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Original article

Molecular and functional characterization of novel hypertrophic cardiomyopathy susceptibility mutations in *TNNC1*-encoded troponin C ☆

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Abstract

Hypertrophic Cardiomyopathy (HCM) is a common primary cardiac disorder defined by a hypertrophied left ventricle, is one of the main causes of sudden death in young athletes, and has been associated with mutations in most sarcomeric proteins (tropomyosin, troponin T and I, and actin, etc.). Many of these mutations appear to affect the functional properties of cardiac troponin C (cTnC), i.e., by increasing the Ca^{2+}-sensitivity of contraction, a hallmark of HCM, yet surprisingly, prior to this report, cTnC had not been classified as a HCM-susceptibility gene. In this study, we show that mutations occurring in the human cTnC (HcTnC) gene (*TNNC1*) have the same prevalence (~0.4%) as well established HCM-susceptibility genes that encode other sarcomeric proteins. Comprehensive open reading frame/splice site mutation analysis of *TNNC1* performed on 1025 unrelated HCM patients enrolled over the last 10 years revealed novel missense mutations in *TNNC1:* A8V, C84Y, E134D, and D145E. Functional studies with these recombinant HcTnC HCM mutations showed increased Ca^{2+} sensitivity of force development (A8V, C84Y and D145E) and force recovery (A8V and D145E). These results are consistent with the HCM functional phenotypes seen with other sarcomeric-HCM mutations (E134D showed no changes in these parameters). This is the largest cohort analysis of *TNNC1* in HCM that details the discovery of at least three novel HCM-associated mutations and more strongly links *TNNC1* to HCM along with functional evidence that supports a central role for its involvement in the disease. This study may help to further define *TNNC1* as an HCM-susceptibility gene, a classification that has already been established for the other members of the troponin complex.

Keywords: Troponin C; TnC; Hypertrophic cardiomyopathy; HCM; Mutation; Calcium; Genetics

1. Introduction

A leading cause of sudden cardiac death in the young is hypertrophic cardiomyopathy (HCM) which affects approximately 1 in 500 individuals and is defined clinically as thickening of the left ventricle and septum in the absence of any identifiable cause [1–3]. Through initial linkage studies and subsequent hypotheses that HCM was a disease of the sarcomere, investigations over the past two decades have led to the identification of hundreds of HCM-associated mutations scattered among the various sarcomeric genes [4–10]. This is

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reflected in the commercially-available clinical genetic tests for HCM which scan for mutations in the genes encoding β-myosin heavy chain, myosin binding protein C, cardiac troponin I, cardiac troponin T, α-tropomyosin, cardiac actin, regulatory myosin light chain, and ventricular myosin light chain. Despite these tremendous advances, approximately 20% of patients with reverse curve-HCM and nearly 90% of the patients with sigmoidal-HCM are genotype negative with respect to the genetic test panel for sarcomeric/myofilament HCM [11–13]. Notably absent from this list is the *TNNC1*-encoded human cardiac troponin C (HcTnC) which has yet to be firmly associated with HCM [14,15]. To date, only one mutation in TnC has been linked to a 60 year old HCM patient [16]. In a small cohort-based study, the authors did not find the L29Q TnC mutation in any other patient (the number of HCM patients screened was not reported) nor in 96 healthy volunteers. They concluded that additional studies would be necessary to elucidate whether TnC should be considered in fact a disease gene for HCM [16].

Cardiac troponin is a heterotrimeric complex comprised of a Ca^{2+}-binding subunit TnC, an inhibitory subunit troponin I (TnI) encoded by *TNNI3*, and an elongated troponin T (TnT) encoded by *TNNT2*. TnC acts as a cytosolic Ca^{2+} sensor which, when bound to the divalent cation at the single Ca^{2+}-specific binding site, strengthens its interaction with TnI and transversely weakens the inhibitory function of TnI causing its release from actin. The troponin-tropomyosin complex then shifts deeper into the actin groove thereby exposing the myosin binding sites on actin making them available for contraction (for review see [17]). cTnC belongs to the EF-hand superfamily of Ca^{2+} binding proteins and consists of N and C terminal globular domains that are connected through a flexible linker. Each globular domain has a pair of EF-hand helix-loop helix Ca^{2+} binding motifs [18,19]. The C-terminus (also called the structural domain) contains two high affinity Ca^{2+} binding sites III and IV ($\sim 10^7$ M^{-1}) that also binds to Mg^{2+} competitively with low affinity ($\sim 10^3$ M^{-1}). The N-terminus contains only one functional low affinity "Ca^{2+}-specific" regulatory Ca^{2+} binding site II ($\sim 10^5$ M^{-1}) [20–22]. The N-terminus is considered the regulatory domain since Ca^{2+} binding initiates muscle contraction [23,24]. In this manner, TnC represents a critical molecular switch through which defects in the primary sequence of the protein may disrupt the TnC-Ca^{2+} regulation process.

At least 90% of HCM Tn mutations (TnT and TnI), that have been investigated *in situ* cause an increase in the Ca^{2+} sensitivity of force development resulting in an increased force at sub-maximal Ca^{2+} concentrations [25–27]. The same functional phenotype has also been observed in transgenic mice containing Tn mutations related to HCM when compared to the WT. There seems to be a correlation between the change in Ca^{2+} sensitivity of force development and the time of onset of disease and prognosis [25–27].

Only one HCM-associated TnC mutation (L29Q) has been functionally studied by two different groups. Schmidtmann et al., showed, using a reconstituted fast skeletal system containing cardiac troponin complex, a decrease in the Ca^{2+} sensitivity measured by ATPase activity and *in vitro* motility assays [28]. However, Liang et al showed an increase in the Ca^{2+} sensitivity of force development measured in TnC-depleted mouse skinned cardiac myocytes reconstituted with recombinant mouse cardiac TnC [29].

Because a 7 year span has elapsed since the first report of an HCM-associated mutation in cTnC and no subsequent reports have shown any linkage between HCM and cTnC, we sought to determine whether genetic perturbations in *TNNC1* may play a role in the pathogenesis of HCM in a large cohort-based study. We report four novel missense mutations in HcTnC – A8V, C84Y, E134D, and D145E – which at least three alter the Ca^{2+} sensitivity of contraction when reconstituted into TnC-depleted porcine cardiac muscle fibers. Furthermore, these results suggest a role for calcium mishandling in the pathogenesis of sarcomeric-HCM and warrants further scrutiny.

2. Methods

2.1. Study population

Between April 1997 and April 2007, 1025 unrelated patients, evaluated in the Hypertrophic Cardiomyopathy Clinic at Mayo Clinic, Rochester, Minnesota, consented to genetic testing. Following receipt of written consent for this Mayo Foundation Institutional Review Board-approved protocol, DNA was extracted from peripheral blood lymphocytes using the Purgene DNA extraction kit (Gentra, Inc, Minneapolis, MN). Clinical data was collected on all patients including physical examination, pertinent personal and family history, 12-lead electrocardiogram (ECG) analysis, and echocardiographic testing to determine maximum left ventricular wall thickness (MLVWT) and maximum left ventricular outflow tract gradient (MLVOT). Each of the subjects met the clinical diagnostic criteria for HCM of a MLVWT greater than 13 mm in the absence of other confounding diagnoses.

2.2. Troponin C mutational analysis

All six *TNNC1* exons, with flanking intronic regions and splice junction, were amplified by PCR using oligonucleotide primers. Each amplicon was evaluated for mutations using denaturing high performance liquid chromatography (DHPLC, Transgenomic, Omaha, NE), and samples with an abnormal elution profile were directly sequenced (ABI Prism 377, Applied Biosystem, Foster City, CA) to characterize the difference between the wild type and variant alleles. Primer sequences, PCR, and DHPLC conditions are available upon request. Using previously published conditions, *TNNC1*-positive subjects were analyzed for mutations in 15 established HCM-susceptibility genes including the eight genes that comprise the commercially-available genetic test for sarcomeric-HCM.

2.3. Site-directed mutagenesis, expression, and protein purification of human cardiac troponin C

The cDNA for human cardiac TnC (HcTnC) was cloned previously in our laboratory by RT-PCR using human heart total