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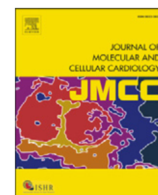

Original article

Dose-dependent diastolic dysfunction and early death in a mouse model with cardiac troponin mutations ☆

Yuejin Li [a], Lei Zhang [b], Pierre-Yves Jean-Charles [a], Changlong Nan [a], Guozhen Chen [b], Jie Tian [b], J.-P. Jin [c], Ira J. Gelb [a], Xupei Huang [a,b,*]

[a] Charles E. Schmidt College of Medicine, Florida Atlantic University, Boca Raton, FL 33431, USA
[b] Division of Cardiology, Children's Hospital, Chongqing Medical University, Chongqing 400014, China
[c] Department of Physiology, Wayne State University College of Medicine, Detroit, MI 48201, USA

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ABSTRACT

Our aim was to explore the dose-dependent diastolic dysfunction and the mechanisms of heart failure and early death in transgenic (TG) mice modeling human restrictive cardiomyopathy (RCM). The first RCM mouse model ($cTnI^{193His}$ mice) carrying cardiac troponin I (cTnI) R193H mutation (mouse cTnI R193H equals to human cTnI R192H) was generated several years ago in our laboratory. The RCM mice manifested a phenotype similar to that observed in RCM patients carrying the same cTnI mutation, i.e. enlarged atria and restricted ventricles. However, the causes of heart failure and early death observed in RCM mice remain unclear. In this study, we have produced RCM TG mice ($cTnI^{193His}$-L, $cTnI^{193His}$-M and $cTnI^{193His}$-H) that express various levels of mutant cTnI in the heart. Histological examination and echocardiography were performed on these mice to monitor the time course of the disease development and heart failure. Our data demonstrate that cTnI mutation-caused diastolic dysfunction is dose-dependent. The key mechanism is myofibril hypersensitivity to Ca^{2+} resulting in an impaired relaxation in the mutant cardiac myocytes. Prolonged relaxation time and delay of Ca^{2+} decay observed in the mutant cardiac myocytes are correlated with the level of the mutant protein in the heart. Markedly enlarged atria due to the elevated end-diastolic pressure and myocardial ischemia are observed in the heart of the transgenic mice. In the mice with the highest level of the mutant protein, restricted ventricles and systolic dysfunction occur followed immediately by heart failure and early death. Diastolic dysfunction caused by R193H troponin I mutation is specific, showing a dose-dependent pattern. These mouse models are useful tools for the study of diastolic dysfunction. Impaired diastole can cause myocardial ischemia and fibrosis formation, resulting in the development of systolic dysfunction and heart failure with early death in the RCM mice with a high level of the mutant protein in the heart.

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1. Introduction

Cardiomyopathy is a disorder that primarily affects cardiac muscles resulting in cardiac dysfunctions [1–3]. Among various types of cardiomyopathies, hypertrophic cardiomyopathy (HCM) and restrictive cardiomyopathy (RCM) share a similar key feature characterized by a diastolic dysfunction [4–6]. Unlike HCM, RCM is rare accounting roughly for 2–5% of all inherited cardiomyopathies. However, the prognosis of the disease, especially in young patients with RCM, is poor, as this condition often leads to heart failure and early death [7]. Treatment of RCM is difficult and often ineffective [8,9]. A linkage study had, for the first time, demonstrated that idiopathic RCM can be a part of clinical expression of six cardiac troponin I (cTnI) mutations [5]. Among those mutations, two of the mutations (K178E and R192H) identified in young individuals were de novo mutations with the worst clinical phenotype [5]. The first transgenic (TG) mouse model ($cTnI^{193His}$ mice) carrying the RCM cTnI mutation (Arg193His in mouse cTnI protein that equals to human cTnI R192H) was generated several years ago in our laboratory [10]. The RCM TG mice manifested a phenotype similar to that observed in RCM patients carrying the same cTnI mutation, i.e. markedly enlarged atria and restricted ventricles due to the increased ventricular pressure and stiffness [11]. The cell-based studies further revealed that the key defect in RCM cardiac myocytes was impaired relaxation that

Abbreviations: RCM, restrictive cardiomyopathy; HCM, hypertrophic cardiomyopathy; TnI, troponin I; TG, transgenic; IVRT, isovolumetric relaxation time; IVCT, isovolumetric contraction time; EF, ejection fraction; FS, fractional shortening; LVEDD, left ventricle end diastole dimension; LVEDV, left ventricle end diastole volume.

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* Corresponding author at: Department of Biomedical Science, Charles E. Schmidt College of Medicine, Florida Atlantic University, Boca Raton, FL 33431, USA. Tel.: +1 561 2972443; fax: +1 561 297 2221.
E-mail address: xhuang@fau.edu (X. Huang).

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resulted from the myofibril hypersensitivity to Ca^{2+} [12,13]. Although the RCM mouse model is well characterized, several questions still remain. The questions we aim to address in this study are: 1) What is the cause of the varied severity of the disease observed in patients suffering from cardiomyopathies caused by myofibril protein mutations? Is the cardiac dysfunction caused by cTnI mutation dose-dependent, i.e. does the impaired relaxation depend on the level of the mutant protein in the heart? 2) We observed that most RCM cTnI TG mice had early onset of impaired relaxation followed by heart failure at a later stage. What is the mechanism that correlates the early diastolic dysfunction to late stage heart failure? 3) What are the causes of death in RCM cTnI TG mice? Is it congestive heart failure, arrhythmia, or both?

Clinically, these questions have not been well answered to date. This is because of the lack of quantitative data of the mutant protein in diseased hearts due to the limited cardiac samples from patients, and the lack of specific antibodies that differentiate mutant proteins from wild type proteins. In the present study, we have produced RCM cTnI TG mice that express various levels of mutant cTnI in the heart by crossing our cTnI knockout mice with RCM TG $cTnI^{193His}$ mice. Histological examination and echocardiography were performed on these TG mice at different time points to monitor the progress of the disease and to determine simultaneously the myocardial ischemia, fibrosis and the damage-caused cardiac dysfunction and heart failure. Our data demonstrate, for the first time, that cTnI mutation-caused diastolic dysfunction is dose-dependent and myocardial ischemia caused by impaired diastole is probably associated with the late development of systolic dysfunction resulting eventually in heart failure and early death in RCM.

2. Methods

This investigation conforms to the Guide for the Care and Use of Laboratory Animals (NIH Pub. No. 85-23, revised 1996) and was in accordance with the protocols approved by the Institutional Animal Care and Use Committees at Florida Atlantic University.

2.1. Animals

The cTnI gene knockout mice (C57BL/6) were generated previously and are well characterized [14]. The heterozygous cTnI knockout ($cTnI^{+/-}$) mice are maintained in our laboratory for more than a decade. These mice were paired with transgenic $cTnI^{193His}$ mice (C57BL/6) that exhibit a RCM phenotype [10]. By crossing $cTnI^{+/-}$ mice with $cTnI^{193His}$ mice, we produced $cTnI^{193His}/cTnI^{+/-}$ double transgenic mice. By crossing these double transgenic mice, we obtained the transgenic mice expressing various levels of cTnI R193H in the heart: $cTnI^{193His}$-H mice that express only mutant cTnI R193H at a wild type cTnI null background and $cTnI^{193His}$-M mice that express about 40% of mutant cTnI with 60% of wild type cTnI. The $cTnI^{193His}$-L is our original transgenic mouse line that expresses about 20% of mutant cTnI in the heart. The wild type (WT, C57BL/6) mice were used as controls in the study and they have the same genomic background as the transgenic mice. A PCR-based assay was performed to determine the genotypes and the mutant protein replacement rate in most of the experimental animals was confirmed by Western blotting assays after the experiments.

2.2. Determination of the replacement rate of the mutant cTnI in the heart by Western blotting

Cardiac myofilament proteins were extracted and the incorporation rate of wild type and mutant cTnI in cardiac myofibrils were determined by Western blotting as described previously [11,12]. Briefly, cardiac myofibril proteins were examined on SDS-PAGE and Western blotting using two mouse monoclonal antibodies (mAb). TnI-1 antibody is specific for the C-terminal epitope of TnI involving the residue of R193 and 4H6 antibody recognizes an epitope in the middle region of cTnI polypeptide chain. The cTnI R193H mutation destroyed the TnI-1 epitope but not the 4H6 epitope, allowing the distinction between cTnI WT and R193H. For Western blotting, cardiac ventricles were homogenized in SDS-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer containing 2% SDS to extract myofilament proteins. The protein bands were transferred to a nitrocellulose membrane using a Bio-Rad Lab semidry electrotransfer apparatus. The nitrocellulose membrane was blocked with 1% bovine serum albumin in Tris-buffered saline and incubated with mAbs diluted in TBS-T containing 0.1% bovine serum albumin. Antibodies on immunoblots were visualized by enhanced chemiluminescence (ECL detection kit from GE Healthcare). Cardiac TnT (cTnT) was used as an internal control to normalize the protein sample loading. For quantifications, the protein bands

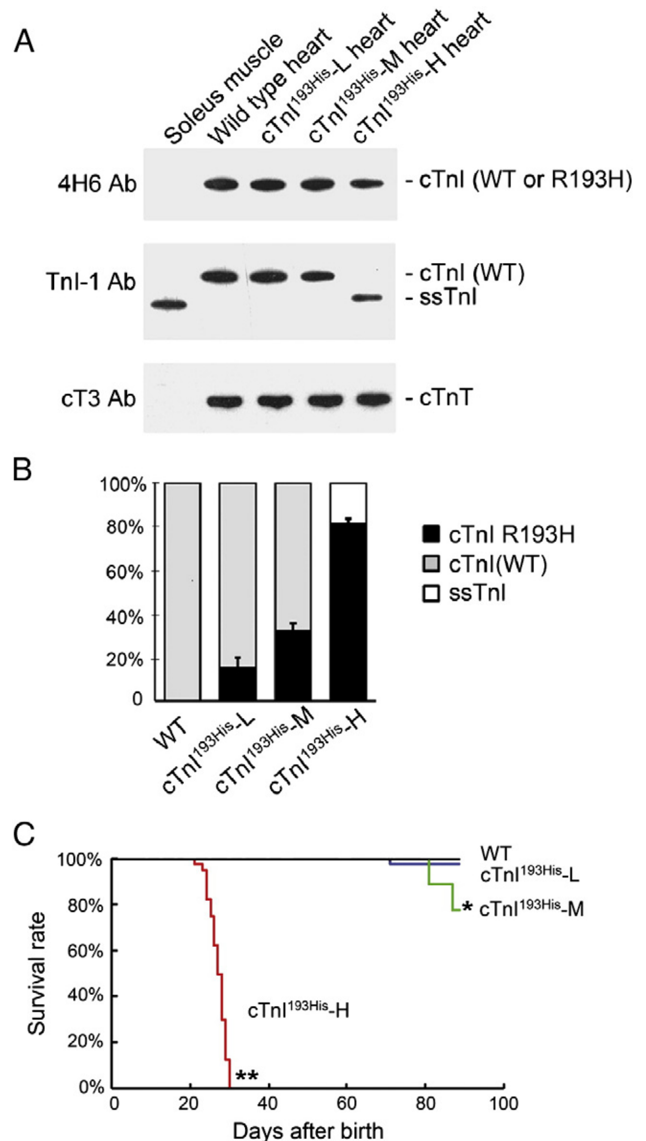

Fig. 1. Determination of the mutant cTnI (cTnI R193H) replacement in the hearts from various RCM transgenic lines. (A) Western blotting analysis using two specific monoclonal antibodies, mAb 4H6 recognizes wild type (WT), cTnI R193H, but not slow skeletal TnI (ssTnI) from soleus muscle; mAb TnI-1 recognizes WT cardiac and skeletal TnI but not cTnI R193H. Cardiac troponin T (cTnT) was used as protein loading control. (B) Summary of Western blotting results and values are expressed as means ± SD from 4 separate experiments. (C) Kaplan–Meier survival curves for WT (n = 18), $cTnI^{193His}$-L (n = 18), $cTnI^{193His}$-M (n = 12) and $cTnI^{193His}$-H (n = 17) mice. The log-rank test indicates statistically significant differences among the four survival curves ($P < 0.05$). The observation was performed for 90 days and 100% death was observed in $cTnI^{193His}$-H mice at age of 1 month.

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