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Expression of Tropomyosin 2 Gene Isoforms in Human Cardiac Tissue

Research Article

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Abstract

Previous studies have shown that although the transcript levels of TPM1α and TPM1k are expressed in human hearts in comparable levels, the level of TPM1α protein is ~90%. The proteins of TPM1κ and TPM2α are about 5% of the total sarcomeric TM. The TPM2 gene is known to generate three alternatively spliced isoforms, which are designated as TPM2α, TPM2β, and TPM2γ. The expression level of TPM2β and TPM2γ in human hearts is unknown. Using a series of primers pairs and probes for RNA PCR, we found that both TPM2α and β but not γ were expressed in fetal and adult heart tissue, with about the same amounts of each isoform in fetal hearts and more β than α in adult hearts. Four new isoforms of TPM2 RNA were identified (TPM2δ - η). Most of these were present in very small amounts in both the fetal and adult hearts with the exception of TPM2ξ, which was present at about 40% of the level of TPM2α in adult heart tissue. Western blot analyses using a series of anti-tropomyosin antibodies indicate that TPM2 protein is present in both fetal and adult hearts at about the same levels as TPM1κ and much less than TPM1α. We are unsure about the expression of TPM2δ, TPM2ζ, and TPM2η proteins in fetal and adult human hearts. The exact function of these new TPM2 isoforms in heart and their role(s) in cardiac disease remain to be elucidated.

Key Words: Alternatively Spliced; RT-PCR; Cloning & Sequencing; Westernblot Analyses.

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Introduction

Tropomyosin (TM) is a component of myofibrils, the contractile apparati of striated muscle cells [1-6]. Recent associations of TM mutations with various myopathies in humans have sparked renewed interest in the structural/functional relationships in TM [7-10]. Vertebrate TMs are expressed from four tropomyosin genes designated as TPM1, TPM2, TPM3, and TPM4 [1-6] except for fish where there are six TPM genes [11]. More than twenty distinct isoforms are generated via a complex pattern of alternative RNA splicing and alternative promoters. However, the functional significance of this isoform diversity is poorly understood. Also, it is not clear whether specific isoforms are required for assembly and integration into distinct actin-containing structures (Helfman et al. (1999). Helfman et al. using GFP tagged TM have shown that both muscle and nonmuscle isoforms can be incorporated into the I-band of neonatal rat cardiac myocytes (NRCs). However, the functional role of each of the non-muscle isoforms in cardiac contractility is yet to be elucidated.

TPM1 gene in vertebrates was known to form nine different alternatively spliced TM isoforms. We were the first to report the tenth TPM1 isoform, which happens to be sarcomeric isoform and is designated as TPM1κ [13,14,15]. Cooley and Bergstrom (2001) also reported several new isoforms of the TPM1 gene [16]. The *TPM2* gene is known to produce three TM isoforms, TPM2α (muscle isoform), TPM2β (TM-1 or nonmuscle isoform), and TPM2γ (in HeLa cells) via alternate splicing. To the best of our knowledge, an extensive analyses of isoform diversity of the TPM2 gene has not yet been reported. In this study, we have decided to explore this issue and search for the presence of novel TPM2 RNA and protein expression in adult and fetal human hearts. We have detected, cloned, and sequenced four new alternatively spliced isoforms of the *TPM2* gene from human adult and fetal hearts hearts. These are designated as TPM2δ, TPM2ε, TPM2ζ, and TPM2η. While preparing this manuscript, predicted sequences of several potential TPM2 isoforms including our TPM2ε and TPM2η have been submitted in the Genbank in 2014. Hence, our claim that this is the first report of the expression of TPM2δ, TPM2ε, TPM2ζ, and TPM2η in human cardiac muscles is well justified. Furthermore, using Western blot analyses and a variety of anti-tropomyosin antibodies, we have made an effort to detect the protein expression of all TM isoforms in fetal and adult human hearts.

Materials and Methods

Human adult and fetal cardiac total RNA was obtained from Zyagen (San Diego, CA) and similar protein samples were obtained from Imgenex (San Diego, CA). The samples represent in each case material from one adult heart and 5 fetal hearts.

Human skeletal muscle protein was obtained from Imgenex. The non-malignant human breast epithelial cell line MCF10A and the normal human B-lymphocytic cell line HCC 1143 (BL) were obtained from ATCC (Manassas, VA). Total cellular RNA and protein were prepared from the cell lines, as previously described. For

RT-PCR, 0.5 µg of RNA in a total volume of 40 µl was used to synthesize cDNA with SuperScript[R] II (Life Technologies, Grand Island, NY) and oligo-dT primers following the manufacturer's specifications. For each PCR 3 µl of cDNA was used, GAPDH housekeeping gene RNA was amplified as previously described. RT-PCR for TPM2 RNA isoforms utilized the following primers located in different exons of the complete TPM2 RNA transcript (Fig 1).

exon 1: 5´ - ATGGACGCCATCAAGAAGAA - 3´ (+)
exon 9a: 5´- CTTGTACTTCATCTTCTGGGCATAG - 3´ (-)
exon 9b: 5´ - CACATGCAGTGGTGAATCA - 3´ (-)
exon 9d: 5´ - TGGGGCTGGCCCTCACAGGTT - 3´ (-)
TPM1α and TPM1κ primers and probes were as previously described.

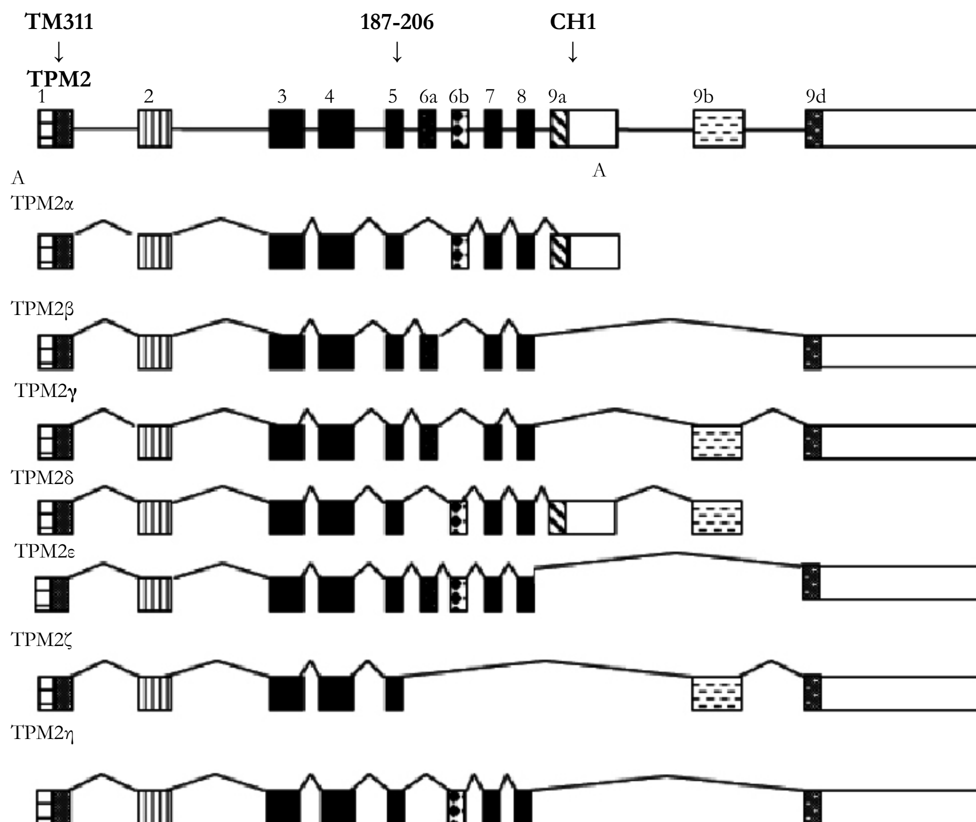
Amplified DNA was detected using the following probes.
exon 5: 5´ - AGAGGGCTGAGGTGGCCGAGAGCCG - 3´ (+)
exon : 5´ - CCTGGCACTGAGCCCACCCACAA - 3´ (+)

cDNA was amplified and detected using Southern blot hybridization, as previously described.

Amplified products were ligated and cloned into the TA cloning vector (Life Technologies) following our published protocol (13). Positive clones were identified using the above specific probes, as previously described. Vectors were grown in E. coli, and the DNA was extracted using Qiagen mini-prep kit (Valencia, CA). The isolated DNA was then sequenced (Cornell University Life Science Core Laboratories Center, Ithaca, NY). Each colony was sequenced twice in both directions. The relative amounts of each TPM2 isoform was determined by comparing their Southern blot hybridization signals to that of the GAPDH of reference housekeeping gene signal, as previously described. The frequency of various TPM2 isoforms was determined by sequencing DNA from 100 different colonies from fetal heart cDNA, and 5 different colonies from adult heart cDNA originally amplified with the TPM2 exon 1 and exon 9d primers.

LDS sample buffer and sample reducing agent (Life Technologies) were added to the protein extracts, and SDS PAGE was carried out following our published protocol. About 10 µg of protein extracts from human adult and fetal hearts, human skeletal muscle and the cell lines MCF 10A and HCC 1143 (BL) were heated at 70°C and subsequently loaded into Novex NUPAGE 4-12% BIS-TRIS gels in MOPS running buffer with antioxidant added (Life Technologies), and run for 1 h at 200 V in a Cell Sure Lock Mini-Cell apparatus (Life Technologies). The gels were transferred to nitrocellulose membranes using the same apparatus, under transfer buffer (Life Technologies) supplemented with methanol and antioxidant. Ponceau reversible staining was done to test loading consistency and transfer efficiency. The blots were blocked in 5% dry milk powder (Nestle HealthCare Nutrition, Inc., Florham Park, NJ), in TBST (10x TBS, 0.05% Tween-20) overnight at 48°C. Primary antibody incubations were carried out for 1 h at room temperature. Blots were washed in TBST after both primary and secondary antibody incubations. Chemiluminescence was accomplished using ECL detection reagents (Amersham, Piscataway, NJ) and exposing the blots to x-ray film (Fuji Film, FP-3000B), following the manufacturer's protocol. Antibodies were diluted in the 5% milk powder blocking solution. Primary antibodies included TM311 (Sigma-Aldrich, St. Louis,

Figure 1. Alternative splicing patterns of human TPM2 gene



Exon composition of TPM1 gene [1-6] and alternative splicing that generates 7 isoforms designated as TPM2α, TPM2β, TPM2γ, TPM2δ, TPM2ε, TPM2ζ, and TPM2η. of the seven, TPM2α to TPM2γ were known. Four novel isoforms are TPM2δ to TPM2η are novel isoforms.

MO) anti-TPM1ϰ, CH1 (Sigma) and the anti-TPM2 exon 6a, 187-206 (a kind gift of G.L. Prasad, Department of General Surgery and Cancer Biology, Wake Forest University School of Medicine, Winston-Salem, NC). Secondary antibodies were goat anti-rabbit immunoglobulin HRP and sheep anti-mouse immunoglobulin HRP (GE Healthcare).

Results

Cloning and sequencing of four novel TPM2 isoforms

Both human fetal and adult heart tissue expressed TPM2α and TPM2β but not TPM2γ (Table 1). Four novel TPM2 RNA isoforms were detected by RT-PCR, which we have labeled TPM2δ, TPM2ε, TPM2ζ, and TPM2η (Table 1). These were present in both the adult and fetal heart tissues. The organization and exon content of each of the above TPM2 isoforms is shown in Figure 1. The signal intensities of the Southern blots of each isoforms relative to GAPDH are shown in Table 1. The nucleotide as well as the deduced amino acid sequences of these isoforms are presented in Figure 2. TPM2δ encodes 284 amino acid residues, which are identical with TPM2α. The only difference between TPM2α and TPM2δ is the 3'-UTR. The transcript of the TPM2α ends at exon 9a whereas the transcript of TPM2d includes exon

Table 1: Tropomyosin 2 gene RNA isoform expression in human cardiac tissue

	Relative RNA isoform expression									
Tissue type	**TPM1α**	**TPM1κ**	**TPM2α**	**TPM2β**	**TPM2γ**	**TPM2δ**	**TPM2€**	**TPM2ζ**	**TPM2η**	**GAPDH**
fetal heart	++	++	++	++	-	+	+	+	+	++++
Adult heart	++++	+++	++	+++	-	+	+	++	+	++++
MCF 10A	-	-	-	+++	-	-	-	-	-	++++
HCC 1143 (bL)	-	-	-	-	-	-	-	-	-	++++
The signal intensities on Southern bolt were scored as negative or positive. Positive signal were ranked + to ++++										

Figure 2. Nucleotide and deduced amino acid sequences of novel isofoms of human *TPM2* gene

Top line: Nucleotide sequence. **Bottom line:** Deduced protein sequences
A: TPM2δ, **B:** TPM2ε, **C:** TPM2ζ, and **D:** TPM2η

Table 2: Tropomyosin 2 RNA isform frequencies in human cardiac tissue

		Tropomyosin 2 RNA isform frequencies among CDNA clones					
Sample	Total Colonics	β%	γ%	δ%	€%	ζ%	η%
Fetal Heart	100	96(96)	0	1(1)	1(1)	1(1)	1(1)
Adult Heart	20	17(85)	0	0	0	3(15)	0
MCF 10A	20	20(100)	0	0	0	0(0)	0

Table 3: Tropomyosin 2 protein expression in human cardiac tissue

	TM311$^{\Delta}$						anti-TPM1κ	CH1			187-206
	>40kD	40kD	38kD	36kD	34kD	<34kD		40kD	36kD	34kD	
											TPM2β
		TPM1βκ						TPM1κ			TPM2γ
Tissue type	TPM2γ	TPM2α,δ	TPM2β,η	TPM1a,γ	TPM1δ	TPM2€,ζ	TPM1κ	TPM2α	TPM1α	TPM3α	TPM2€
Fetal heart	-	+	+	++++	-	-	+	+	++++	-	+
Adult heart	-	+	+	++++	-	-	+	+	++++	-	+
Skeletel music	-	+++	-	+++	+++	-	-	+++	+++	+++	+
MCF 10A	-	-	+++	++	-	-	-	-	-	-	++
HCC 1143 (BL)	-	-	-	-	-	-	-	-	-	-	-

9b due to the novel splicing. The splice variant TPM2ε contains both exon 6a and 6b. The transcript stops at exon 9b. This isoform encodes 213 amino acid residues due to the formation of a premature stop codon after exon 6a and at the beginning of exon 6b. The nucleotide as well deduced amino sequences of TPM2ε are identical with those of the predicted splice variant X7 (XM_005251571). TPM2ζ isoform encoding 240 amino acid residues contains exon 1, 2, 3, 4, 5, 9b, and 9d and lacks both 6a and 6b (Figure 2C). The other novel isoform TPM2η encodes 284 amino acid residues, which are identical with TPM2β except for exon 6. TPM2β contains exon 6a whereas, TPM2η contains exon 6b. The amino acid sequence of this isoform is identical with the predicted TPM2 variant X6 (XM_005251570).

Expression of transcripts of the novel TPM2 isoforms

TPM2α and β were equally expressed in fetal heart, while TPM2β was expressed a bit more in adult hearts. TPM2δ, TPM2ε, and TPM2η gave weak signals for adult heart tissue; they and TPM2ζ gave weak signals in the fetal heart tissue. However, TPM2ζ gave a stronger signal in adult heart. In order to be more precise with the relative frequencies of the TPM2 isoforms β through η, we amplified with the common exon 1 and exon 9d primers and analyzed 100 and 20 colonies of cloned cDNA from the fetal heart and adult heart tissue, respectively (Table 2). It should be remembered that these primers would not amplify TPM2α (Figure 1). As can be seen, 96% of the colonies in the fetal heart were TPM2β, none were TPM2γ and the other isoforms were present in 1 colony each. In the adult heart 85% of the colonies were TPM2β and 15% were TPM2ζ. As can be seen, in the adult heart tissue, there is much more TPM1α than TPM1ϰ, TPM2α or TPM2β RNA, but TPM2α and TPM2β RNA is present at about the same or slightly greater amounts as TPM1κ (Table 1). In the fetal heart tissue TPM1α is, again, the more prevalent isoforms while there is slightly more TPM1ϰ than TPM2α and TPM2β.

Western blot analyses of the extracts from fetal and adult human hearts with various anti-tropomyosin antibodies

Table 3 shows Western blot data using the antibodies TM311, anti-TPM1κ, CH1 and TPM2 187-206. The various bands observed and, the known or putative TPM1, TPM2, and/or TPM3 isoforms that should be observed with those antibodies at a particular molecular weight are indicated. TPM1α was the dominant tropomyosin protein in both adult and fetal hearts with much lower levels of TPM1κ present in both as well. TPM2 protein was present in both, as well, in amounts roughly equivalent to TPM1κ. No TPM2γ or TPM2ε or TPM2ζ protein expression was observed. Given the low level TPM2η RNA observed then, by deduction, there is definite expression of TPM2β protein in both fetal and adult hearts at the level of TPM1κ. Given that we have no specific TPM2α antibody and that TPM2α protein comigrates with TPM1κ, we cannot definitively state that TPM2α is expressed.

Discussion

We have identified and characterized four different TPM2 splice variants in human fetal and adult hearts. Due to a lack of isoform-specific antitropomyosin antibodies, we are unsure about the protein expression from these splice variants in human hearts. The muscle type novel isoform TPM2δ and the known TPM2α isoform have distinctly different 3'-UTRs but encode the identical protein containing 284 amino acid residues. Currently, we do not know whether these two isoforms are expressed in the same cardiac tissues for example, myocytes, or fibroblasts or any other cell types. As the 3'-UTRs are known to play a critical role in the translation of a variety of mRNAs [17-20], we can speculate that these two mRNAs may be differentially translated in different cardiac cell types for example myocytes, fibroblasts, etc. depending on their specific requirements. Before drawing a definitive conclusion, it is an essential pre-requisite to determine the exact expres-

sion patterns of these two mRNAs in different cardiac cell types.

In a classical sense, three of the four novel isoform TPM2ε, TPM2ζ, and TPM2η are non-muscle isoforms because none of them contain exon9a that encodes the troponin binding domain of sarcomeric TMs. Troponins are an indispensable components of thin filaments in sarcomere in muscle tissues [1-5]. However, the permanent cellular constituents of the heart include myocytes, cardiac fibroblasts, endothelial cells, and vascular smooth muscle cells [2]. Therefore, these novel TPM2 isoforms may be expressed in cardiac cell types other than cardiomyoctes. Current dogma states that fibroblasts make up the largest cell population of the heart [21]. Again, cardiac fibroblasts play a critical role in maintaining normal cardiac function, as well as in cardiac remodeling during pathological conditions such as myocardial infarct and hypertension. Hence, it is not illogical to speculate that some of these isoforms may play a critical role(s) in heart development/function.

Helfman et al. [12] using GFP or other tagged non-muscle TM isoforms (TM-1, -2, -3, -4, -5(NM1), -5a or -5b) and striated muscle (skeletal muscle α-TM) isoforms have shown that these non-muscle TM isoforms are incorporated into actin filaments in neonatal rat cardiomyocytes (NRC). All of these non-muscle TM isoforms, were localized into the I-band of NRCs. In other word, these researchers showed that specific isoforms characteristic of nonmuscle cells can incorporate into the myofibrillar apparatus of cardiomyocytes. Incorporation of these GFP non-muscle TM fusion proteins into the myofibers exhibited sarcomeric shortening and cell beating. However, they did not address whether the nonmuscle isoforms can substitute functionally for sarcomeric TM proteins or not. Following their lead, we are planning to study the myofibrillogenesis in axolotl hearts with GFP. TPM2η or other novel TPM2 isoforms. Mexican axolotl (*Ambystoma mexicanum*) is a unique animal model for studying structural/functional relationships of various myofibrillar proteins especially tropomyosin. Some of these animals carry a genetic mutation in gene "c" where "c" stands for cardiac lethal. The homozygous (c/c) mutant axolotl embryos form hearts that do not beat. As a result, the embryos die after hatching due to a lack of circulation [22-25]. The mutant hearts are deficient in sarcomeric TM protein and do not form organized myofibrils. However, the transfection of TPM1α or TPM1κ expression construct into mutant axolotl hearts allows the ectopic expression of TM protein and thereby helps to form myofibrils and let the mutant hearts beat *in situ*. We will also perform the transfection assays in normal axolotl hearts, which will allow us to understand whether the transfected novel TM isoforms can incorporate into organized myofibrils with help of endogenous saromeric TM proteins [12,26]. Transfection assays with mutant axolotl hearts will allow us to study whether an ectopically expressed non-muscle TM protein alone can form the myofibrils as was observed with sarcomeric tropomyosin or an ectopic overexpression of non-muscle TM disarrays the organized myofibrils that subsequently affect the contractility of the normal axolotl hearts *in situ*.

It is worth mentioning at this point that Assinder et al (2010) reported the expression of a novel TPM2 splice variant (identical with our TPM2ε) in human prostate cancer tissues, which is absent from the normal human prostate epithelial primary cells. By Western blot analyses using TM311 antibodies they also reported the expression of a ~25kDa TM protein in human prostate tissue extracts [27]. Interestingly, TPM2ε isoform encodes a ~25 kDa protein due to the presence of a premature stop codon immediately after codon 6a. Most importantly, the ~25 kDa protein is not present in normal prostate tissues. At this juncture, we would like to point out that in a separate study, we have also detected the expression of TPM2ε transcript in three different human breast cancer cell lines but not in normal breast cell line (MCF10) (unpublished). In addition, we have detected the expression of TPM2η in one breast cancer cell line but not in normal human breast cell line (unpublished results). Hence, one cannot rule out the possibility of the association of development of cancer cell phenotype in humans.

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