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Tropomyosin 1: Multiple roles in the developing heart and in the formation of congenital heart defects

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ABSTRACT

Tropomyosin 1 (TPM1) is an essential sarcomeric component, stabilising the thin filament and facilitating actin's interaction with myosin. A number of sarcomeric proteins, such as alpha myosin heavy chain, play crucial roles in cardiac development. Mutations in these genes have been linked to congenital heart defects (CHDs), occurring in approximately 1 in 145 live births. To date, *TPM1* has not been associated with isolated CHDs. Analysis of 380 CHD cases revealed three novel mutations in the *TPM1* gene; IVS1 + 2T > C, I130V, S229F and a polyadenylation signal site variant GATAAA/AATAAA. Analysis of IVS1 + 2T > C revealed aberrant pre-mRNA splicing. In addition, abnormal structural properties were found in hearts transfected with TPM1 carrying I130V and S229F mutations. Phenotypic analysis of TPM1 morpholino-treated embryos revealed roles for *TPM1* in cardiac looping, atrial septation and ventricular trabeculae formation and increased apoptosis was seen within the heart. In addition, sarcomere assembly was affected and altered action potentials were exhibited. This study demonstrated that sarcomeric *TPM1* plays vital roles in cardiogenesis and is a suitable candidate gene for screening individuals with isolated CHDs.

1. Introduction

The heart is first seen as a tube early in development, which undergoes a series of complex processes to form a four-chambered structure. Looping of the chicken primitive heart tube is initiated from Hamburger and Hamilton (HH) 10 (human gestation day 22) [1,2]. This tube subsequently divides into chambers by septation, initiated in the primitive single atrium from the dorso-cranial wall around HH14 (4th week in human) followed by septation of the common ventricle [3]. In humans, congenital heart defects (CHDs) occur in approximately 1 in 145 live births [4]. Although most cases of CHDs are thought to have complex etiology, some cases segregate through families following Mendelian patterns of inheritance. Studying the genetic causes of the Mendelian variety could potentially unravel the biology of this disease, which, in turn, could be applied to the prevention and treatment of complex-etiology CHD. The molecular genetics of Mendelian CHDs are being elucidated, with mutations found in several genes, including transcription factors NKX2 homeobox 5 (*NKX2.5*) and T-box 5 (*TBX5*), and structural proteins myosin heavy chain 6 (MYH6), MYH7 and alpha cardiac actin [5–7].

The cardiac conduction system (CCS) also appears early in cardiogenesis with the development of a primordial pacemaker, and subsequent formation continues with differentiation of the Purkinje fibres still ongoing around birth. The CCS is also susceptible to defects with associated mutations in several genes; malformations may occur in isolation or in association with structural defects such as atrial septal defects (ASDs) [8]. Although much progress has been made in the understanding of cardiogenesis and its contribution to CHDs and CCS malformations, the causative genetic determinants remain unknown in most cases.

Cardiomyopathies are contractile diseases of the heart, which are associated with heart enlargement and dysfunction. The most common types are hypertrophic and dilated cardiomyopathy (HCM and DCM, respectively). HCM is defined as a notable thickening of the ventricular

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wall and DCM by ventricular dilatation and decreased contractile function [9]. Mutations in a range of sarcomeric genes have been associated with both HCM and DCM, including *Tropomyosin 1* (*TPM1*) [10,11]. Further, a number of genes encoding structural proteins have been associated with both cardiomyopathies and CHDs in the same individuals, such as *TPM1*, *MYH7* and *alpha cardiac actin* [6,12–14]. Fewer sarcomeric genes, such as *MYH6* and *alpha cardiac actin*, have been associated with isolated CHDs [5,7,15], or with the developing heart [16].

Tropomyosin regulates contraction of the sarcomere, through direct interaction with actin and troponin T [17]. In the presence of elevated levels of intracellular calcium (Ca^{2+}), Ca^{2+} binds to troponin C of the troponin complex inducing conformational changes that moves tropomyosin across the actin filament, exposing the myosin-binding sites, allowing the globular myosin heads to interact. This produces movement of the thick and thin filaments relative to each other, resulting in sarcomere shortening and hence muscle contraction [18,19]. Despite this essential interaction, roles for TPM1 in the developing heart are poorly understood, with isolated CHD causative mutations previously not described within the literature. Because of the aforementioned involvement of other genes encoding components of the thick and thin filaments of the cardiac sarcomere in both cardiomyopathy and heart development, we aimed to screen *TPM1* in a cohort of patients with diagnosed CHDs (presented in this report). Our initial results prompted us to investigate developmental roles of TPM1 and its associated thin filament protein cardiac Troponin T (cTNT) [16].

Here we report the screening of the *TPM1* gene in 380 patients with various CHDs. Four variants were detected. A splice donor site mutation resulted in abnormal splicing of pre-mRNA, while two non-synonymous mutations failed to incorporate with the sarcomeric protein cardiac troponin T in the sarcomere. Additionally, we detected a polyadenylation site variant. We showed that upon treatment with TPM1-specific morpholino oligonucleotides, the atrial septum and ventricular trabeculae developed abnormally. Additionally, some hearts showed abnormal looping. Although mature sarcomeres formed normally, there were fewer mature structures and increased apoptosis in the TPM1 morpholino-treated hearts. Further, the action potential (AP) of the cardiomyocytes was affected. These data suggest that TPM1 is essential for normal heart development and contractile function. Screening detected four variants in the *TPM1* gene, of which three we predict would lead to a pathological phenotype; we therefore consider *TPM1* a gene worthy of screening patients afflicted with CHDs or conduction anomalies.

2. Materials and methods

2.1. Phenotyping of patients

Patients with any form of CHD were recruited. All volunteered to participate and were under the routine care of the East Midlands Congenital Heart Centre, Leicester, UK. All patients had their CHD diagnosed fully by echocardiography and any other diagnostic modalities needed. All phenotyping, non-cardiac diagnoses and family histories were confirmed by one of the authors (Bu'Lock) who is an experienced Consultant Congenital and Paediatric Cardiologist.

2.2. Mutational dHPLC analysis

dHPLC analysis was completed as previously described [20] under consent from participants and according to local ethics committees. Samples of peripheral blood were collected from all participants. Genomic DNA was purified from the blood samples using QIAmp DNA blood Midi Kit (Qiagen). Control DNA from anonymous human UK blood donors was obtained from the European Collection of Cell Culture (Salisbury). The mutational analysis by dHPLC was performed by designing 14 PCR assays to cover the exons of the *TPM1* gene and their surrounding sequences (see Table S1). The melting temperature (Tm) for each amplicon was calculated with the following formula: Tm = 63.728 + (0.41 × %GC) − (600/length) where %GC = percentage GC of the primer; length = length of the primer in nucleotides. The annealing temperature used was the average of the two primer Tms + 3 °C.

PCR products were obtained by standard protocols from patient genomic DNA. After amplification, a final hybridization step was carried out, starting at 95 °C and reducing the temperature by 1.5 °C per minute to 25 °C in order to favour the formation of heteroduplexes. The sequence of each amplicon was analysed using the Navigator software (Transgenomic) to determine the melting profile of each DNA fragment and select the optimal temperatures for dHPLC. PCR products were processed using the dHPLC WAVE System (Transgenomic). PCR products from samples showing a dHPLC trace suggestive of variation were amplified again and sequenced using standard protocols. Potentially deleterious variants were screened by dHPLC in 380 individuals with a CHD and 384 ethnically matched control subjects.

2.3. Homology molecular model of the S229F TPM1 protein

The molecular model of the S229F TPM1 mutant protein was made by comparing human wild-type TPM1 protein with the known structure of other tropomyosin proteins [21] (Protein Data Bank ID:1C1G). Images were prepared using PyMOL Molecular Graphics System software v0.99 (DeLano Scientific).

2.4. Splicing-donor site mutation of TPM1

Genomic DNA was obtained from patient A with a heterozygous splicing-donor site mutation at exon1-intron1 (described in Section 2.2). DNA was also obtained from a control subject. PCR products were obtained and subcloned into pcDNA3.1(+) (Invitrogen). COS7 cells were transfected with each construct using Polyfect Transfection reagent (Qiagen) and incubated for 48 h. Following RNA extraction, reverse transcription was performed using 0.5 μg of RNA per reaction. A primer pair specific to *TPM1* 5′ upstream and exon2a were used to detect *TPM1* products. Full procedure is available in Supplementary material and methods.

2.5. Missense mutations of TPM1α

Full length human *TPM1α* cDNA was amplified using specific primers. The *TPM1α* product was cloned using the StrataClone PCR cloning set (Agilent Technologies) and subcloned into pEGFP-C1 vector (Clontech). The I130V and S229F missense mutations were introduced to the *TPM1α* sequence using site-directed mutagenesis using primers in Table S2. See Supplementary materials and methods for further information.

2.6. Chick embryo care and maintenance

White fertile chick eggs (*Gallus gallus*, Dekalb White strain; Henry Stewart) were incubated in a humidified chamber at 38 °C under constant rotation [1]. Before treatment, the embryo was exposed *in ovo*, and 3–5 ml of albumin was removed with a syringe to separate the embryo from the overlying allantoic and vitelline membranes, and both membranes were carefully removed. After treatment, eggs were resealed with masking tape and reincubated until harvesting. Studies were performed within national (UK Home Office) and institutional ethical regulations.

2.7. Transfection of embryonic chick hearts with TPM1 missense mutant constructs

Fertile chick eggs were incubated, as described in Section 2.6, until HH10/11 [1], approximately 48 h. Following a published protocol [22], a transfection mixture was prepared as follows: 4 μg of either GFP/