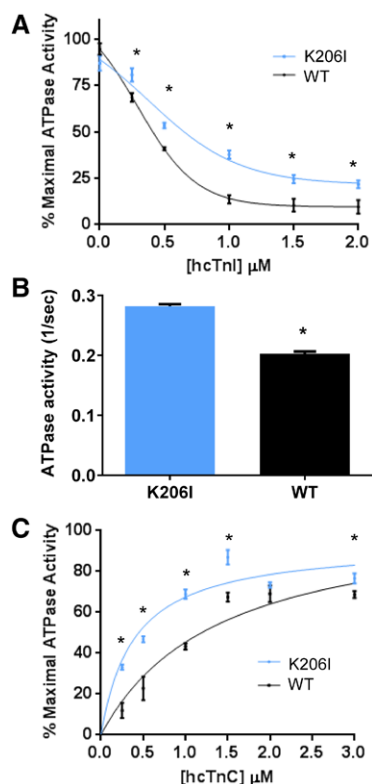


Figure 6. Human cardiac troponin I (hcTnI) regulatory activity in the presence of a point mutation K206I. Mean \pm SEM, n=4 to 8. * P <0.05 wildtype (WT) vs K206I. **A**, hcTnI inhibition of ATPase activity at low calcium (pCa>9.0). The assay contained 0.2 μ mol/L skeletal-S1, 7 μ mol/L actin, 1 μ mol/L tropomyosin (TM), and varying amounts of hcTnI. **B**, Same conditions as above except there was no TM present, and the hcTnI concentration was 1 μ mol/L. **C**, Neutralization of the inhibitory effect of hcTnI by increasing human cardiac troponin C (hcTnC) in the presence of high calcium (pCa 4.5) on ATPase activity. The assay contained 0.2 μ mol/L skeletal S1, 7 μ mol/L actin, 1 μ mol/L tropomyosin, 1 μ mol/L hcTnI, and varying amounts of hcTnC.

ATPase rate regulated by WT versus K206I is expected in view of the promotion of force and ATPase rate in the presence of K206I. That is, with WT, the effect of EGCG was unopposed, whereas with K206I, the inhibitory effect was partially offset by the increase in ATPase rate associated with K206I. In contrast to the results with ATPase measurements, in the case of the force measurements, differences in effects of EGCG on tension developed by fibers regulated by WT versus K206I were not as great as in the case of the ATPase measurements (Figures 3 and 5; Tables 2 and 3). We speculate that the effects of the charge change in the MD of hcTnI may also be greater in the ATPase activity versus force measurements because of the unloaded versus loaded systems and the absence of other regulating proteins particularly the Z-disk proteins.

The EGCG experiments indirectly support the premise of increased interaction between K206I and hcTnC, as EGCG binds to the carboxyl terminus of hcTnC near sites III and IV but not directly affecting Ca²⁺ binding to site II. This evidence also indicates that there are likely little differences in Ca²⁺ binding because of the mutation. The EGCG concentration used was based on our dose-response curves (Figures 4 and 5A and 5B), which predicted restoration of K206I and K206I/

WT (in both ATPase and force measurements) Ca²⁺ sensitivity at 20 μ mol/L. However, previously published reports indicated no effects <25 μ mol/L EGCG in skinned cardiac myofibers.^{15,28,31} In our ATPase experiments, we used reconstituted thin filaments from individually purified proteins and added myosin-S1 in vitro to carry out the ATPase assay. So it is likely that in our more simple system, the ChcTnC-binding partner for EGCG is more accessible allowing for a lower effective dose. In our pCa-force measurements, we saw significant effects of EGCG at 20 μ mol/L, and Papadaki et al³² found 10 μ mol/L EGCG to be effective in isolated myofibrils, suggesting that experimental design may contribute to variable efficacy in EGCG.

Restoration of myofilament Ca²⁺ sensitivity to near WT levels has been suggested as an approach to treatment for HCM. An HCM mouse model expressing a mutation in one of the other minor sarcomeric genes—Glu180Gly in *TPMI* encoded α -tropomyosin—demonstrated increased myofilament Ca²⁺ sensitivity, and rescue of the phenotype occurred when crossed with a chimeric α - β -tropomyosin transgenic mouse with a decreased myofilament Ca²⁺ sensitivity.³³ A transgenic mouse model mimicking a human restrictive cardiomyopathy (cTnI^{Arg193His}) with increased Ca²⁺ sensitivity was also rescued by crossing with a mouse transgene (N-terminus of cTnI truncated) thereby decreasing the Ca²⁺ sensitivity.³⁴ Recently, Alves et al³⁵ proposed that reduction in myofilament sensitivity to Ca²⁺ corrects abnormal relaxation and can delay or prevent HCM. Genetic rescue of a disease phenotype does not elucidate whether intervention during disease progression would be beneficial. It has been suggested that treatment with EGCG may be able to restore Ca²⁺ sensitivity during disease progression.³²

Although green tea may be a therapeutic treatment by affecting Ca²⁺ sensitivity, it has been linked to adverse events.³⁶ In addition, our study indicate 20 μ mol/L EGCG as an effective concentration and has shown cardioprotection by enhancing shortening,³⁷ but would likely require dietary supplements. A higher concentration of EGCG (50 μ mol/L) has also been demonstrated to afford cardioprotection, but increases in incidences of arrhythmia and diastolic dysfunction have been reported.³⁷ The Ohsaki and Japanese Public Health Center studies showed an association with regular green tea consumption and reduced mortality because of cardiovascular disease versus diets with less green tea.^{38,39}

Table 3. Mechanical Characteristics of Tn-Exchanged Skinned Fibers

	Maximum, mN/mm ²	pCa50	Hill Slope
K206I/WT	34.35 \pm 0.83	5.58 \pm 0.02*	2.04 \pm 0.19
WT	31.93 \pm 0.94	5.42 \pm 0.02	2.25 \pm 0.23
K206I/WT w/EGCG	31.03 \pm 0.79†,‡	5.40 \pm 0.02†,‡	1.82 \pm 0.14*
WT w/EGCG	27.34 \pm 0.72*,†	5.20 \pm 0.02*,†	1.97 \pm 0.15

Data are presented as mean \pm SEM. P <0.05 as indicated by the symbols (*, †, and ‡) in the table within each parameter, and n=6–7. Note the Ca²⁺ sensitivity is restored back to near WT levels when treated with 20 μ mol/L EGCG (see also Figure 5C). EGCG indicates (-)-epigallocatechin gallate; and WT, wildtype.

*Significantly different vs WT.

†Significantly different vs K206I/WT.

‡Significantly different vs WT w/EGCG.

In summary, this is the first report to describe this newly found hcTnI K206I mutation associated with severely arrhythmogenic HCM. These studies provide evidence in support of K206I-*TNNI3* as an HCM pathogenic missense mutation. We have provided direct evidence for the first time that hcTnI K206I induces increased Ca²⁺ sensitivity in a reconstituted thin filament system with restoration of Ca²⁺ sensitivity to WT levels with EGCG treatment. The impaired relaxation caused by K206I may be partly explained by induction of a weakened interaction with actin and intrinsic inhibitory activity while increasing the interaction of hcTnI with hcTnC leading to increased Ca²⁺ sensitivity.

Sources of Funding

This work was supported by National Institutes of Health (NIH) PO1 HL 062426 (project 1, 4, core B, core C; C.M. Warren, Dr Solaro, Dr de Tombe, Dr Kobayashi, and Dr Wolska), NIH RO1 HL22231 (Dr Solaro), and the Mayo Clinic Windland Smith Rice Comprehensive Sudden Cardiac Death Program (Dr Ackerman).

Disclosures

Dr Ackerman is a consultant for Boston Scientific, Gilead Sciences, Medtronic, and St. Jude Medical and receives royalties from Transgenomics for FAMILION-LQTS and FAMILION-CPVT. None of these entities provided financial support for this study. The other authors report no conflicts.

References

- Kobayashi T, Solaro RJ. Calcium, thin filaments, and the integrative biology of cardiac contractility. *Annu Rev Physiol*. 2005;67:39–67. doi: 10.1146/annurev.physiol.67.040403.114025.
- Lu QW, Wu XY, Morimoto S. Inherited cardiomyopathies caused by troponin mutations. *J Geriatr Cardiol*. 2013;10:91–101. doi: 10.3969/j.issn.1671-5411.2013.01.014.
- Willott RH, Gomes AV, Chang AN, Parvatiyar MS, Pinto JR, Potter JD. Mutations in troponin that cause HCM, DCM AND RCM: what can we learn about thin filament function? *J Mol Cell Cardiol*. 2010;48:882–892. doi: 10.1016/j.yjmcc.2009.10.031.
- Dong WJ, Xing J, Villain M, Hellinger M, Robinson JM, Chandra M, et al. Conformation of the regulatory domain of cardiac muscle troponin C in its complex with cardiac troponin I. *J Biol Chem*. 1999;274:31382–31390.
- Li MX, Spyropoulos L, Sykes BD. Binding of cardiac troponin-I147-163 induces a structural opening in human cardiac troponin-C. *Biochemistry*. 1999;38:8289–8298. doi: 10.1021/bi9901679.
- Takeda S, Yamashita A, Maeda K, Maeda Y. Structure of the core domain of human cardiac troponin in the Ca(2+)-saturated form. *Nature*. 2003;424:35–41. doi: 10.1038/nature01780.
- Akhter S, Bueltmann K Jr, Huang X, Jin JP. Restrictive cardiomyopathy mutations demonstrate functions of the C-terminal end-segment of troponin I. *Arch Biochem Biophys*. 2014;552:553–10. doi: 10.1016/j.abb.2013.12.001.
- Galińska A, Hatch V, Craig R, Murphy AM, Van Eyk JE, Wang CL, et al. The C terminus of cardiac troponin I stabilizes the Ca²⁺-activated state of tropomyosin on actin filaments. *Circ Res*. 2010;106:705–711. doi: 10.1161/CIRCRESAHA.109.210047.
- Rarick HM, Tu XH, Solaro RJ, Martin AF. The C terminus of cardiac troponin I is essential for full inhibitory activity and Ca²⁺ sensitivity of rat myofibrils. *J Biol Chem*. 1997;272:26887–26892.
- Zhou Z, Li KL, Rieck D, Ouyang Y, Chandra M, Dong WJ. Structural dynamics of C-domain of cardiac troponin I protein in reconstituted thin filament. *J Biol Chem*. 2012;287:7661–7674. doi: 10.1074/jbc.M111.281600.
- Chiou KR, Chu CT, Chang MJ. Detection of mutations in symptomatic patients with hypertrophic cardiomyopathy in Taiwan. *J Cardiol*. 2015;65:250–256. doi: 10.1016/j.jjcc.2014.05.010.
- Bos JM, Will ML, Gersh BJ, Krusselbrink TM, Ommen SR, Ackerman MJ. Characterization of a phenotype-based genetic test prediction score for unrelated patients with hypertrophic cardiomyopathy. *Mayo Clin Proc*. 2014;89:727–737. doi: 10.1016/j.mayocp.2014.01.025.
- Kapplinger JD, Landstrom AP, Bos JM, Salisbury BA, Callis TE, Ackerman MJ. Distinguishing hypertrophic cardiomyopathy-associated mutations from background genetic noise. *J Cardiovasc Transl Res*. 2014;7:347–361. doi: 10.1007/s12265-014-9542-z.
- Tadano N, Yumoto F, Tanokura M, Ohtsuki I, Morimoto S. EGCG, a major polyphenol in green tea, binds to the C-lobe of cardiac troponin and desensitizes cardiac muscle contraction to Ca²⁺. *Biophys J*. 2005;88:314. Abstract.
- Tadano N, Du CK, Yumoto F, Morimoto S, Ohta M, Xie MF, et al. Biological actions of green tea catechins on cardiac troponin C. *Br J Pharmacol*. 2010;161:1034–1043. doi: 10.1111/j.1476-5381.2010.00942.x.
- Kobayashi T, Solaro RJ. Increased Ca²⁺ affinity of cardiac thin filaments reconstituted with cardiomyopathy-related mutant cardiac troponin I. *J Biol Chem*. 2006;281:13471–13477. doi: 10.1074/jbc.M509561200.
- Karam CN, Warren CM, Rajan S, de Tombe PP, Wiecek DF, Solaro RJ. Expression of tropomyosin-k induces dilated cardiomyopathy and depresses cardiac myofibril tension by mechanisms involving cross-bridge dependent activation and altered tropomyosin phosphorylation. *J Muscle Res Cell Motil*. 2011;31:315–322. doi: 10.1007/s10974-010-9237-2.
- Solaro RJ, Gambassi G, Warsaw DM, Keller MR, Spurgeon HA, Beier N, et al. Stereoselective actions of thiazidiazones on canine cardiac myocytes and myofilaments. *Circ Res*. 1993;73:981–990.
- Kimura A, Harada H, Park JE, Nishi H, Satoh M, Takahashi M, et al. Mutations in the cardiac troponin I gene associated with hypertrophic cardiomyopathy. *Nat Genet*. 1997;16:379–382. doi: 10.1038/ng0897-379.
- Maron BJ, Yeates L, Semsarian C. Clinical challenges of genotype positive (+)-phenotype negative (-) family members in hypertrophic cardiomyopathy. *Am J Cardiol*. 2011;107:604–608. doi: 10.1016/j.amjcard.2010.10.022.
- Köhler J, Chen Y, Brenner B, Gordon AM, Kraft T, Martyn DA, et al. Familial hypertrophic cardiomyopathy mutations in troponin I (K183D, G203S, K206Q) enhance filament sliding. *Physiol Genomics*. 2003;14:117–128. doi: 10.1152/physiolgenomics.00101.2002.
- Takahashi-Yanaga F, Morimoto S, Harada K, Minakami R, Shiraishi F, Ohta M, et al. Functional consequences of the mutations in human cardiac troponin I gene found in familial hypertrophic cardiomyopathy. *J Mol Cell Cardiol*. 2001;33:2095–2107. doi: 10.1006/jmcc.2001.1473.
- Ahmad F, Seidman JG, Seidman CE. The genetic basis for cardiac remodeling. *Annu Rev Genomics Hum Genet*. 2005;6:185–216. doi: 10.1146/annurev.genom.6.080604.162132.
- Murakami K, Yumoto F, Ohki SY, Yasunaga T, Tanokura M, Wakabayashi T. Structural basis for Ca²⁺-regulated muscle relaxation at interaction sites of troponin with actin and tropomyosin. *J Mol Biol*. 2005;352:178–201. doi: 10.1016/j.jmb.2005.06.067.
- Triplet B, Van Eyk JE, Hodges RS. Mapping of a second actin-tropomyosin and a second troponin C binding site within the C terminus of troponin I, and their importance in the Ca²⁺-dependent regulation of muscle contraction. *J Mol Biol*. 1997;271:728–750. doi: 10.1006/jmbi.1997.1200.
- Foster DB, Noguchi T, VanBuren P, Murphy AM, Van Eyk JE. C-terminal truncation of cardiac troponin I causes divergent effects on ATPase and force: implications for the pathophysiology of myocardial stunning. *Circ Res*. 2003;93:917–924. doi: 10.1161/01.RES.0000099889.35340.6F.
- Tachampa K, Kobayashi T, Wang H, Martin AF, Biesiadecki BJ, Solaro RJ, et al. Increased cross-bridge cycling kinetics after exchange of C-terminal truncated troponin I in skinned rat cardiac muscle. *J Biol Chem*. 2008;283:15114–15121. doi: 10.1074/jbc.M801636200.
- Liou YM, Kuo SC, Hsieh SR. Differential effects of a green tea-derived polyphenol (-)-epigallocatechin-3-gallate on the acidosis-induced decrease in the Ca(2+) sensitivity of cardiac and skeletal muscle. *Pflugers Arch*. 2008;456:787–800. doi: 10.1007/s00424-008-0456-y.
- Robertson IM, Li MX, Sykes BD. Solution structure of human cardiac troponin C in complex with the green tea polyphenol, (-)-epigallocatechin 3-gallate. *J Biol Chem*. 2009;284:23012–23023. doi: 10.1074/jbc.M109.021352.
- Botten D, Fugallo G, Fraternali F, Molteni C. A computational exploration of the interactions of the green tea polyphenol (-)-Epigallocatechin 3-Gallate with cardiac muscle troponin C. *PLoS One*. 2013;8:e70556. doi: 10.1371/journal.pone.0070556.
- Fuchs F, Grabarek Z. The green tea polyphenol (-)-epigallocatechin-3-gallate inhibits magnesium binding to the C-domain of cardiac troponin C. *J Muscle Res Cell Motil*. 2013;34:107–113. doi: 10.1007/s10974-013-9338-9.
- Papadaki M, Vikhorev PG, Marston SB, Messer AE. Uncoupling of myofilament Ca²⁺ sensitivity from troponin I phosphorylation by mutations can be reversed by epigallocatechin-3-gallate. *Cardiovasc Res*. 2015;108:99–110. doi: 10.1093/cvr/cvv181.
- Jagatheesan G, Rajan S, Petrashevskaya N, Schwartz A, Boivin G, Arteaga GM, et al. Rescue of tropomyosin-induced familial hypertrophic

- cardiomyopathy mice by transgenesis. *Am J Physiol Heart Circ Physiol*. 2007;293:H949–H958. doi: 10.1152/ajpheart.01341.2006.
34. Li Y, Charles PY, Nan C, Pinto JR, Wang Y, Liang J, et al. Correcting diastolic dysfunction by Ca²⁺ desensitizing troponin in a transgenic mouse model of restrictive cardiomyopathy. *J Mol Cell Cardiol*. 2010;49:402–411. doi: 10.1016/j.yjmcc.2010.04.017.
 35. Alves ML, Dias FA, Gaffin RD, Simon JN, Montminy EM, Biesiadecki BJ, et al. Desensitization of myofilaments to Ca²⁺ as a therapeutic target for hypertrophic cardiomyopathy with mutations in thin filament proteins. *Circ Cardiovasc Genet*. 2014;7:132–143. doi: 10.1161/CIRCGENETICS.113.000324.
 36. Sarma DN, Barrett ML, Chavez ML, Gardiner P, Ko R, Mahady GB, et al. Safety of green tea extracts: a systematic review by the US Pharmacopeia. *Drug Saf*. 2008;31:469–484.
 37. Bao L, Lu F, Chen H, Min Q, Chen X, Song Y, et al. High concentration of epigallocatechin-3-gallate increased the incidences of arrhythmia and diastolic dysfunction via β_2 -adrenoceptor. *J Food Sci*. 2015;80:T659–T663. doi: 10.1111/1750-3841.12803.
 38. Kuriyama S, Shimazu T, Ohmori K, Kikuchi N, Nakaya N, Nishino Y, et al. Green tea consumption and mortality due to cardiovascular disease, cancer, and all causes in Japan: the Ohsaki study. *JAMA*. 2006;296:1255–1265. doi: 10.1001/jama.296.10.1255.
 39. Saito E, Inoue M, Sawada N, Shimazu T, Yamaji T, Iwasaki M, et al.; JPHC Study Group. Association of green tea consumption with mortality due to all causes and major causes of death in a Japanese population: the Japan Public Health Center-based Prospective Study (JPHC Study). *Ann Epidemiol*. 2015;25:512–518.e3. doi: 10.1016/j.annepidem.2015.03.007.

CLINICAL PERSPECTIVE

Since the first hypertrophic cardiomyopathy (HCM) mutation in *TNNI3*-encoded cardiac troponin I (*TNNI3*) was discovered about 18 years ago, there have been ≈ 30 more HCM variants discovered as well as 4 variants associated with dilated cardiomyopathy. We recently identified a family carrying a novel heterozygous mutation in *TNNI3* identified as p.Lys206Ile (also p.K206I, c. 617 A>T) in a 13-year-old male and his sister. In our study, we successfully demonstrated the variant's association to HCM by showing a known pathogenesis, namely that sarcomeres in hearts stressed by familial HCM, is sensitized to Ca²⁺. However, sarcomeres with mutations linked to dilated cardiomyopathy are generally desensitized to Ca²⁺. This indicates the possibility of a personalized medicine approach to treat HCM versus dilated cardiomyopathy. Herein, we successfully desensitized the sarcomeres to Ca²⁺ by treating them with a green tea catechin (EGCG). With this treatment, sarcomere response to Ca²⁺ was returned to near wild-type levels. Our data thus illustrate the possibility of precision in medical intervention in the larger context of cardiomyopathy. More studies are required to determine whether a new generation of Ca²⁺ desensitizers based on the green tea catechin EGCG may be developed with even more potency and specificity.

Green Tea Catechin Normalizes the Enhanced Ca²⁺ Sensitivity of Myofilaments Regulated by a Hypertrophic Cardiomyopathy–Associated Mutation in Human Cardiac Troponin I (K206I)

Chad M. Warren, Chehade N. Karam, Beata M. Wolska, Tomoyoshi Kobayashi, Pieter P. de Tombe, Grace M. Arteaga, J. Martijn Bos, Michael J. Ackerman and R. John Solaro

Circ Cardiovasc Genet. 2015;8:765-773; originally published online November 9, 2015;

doi: 10.1161/CIRCGENETICS.115.001234

Circulation: Cardiovascular Genetics is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

Copyright © 2015 American Heart Association, Inc. All rights reserved.

Print ISSN: 1942-325X. Online ISSN: 1942-3268

The online version of this article, along with updated information and services, is located on the World Wide Web at:

<http://circgenetics.ahajournals.org/content/8/6/765>

Data Supplement (unedited) at:

<http://circgenetics.ahajournals.org/content/suppl/2015/11/09/CIRCGENETICS.115.001234.DC1>

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in *Circulation: Cardiovascular Genetics* can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the [Permissions and Rights Question and Answer](#) document.

Reprints: Information about reprints can be found online at:
<http://www.lww.com/reprints>

Subscriptions: Information about subscribing to *Circulation: Cardiovascular Genetics* is online at:
<http://circgenetics.ahajournals.org/subscriptions/>

SUPPLEMENTAL MATERIAL

Methods

Protein Purification, Reconstitution and Western blotting

The mutation at codon 206 of human cardiac troponin I (hcTnI) was changed from AAA (Lys) to ATC (Ile) with Agilent's QuikChange lightning kit following manufacturer's recommendations. The mutant K206I hcTnI and wildtype (WT) hcTnI were both over-expressed with vector pET17b in BL21-DE3 cells from Novagen and purified as described previously.¹ The human cardiac troponin T (hcTnT), myc-tagged hcTnT, and human cardiac troponin C (hcTnC) were expressed with vectors pSBET (hcTnT) and pET3d (hcTnC) in BL21-DE3 cells from Novagen and purified as previously described.^{2,3} Troponin subunits were reconstituted into a complex as previously described.^{2,4} Actin and cardiac myosin-S1 subfragment were all purified from bovine cardiac tissue, and the skeletal myosin-S1 subfragment was purified from rabbit skeletal tissue as previously described.^{5,6} Tropomyosin was purified from bovine cardiac tissue as previously described⁴ with an additional final chromatographic step using Q-Sepharose FF in a 1.6XK column with AKTA-Purifier system from GE Healthcare. Protein purity and relative qualitative stoichiometry was determined by 15% SDS-PAGE and stained with Coomassie R-250 as previously described.⁷

To determine the exchange efficiency of the exogenous troponin complex a 12%T, 3.4%C SDS-PAGE followed by Western blot analysis was performed as previously described except the SDS-PAGE was performed in a SE600 large gel format box.⁷ The exogenous troponin complex was reconstituted with a myc-tagged hcTnT

(molecular weight difference of about 1200Da) in order to separate from the endogenous from the exogenous troponin. The primary monoclonal antibody was CT3 (developmental Studies Hybridoma Bank, Iowa City, IA) with 1:500 dilution, and the secondary antibody was anti-mouse IgG Fab specific peroxidase conjugate with 1:80,000 dilution (Sigma Chemical Co.). The blots were developed using an enhanced ECL kit from Thermo (Supersignal West Femto) and imaged with a Chemidoc (BioRad). The densitometric analysis of the bands was performed with Image Lab v5.0 software (BioRad).

Acto-Myosin-S1 ATPase Assay

The micro-ATPase assay was performed as previously described with some minor modifications.² For all experiments, the actin and tropomyosin were procured from bovine cardiac tissue. Briefly, the reaction conditions with cardiac myosin-S1 (0.2 μ M) were: actin (7 μ M), tropomyosin (1.5 μ M), and troponin complex (1.5 μ M); either WT or mutant form of troponin I K206I was used to make the troponin complex and for the K206I/WT group mixed 1:1 to yield 50%. The reaction with cardiac S1 was carried out at 30°C. The reaction conditions were similar for skeletal myosin-S1, except the actin concentration was 6 μ M and the temperature was 25°C due to big differences in the ATPase rates between fast-rabbit-skeletal myosin-S1 and slow-bovine-cardiac myosin-S1. Cardiac myosin-S1 has much slower rates compared to skeletal myosin-S1, and thus more actin and a higher temperature were required to get reliable rates. In all experiments the buffer was: 35mM NaCl, 5mM MgCl, and 20mM MOPS pH 7.0. The free Ca²⁺ concentration was calculated using WebmaxC standard.⁸ ATPase activity

was determined from a time course of inorganic phosphate release every 3 min up to 18 min total after starting the reaction with 1mM ATP. The inorganic phosphate levels were determined by malachite green.²

Under the same conditions as described above, the effects of (-)-epigallocatechin gallate (EGCG) (Sigma-Aldrich St. Louis, MO), a green tea catechin, were tested to determine if dysfunction could be restored. The EGCG was dissolved in water, added to the reaction, and incubated for 10 min. The conditions were the same when determining hcTnI inhibition as above except only skeletal myosin-S1 (0.2 μ M) was used along with 7 μ M actin and 1 μ M tropomyosin at 25°C. The hcTnI concentration was varied from 0.0 μ M to 2.0 μ M to test inhibition ability of the mutant K206I vs WT. We also tested hcTnI inhibition in the absence of tropomyosin and at 1 μ M of hcTnI to determine if actin alone could inhibit. In experiments where hcTnC concentration was varied to determine neutralization of inhibition by hcTnI (1 μ M), the conditions were the same where tropomyosin was present and the hcTnC concentration was varied from 0.0 μ M to 3.0 μ M. Due to the low inherent rates of cardiac myosin-S1 only the skeletal myosin-S1 was used for the hcTnI inhibition, hcTnC competition and EGCG experiments. The skeletal myosin-S1 yielded much higher rates with a larger dynamic range.

Measurements of Ca^{2+} -dependent activation of force in troponin-exchanged skinned fiber bundles

Fiber bundles dissected from the papillary muscles of FVBN mice (3-4months), were detergent-extracted (skinned) in high relaxing (HR) buffer (10 mM EGTA, 41.89 mM K-Prop, 100 mM BES, 6.75 mM MgCl₂, 6.22 mM Na₂ATP, 10 mM Na₂CrP, 5 mM NaAzide, pH 7) containing 1% Triton X-100 at 4⁰C for 2 hours. Troponin exchange was carried out at 4⁰C by overnight incubation of the fibers with either 100% WT (23μM) or 65% K206I (23 μM) mixed with 35% WT (65% K206I/WT) troponin in exchange buffer (200mM KCl, 5mM MgCl₂, 5mM EGTA, 1mM DTT, 20mM MOPS, 1:100 (v/v) protease inhibitor cocktail [Sigma Chemical Co. St Louis, Mo], pH 6.5).³ Measurement of steady-state isometric tension was conducted as described previously.⁹ Briefly, fibers were mounted between a force transducer and a micromanipulator, and the sarcomere length (SL) was adjusted at 2.2μm using laser diffraction patterns. The exchanged fibers were then subjected to a range of pCa solutions (8-4.5), with or without incubation with 20 μM EGCG. The EGCG was prepared in water and was able to be washed out from the fiber; hence any rundown, which was less than 10% was not due to the EGCG. For the dose-response experiments, exchanged fibers (SL=2.2μm) were activated at either pCa5.6 (pCa₅₀) or pCa4.5 in the presence of different concentrations of EGCG. The following protease inhibitors were added to HR and pCa solutions: pepstatin A (2.5 μg/ml), leupeptin (1 μg/ml), and phenylmethylsulfonyl fluoride (PMSF) (50 μmol/L). All experiments were carried out at 23⁰C.

REFERENCES

1. Kobayashi T, Yang X, Walker LA, Van Breemen RB, Solaro RJ. A non-equilibrium isoelectric focusing method to determine states of phosphorylation of cardiac troponin I: identification of Ser-23 and Ser-24 as significant sites of phosphorylation by protein kinase C. *J Mol Cell Cardiol.* 2005;38:213-218.
2. Kobayashi T, Solaro RJ. Increased Ca²⁺ affinity of cardiac thin filaments reconstituted with cardiomyopathy-related mutant cardiac troponin I. *J Biol Chem.* 2006;281:13471-13477.
3. Sumandea MP, Pyle WG, Kobayashi T, de Tombe PP, Solaro RJ. Identification of a functionally critical protein kinase C phosphorylation residue of cardiac troponin T. *J Biol Chem.* 2003;278:35135-35144.
4. Greaser ML, Gergely J. Reconstitution of troponin activity from three protein components. *J Biol Chem.* 1971;246:4226-4233.
5. Pardee JD, Spudich JA. Purification of muscle actin. *Methods Enzymol.* 1982;85 Pt B:164-181.
6. Swartz DR, Moss RL. Influence of a strong-binding myosin analogue on calcium-sensitive mechanical properties of skinned skeletal muscle fibers. *J Biol Chem.* 1992;267:20497-20506.

7. Layland J, Cave AC, Warren C, Grieve DJ, Sparks E, Kentish JC, et al. Protection against endotoxemia-induced contractile dysfunction in mice with cardiac-specific expression of slow skeletal troponin I. *FASEB J.* 2005;19:1137-1139.
8. Bers DM, Patton CW, Nuccitelli R. A practical guide to the preparation of Ca(2+) buffers. *Methods Cell Biol.* 2010;99:1-26.
9. Karam CN, Warren CM, Rajan S, de Tombe PP, Wieczorek DF, Solaro RJ. Expression of tropomyosin-kappa induces dilated cardiomyopathy and depresses cardiac myofilament tension by mechanisms involving cross-bridge dependent activation and altered tropomyosin phosphorylation. *J Muscle Res Cell Motil.* 2011;31:315-322.

Table 1S. Summary for the Ca²⁺-dependent cardiac acto-myosin-S1 ATPase assays.

	Min (1/sec)	Max (1/sec)	pCa50	Hill Slope
K206l	0.05 ± 0.01	0.34 ± 0.01	5.86 ± 0.01*	2.50 ± 0.13
K206l /WT	0.07 ± 0.01*,†	0.33 ± 0.01	5.71 ± 0.01*	2.50 ± 0.17
WT	0.04 ± 0.01	0.32 ± 0.01	5.56 ± 0.02	2.45 ± 0.21

Data are presented as mean ± SEM., p-value < 0.05 as indicated by the symbols (* and †) in the table within each parameter, and n=5. See Figure 3A.

*significantly different vs WT

† significantly different vs K206l