

## Genetic Variation Screening of *TNNT2* Gene in a Cohort of Patients With Hypertrophic and Dilated Cardiomyopathy

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### Summary

Mutations in troponin T (*TNNT2*) gene represent the important part of currently identified disease-causing mutations in hypertrophic (HCM) and dilated (DCM) cardiomyopathy. The aim of this study was to analyze *TNNT2* gene exons in patients with HCM and DCM diagnosis to improve diagnostic and genetic consultancy in affected families. All 15 exons and their flanking regions of the *TNNT2* gene were analyzed by DNA sequence analysis in 174 patients with HCM and DCM diagnosis. We identified genetic variations in *TNNT2* exon regions in 56 patients and genetic variations in *TNNT2* intron regions in 164 patients. Two patients were found to carry unique mutations in the *TNNT2* gene. Limited genetic screening analysis is not suitable for routine testing of disease-causing mutations in patients with HCM and DCM as only individual mutation-positive cases may be identified. Therefore, this approach cannot be recommended for daily clinical practice even though, due to financial constraints, it currently represents the only available strategy in a majority of cardio-centers.

### Key words

Cardiomyopathy • Genetic variations (mutation, polymorphism, deletion) • *TNNT2* • Gene

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### Introduction

Cardiomyopathies are generally defined as myocardial disorders in which the heart muscle is structurally and functionally abnormal, in the absence of coronary artery disease, hypertension, valvular disease and congenital heart disease sufficient to cause the observed myocardial abnormality (Elliott *et al.* 2008). According to the morphological and functional phenotype the diagnosis of hypertrophic and dilated cardiomyopathy can be established. Hypertrophic cardiomyopathy (HCM) is an autosomal dominant cardiac disorder (Marian and Roberts 1995) with a prevalence of 0.2 % in the general population (Richard *et al.* 2003). More than 70 % of HCM cases are familial (Friedrich *et al.* 2009). Hypertrophic cardiomyopathy represents one of the the most frequent causes of sudden cardiac death in the young, especially in competitive athletes (Erdmann *et al.* 2003, Ehlermann *et al.* 2008) and a major cause of morbidity and mortality in the elderly (Michels *et al.* 2007).

The major morphological features of the disease are left ( $\pm$  right) ventricular hypertrophy with predominant involvement of the interventricular septum (Fokstuen *et al.* 2008), myocyte disarray and interstitial myocardial fibrosis (Friedrich *et al.* 2009). However, the mechanism of HCM development on the cellular and molecular level has not yet been fully described. Myocardial hypertrophy could develop as a result of reduced contractile function. However, studies conducted

in humans and animals with sarcomeric protein mutations showed that the velocity and force of myocyte contraction were in fact increased (Redwood *et al.* 1999). Furthermore, some authors have suggested that abnormal myocardial bioenergetics importantly contribute to the HCM phenotype (Ashrafian *et al.* 2003).

Dilated cardiomyopathy (DCM) is an inherited or acquired disease characterized by left ventricular dilatation and reduced systolic function. DCM represents the third most common cause of heart failure and the most frequent cause of heart transplantation. It accounts for approximately 3 % of all sudden cardiac deaths in young athletes (Gilbert *et al.* 1993, Maron *et al.* 1996, Kamisago *et al.* 2000). Importantly, 30-50 % of all cases are diagnosed as a familial form of DCM (Burkett and Hershberger 2005).

Recent studies have reported 27 genes associated with HCM and 32 genes, involving 2 X-linked genes, associated with DCM (reviewed in Tester *et al.* 2011 and Hershberger *et al.* 2011). In the vast majority of cases these genes encode for sarcomeric contractile proteins (García-Castro *et al.* 2009, Landstrom *et al.* 2011):  $\beta$ -myosin heavy chain (*MYH7*), myosin binding protein C (*MYBPC3*), troponin T (*TNNT2*), troponin I (*TNNI3*), cardiac  $\alpha$ -actin (*ACTC*) and  $\alpha$ -tropomyosin (*TPMI*). Additionally, genes encoding components of the cardiac Z-disk, calcium ( $\text{Ca}^{2+}$ )-handling, and regulatory proteins have been recently associated with cardiomyopathies (Landstrom *et al.* 2010, Tester *et al.* 2011). Nearly all of the mutations (86 %) are single nucleotide mutations, which can lead to the changes in protein chains. Remaining mutations include small in-frame insertions or deletions and rarely large deletions (Fokstuen *et al.* 2008).

The *TNNT2* gene (OMIM number \*191045) encodes the thin-filament contractile protein cardiac troponin T, which links the troponin complex to tropomyosin in the sarcomere (García-Castro *et al.* 2003). The gene containing 15 exons is located on chromosome 1q32, and comprises 25kb of genome. *TNNT2* mutations are responsible for about 15 % of all cases of familial HCM (Sehnert *et al.* 2002). *MYH7* and *TNNT2* gene mutations also represent common genetic causes of DCM. According to the recent studies, *MYH7* and *TNNT2* mutations are responsible for about 4-6 % and 3 % of cases of familial DCM, respectively (Hershberger *et al.* 2009).

One of the significant features of cardiomyopathies is a wide phenotypic heterogeneity even

within one family with the same mutation (Michels *et al.* 2007, Friedrich *et al.* 2009). Some genetic analyses have also revealed the presence of clinically healthy individuals carrying the mutant allele (Forissier *et al.* 1996). Moreover, significant proportion of the cardiomyopathy population remains genotype-negative with no biomarker for, or mechanistic explanation of, their disease process. These findings indicate that in addition to the responsible gene, environmental factors and other genetic factors (genes/polymorphisms) can modulate the phenotypic expression of the disease (Forissier *et al.* 1996, Friedrich *et al.* 2009, Landstrom *et al.* 2010).

In the present study, we analyzed *TNNT2* exons in patients with HCM and DCM diagnosis to improve diagnostic and genetic consultancy in affected families.

## Methods

### Patients

174 unrelated Caucasian patients with HCM (n=84) and DCM (n=90), mean age 48.4±15.1 years, were evaluated in the Clinical Department of Cardiology and Angiology, First Faculty of Medicine and General University Hospital, Charles University, Prague, Czech Republic, and were included in this single center study. The diagnosis of HCM and DCM was based on current recommendations (Elliott *et al.* 2008). The clinical characteristic of our patients is provided in Table 1.

**Table 1.** Clinical characteristic of patients with HCM and DCM.

	Hypertrophic cardiomyopathy (n=84)	Dilated cardiomyopathy (n=90)
<i>Age (years)</i>	51 ± 13	48 ± 17
<i>Males</i>	64 (76 %)	72 (80 %)
<i>Positive family history of cardiomyopathy</i>	29 (35 %)	11 (12 %)
<i>NYHA class</i>	2.0 ± 0.7	1.5 ± 0.8
<i>Arterial hypertension</i>	40 (48 %)	39 (43 %)
<i>Diabetes mellitus</i>	9 (11 %)	14 (16 %)
<i>Smoking</i>	27 (32 %)	45 (50 %)

Data are expressed as mean ± standard deviation or as a number and percentage of the subjects.

**Table 2.** Primers and PCR-RFLP conditions - *TNNT2* polymorphisms screening.

Polymorphism	I79N	R92W
<i>Primer sequence, 5'-3'</i>		
<i>forward</i>	atg ggg ctg atg ctg act at	cac cca tct ctc ctc tgg ac
<i>reverse</i>	gcc caa ggt cac aaa atc tc	ctc aca aaa ggg atg gag ga
<i>Annealing T (°C)</i>	59.5	59.5
<i>Restriction enzyme</i>	MboI	MspI
<i>Product size (bp)</i>	180	247
<i>Allele (fragment sizes) (bp)</i>	T (180) / A (115+65)	C (175+72) / T (247)

One hundred and two unrelated healthy Caucasian subjects (mean age 51±10 years, 52 males (51 %) served as controls.

The study was performed in accordance with principles of the Declaration of Helsinki and approved by the Institutional Ethical Committee. All patients gave their informed consent prior to entering the study.

#### Samples

Blood samples were collected *via* puncture of the cubital vein. Tubes with ethylene diamine tetraacetic acid were used for DNA analysis. Blood samples were stored at 4 °C and isolation of DNA was performed by a modified salting out procedure according to Miller *et al.* (1988) within a week of collection.

#### TNNT2 screening

First, screening for mutations in *TNNT2* exons 7 (I76N) and 8 (R92W) was done using restriction fragment length polymorphism (RFLP) analysis.

The DNA was amplified by polymerase chain reaction (PCR) with primers and annealing temperatures summarized in Table 2. PCR product was then digested with corresponding restriction endonuclease (Table 2) according to the manufacturer's recommended protocol. Restriction fragments were separated by electrophoresis in 3 % agarose gel and visualized in UV light after ethidium bromide staining. Results were confirmed by DNA sequencing.

The primers were predicted by Primer3 Input (<http://frodo.wi.mit.edu/>) and restriction enzymes were assessed using NebCutter V2.0 (<http://tools.neb.com/NEBcutter2>).

#### TNNT2 sequencing

The entire coding sequences of *TNNT2* gene

were amplified by PCR with primers and annealing temperatures summarized in Table 3. Products were separated by electrophoresis in 2 % agarose gel, excised from the gel and purified with spin columns (NucleoSpin Extract II, Macherey-Nagel). Both strands of purified DNA were then sequenced in a CEQ 8000 genetic analysis system (Beckman Coulter) according to the manufacturer's protocol. The primers for *TNNT2* exons 9, 11, 12, 13 and 14 were predicted by Primer3 Input (<http://frodo.wi.mit.edu/>), while the remainder was designed according to Seidmann *et al.* ([www.cardiogenomics.org](http://www.cardiogenomics.org)).

## Results

The mutations I79N, R92W, R92G, R92L in the *TNNT2* gene were screened by RFLP analysis and results were then confirmed by DNA sequencing. Within our study group, which consisted of 174 patients (84 patients with HCM and 90 patients with DCM), we identified one mutation in R92W in exon 8 of the *TNNT2* gene in a patient with HCM.

We additionally examined all of the 15 exons and their flanking regions of the *TNNT2* gene in the same group of patients. Using DNA sequence analysis to investigate polymorphisms, small deletions and new mutations and we found genetic variations in exon regions in 56 patients and genetic variations in intron regions in 164 patients (Table 4). We confirmed the presence of a unique mutation in R92W (exon 8) in a single HMC patient and another unique mutation A172S (exon 10) in a DCM patient. Moreover, we analyzed mutations R92W in exon 8 and A172S in exon 10 in the control group consisted of 102 healthy individuals to confirm that these mutations are not associated with healthy subjects.

**Table 3.** Primers and PCR conditions – *TNNT2* sequencing.

<b>Primers predicted by Primer3 Input</b>			
	<b>Primer sequence 5' - 3'</b>	<b>Annealing T (°C)</b>	<b>Product size (bp)</b>
<i>Exon 9</i>	ggc acc att gct tca aga ct tcc caa agt gct ggg att ac	60.1	369
<i>Exon 11</i>	cct gct gta acc ctc aga cc cag ccc aat ctc ttc act cc	59.8	284
<i>Exon 12</i>	tgt ggc agg aag aag agc at tgc cat ggg aaa ata tgt ga	60.3	365
<i>Exon 13</i>	gaa ctt tgc cct ggc agt c cag gga cct gca gca gta tt	60.5	398
<i>Exon 14</i>	ctc ctt ctc ctc ctg cac tg agg agc cag aga agg aaa cc	60.0	301
<b>Primers according to Seidmann <i>et al.</i></b>			
	<b>Primer sequence 5' - 3'</b>	<b>Annealing T (°C)</b>	<b>Product size (bp)</b>
<i>Exon 1</i>	gct gca tgt ggt gtc act atc tcc c cac agc tac ttc tac cca gaa tcc	66.8	358
<i>Exon 2+3</i>	aca agg gaa aag aaa ggg gga tta agt gag gag cag gga cag atg agc	63.9	352
<i>Exon 4+5</i>	cat gag tgg ggc ctg ctt ctt c tgt ggg cat tct cct cca aag ctg	67.0	577
<i>Exon 6</i>	cac tgt gca gat ggg gaa atg ga tcc tct ctc cta ggc ctc tgc t	66.8	200
<i>Exon 7+8</i>	gga tca ggc cct gcc tgt cct gac a gga tga gac aga ctg gcc atc ag	62.6	620
<i>Exon 9+10</i>	gga ggc cgg gca cca ttc ttc aag gga cct gac cta aag tct acc tgc	60.3	1313
<i>Exon 14+15</i>	acc tgg acc tga gcc agt cta gtt tct ctc tct ctc tga	59.9	756

**Table 4.** Genetic variations (mutations, polymorphisms, small deletions) of the *TNNT2* gene in HCM and DCM patients.

<b>Gene</b>	<b>Exon</b>	<b>Amino acid position</b>	<b>dbSNP access number<sup>1</sup></b>	<b>DNA variation</b>	<b>Number of patients</b>	
<i>TNNT2</i>	7	S79S	rs3729845	aTCG→TCA	9	
	8	R92W	CM971501	cCGG→TGG	1	
		I116I	rs3729547	ATC→ATT	41	
	10	A172S	CM043107	gGCC→TCC	1	
	13	K260R	rs3730238	gAAG→AGG	4	
	<b>Intron</b>					
	1	-	rs868407	C→T	74	
2	-	rs45533739	deletion CTTCT	70		
12	-	rs2275861	C→T	20		

<sup>1</sup>dbSNP access number: polymorphisms and deletions are characterized by rs number, mutations by CM number.

**Table 5.** *TNNT2* gene polymorphisms and small deletions in HCM patients.

		Genotypes (%)			Allele frequencies	
<i>Exon 7</i>	S79S	GG	GA	AA	G	A
		89	11	0	0.946	0.054
<i>Exon 8</i>	I116I	TT	CT	CC	T	C
		45	40	15	0.653	0.347
<i>Exon 13</i>	K260R	AA	AG	GG	A	G
		95	5	0	0.975	0.025
<i>Intron 1</i>	C/T	TT	CT	CC	T	C
		49	39	12	0.685	0.315
<i>Intron 2</i>	deletion CTTCT	++	+-	--	+	-
		34	49	17	0.589	0.411
<i>Intron 12</i>	C/T	TT	CT	CC	T	C
		77	23	0	0.883	0.117

The frequencies of remaining *TNNT2* gene polymorphisms from Table 4 correlated with data in the SNP database (dbSNP) of the National Centre for Biotechnology Information (Table 5).

## Discussion

In our study, we analyzed the entire coding sequences of the *TNNT2* gene (15 exons, ~ 6000 nucleotides) in patients with HCM and DCM. Our aim was to improve diagnostic and genetic consultancy in affected families. Within our study population consisting of 174 patients, we were able to identify two patients with unique mutations in *TNNT2* gene (one with HCM and one with DCM) and none of these mutations were presented in our control group of healthy subjects.

The results of our study clearly show both advantages and disadvantages of employing routine clinical genetic screening in subjects suffering from cardiomyopathies. The identification of a unique mutation responsible for phenotypic expression of cardiomyopathy in a given proband requires that genetic analyses be performed on the patient's immediate relatives. Mutation-free relatives will not require regular clinical screening and can be assured that their children will not be affected by this type of cardiomyopathy. On the other hand, the presence of the disease-causing mutation should prompt more frequent check-ups of the affected relative in order to identify early phenotypic expression of the disease and thereby improve the management of the affected person (Charron *et al.* 2010). Importantly, the identification of the

mutation in a proband and, possibly, in their relatives requires that they be given clinical genetic counselling to explain of the risk of transmitting the mutation to their children with the possibility of prenatal genetic diagnosis (Charron *et al.* 2010).

There are clear limitations in the feasibility and practicality of screening for mutations in only one gene. In our opinion, these limitations outweigh the above listed advantages. According to current recommendations, the main genes of interest in HCM and DCM for mutation screening in routine practice comprise both *MYH7* and *TNNT2* genes (Hershberger *et al.* 2009). The mutations in these two genes represent the majority of currently identifiable disease-causing mutations of HCM and DCM (Fowler *et al.* 2009). However, genetic analysis as we have done, which is limited to only a small number of potentially involved exons, does not seem to be applicable. We were able to perform analysis of all 15 exons of *TNNT2* gene, and we have found 2 unique *TNNT2* mutations, one in an HCM patient and one in a DCM patient. However, this number is lower than the frequency of *TNNT2* gene mutations in both cardiomyopathies as reported in current literature (Richard *et al.* 2003, Hershberger *et al.* 2009).

We believe that the rather general definition of DCM in our study cohort may explain this discrepancy, as subjects with chronic myocarditis or other acquired forms of cardiomyopathy were likely included. In a more specifically defined population of patients with otherwise unexplained, idiopathic DCM, the prevalence of *TNNT2* mutations could be higher than what we observed.

Nevertheless, this fact again underscores the important limitations of our approach based on analyses of only a limited number of exons of disease-related mutations. Due to limited financial resources, we focused only on *TNNT2* gene exons. This strategy was employed since *TNNT2* mutations are thought to be responsible for an important form of HCM associated with highly increased risk of sudden cardiac death (Watkins *et al.* 1995).

Nevertheless, even in highly specialized centers for genetic diagnostics of cardiomyopathies, testing is usually limited to the most common causative genes (Cowan *et al.* 2008). This approach may change in the near future as more cost-effective methods capable of wide genome screening, such as chip-based or next generation sequencing, become available.

In conclusion, limited genetic screening analysis is not suitable for routine testing of disease-causing

mutations in patients with HCM and DCM as only individual mutation-positive cases may be identified. Therefore, this approach cannot be recommended for daily clinical practice even though, due to financial constraints, it currently represents the only available strategy in the majority of cardio-centers. More cost-effective methods enabling wide genome screening are promising and should be implemented in genetic analyses of cardiomyopathies in the near future.

### Conflict of Interest

There is no conflict of interest.

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