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Recent Advances on the TPM4 Gene Expression in Humans

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Abstract

Tropomyosin, a coiled-coil dimeric protein, is a component of thin filaments that constitute myofibrils, the contractile apparatus of striated muscles. It is also a component of the actin filament network in non-muscle cells. In vertebrates, except for fish, there are four known TPM genes (TPM1, TPM2, TPM3, and TPM4) each of which can generate a number of TPM isoforms via alternative splicing and/or using alternate promoters. In humans, except for TPM4, the sarcomeric TPM isoform (s) for each TPM gene have been known for long time. Recently, we have cloned and sequenced the sarcomric isoform of the TPM4 gene designated as TPM4a. In addition, using qRT-PCR we have reported the expression of TPM4a in human striated muscles. Also, using 2D Western blot analyses followed by Mass spectra, we have reported the potential TPM4a protein expression in human cardiac tissues. However, much of the role of TPM4a in muscle contraction in human is yet to be elucidated. Although the role of the TPM4 gene in human diseases in not well documented, new information is emerging in this regard. For example, of the TPM4 isoforms TPM4g has been reported as a non-invasive biomarker in prenatal diagnosis of congenital heart defects; mutations in TPM4 have been implicated in Macrothombocytopenia in humans; differential expression of two TPM isoforms TPM4b and TPM4g in human breast cancer cells. However, the role of TPM4-ALK oncogenes in inflammatory myofibroblastic tumors in humans is well documented.

Keywords: TPM isoform; Muscle; Gene expression; Human

Introduction

The tropomyosin (TPM) family of actin-binding proteins is a coiled-coil dimeric protein(s) present in all eukaryotes ranging from yeast to humans. In vertebrates, tropomyosin is expressed in muscle and non-muscle cells. TPM is well known for its role in muscle contraction where it regulates the interaction between actin and myosin. However, its regulatory role in the organization as well as dynamics of cytoskeleton has been acknowledged in recent years.

In vertebrates, except for fish, four TPM genes –TPM1, TPM2, TPM3, and TPM4 are known [1-10]. In zebrafish, there are six TPM genes that include the paralogs of the TPM1 (TPM1-1 & TPM1-2), the paralogs of the TPM4 gene (TPM4-1 & TPM4-2) and single copy TPM2 and TPM3 [4]. Each of the TPM genes is known to generate multiple tropomyosin isoforms via alternate splicing, and/or using different promoters or different polyadenylation sites.

Although this editorial is focused primarily on the diversity, roles in cellular processes and their implications of TPM4 isoforms in human diseases, we would like to start by briefly discussing these aspects of the other three TPM genes.

Isoform diversity of TPM1, TPM2, and TPM3 genes in humans

Of the four TPM genes, TPM1 is the most versatile one as far as the generation of isoforms is concerned. Previously, the human TPM1 gene was known to generate nine different alternatively spliced isoforms (Helfman). Of which, only one was thought to be specific for striated muscle contraction or sarcomeric isoform, which we call TPM1a (Table 1) [1,3,7,8]. We reported the expression of the tenth TPM1 isoform designated as TPM1k in human adult and fetal hearts [11]. TPM1k is the second sarcomeric isoform of the TPM1 gene, and has been found in various animals – amphibian [12], avian [13], and fish (unpublished results in zebrafish), and other mammals [14]. The TPM1 gene also generates one smooth muscle isoform known as TPM1b with exon 2a as in TPM1k and exon 9c/d instead of exon 9a/b, which is the integral part of the two sarcomeric isoforms TPM1a and TPM1k. In addition, four other alternatively spliced isoforms of the TPM1 gene have been reported [9]. Some of them are of high molecular weight, containing mostly 284 amino acid residues, and many of them are of low molecular weight, containing mostly 248 amino acid residues. Although exon compositions and transcript expression of all isoforms are known, the encoded protein expression of all TPM1 isoforms is yet to be established. TPM1l has been shown to be upregulated in human breast cancer cell lines [9].

The TPM2 gene is known to produce one sarcomeric isoform called TPM2a, a smooth muscle isoform TPM2b, and another high molecular weight isoform TPM2g (Table 1) [1-6]. It also generates 4 more alternatively spliced isoforms generating both high molecular and low molecular weight proteins [15]. The sarcomeric isoform TPM2a is expressed in all vertebrates. Several missense mutations in TPM2 have been implicated in in various myopathies in humans [15,16]. Down modulation of TPM2b has been observed in human breast cancer cell lines [15,17].

Via alternate splicing the TPM3 gene generates 12 isoforms, one of which one encodes a high molecular weight protein TPM3a containing 285 amino acid residues. All others are low molecular weight proteins encoding 248 amino acid residues. The exon composition of TPM3a is given in Table 1. TPM3a is known to be involved in slow skeletal muscle contraction and several missense mutations in TPM3a have been implicated in nemaline myopathy in humans [18]. Recently two groups have reported the expression of TPM3a protein in human hearts

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Nomenclature of Various High Molecular wt Isoforms of TM referred to in this article		TPM Gene encoding the Isoforms: New Nomenclature (Old Nomenclature)	Various isoforms of TM currently known as	Exon Composition
Our Nomenclature. Narshi et al. (2005)*	Nomenclature by Geeves et al. (2015)**			
TPM1α	Tpm1.1	TPM1 (αTM)	Striated Muscle	1a,2b,3,4,5.6b,7,8,9a/b
TPM1β	Tpm1.3	TPM1 (α-TM)	Smooth Muscle	1a,2a,3,4,5,6b,7,8,9d
TPM1γ	Tpm1.6	TPM1 (α-TM)	TM-2 Fibroblast	1a,2b,3,4,5,6b,7,8,9d
TPM1δ	Tpm1.7	TPM1 (α-TM)	TM-3 Fibroblast	1a,2b,3,4,5,6a,7,8,9d
TPM1κ	Tpm1.2	TPM1 (α-TM)	Striated/Card	1a,**2a**,3,4,5,6b,7,8,9a/b
TPM2α	Tpm2.2	TPM2 β-TM)	Striated/Sk Muscle	1,2,3,4,5,6b,7,8,9a
TPM2	Tpm2.1	TPM2	Smooth Muscle (TM-1)	1,2,3,4,5,6a,7,8,9d
TPM3α	Tpm3.12	TPM3 (hTMnm)	Sk.Muscle	1a,2b,3,4,5,6b,7,8,9a/b
TPM4α	None	TPM4 (TM)	StrTM4	1a,2,3, 4, 5,6,7,8,9a/b
TPM4	Tpm4.2	TPM4	Smooth muscle (TM4)	1b, 3,4,5,6,7,7,8,9d1
* Reference number, ** Reference number [10]				

Table 1: Nomenclature and exon composition of various human high molecular weight TPM isoforms.

in a low quantity. Our unpublished results also support the expression of TPM3a protein in human hearts [19].

The principal tropomyosin isoforms found in human striated muscles are TPM1a and TPM1k from the TPM1 gene, TPM2a from the TPM2 gene, and TPM3a transcribed from the TPM3 gene. Two groups independently reported that a knockout of the TPM1 gene in mouse is embryonic lethal, suggesting the essential role of TPM proteins in development [20,21]. Both groups showed that ablation of one allele resulted in heterozygous knockout mice. In other words, one of the two alleles of the TPM1 gene is functional. This ablation caused a ~50% reduction of TPM1a transcripts in the targeted mouse hearts. Despite the ablation of one TPM1 allele a concomitant increase in transcript level from other sarcomere TM isoforms, for example, TPM2b was not seen. Interestingly, there was no reduction of TPM1a protein in ablated mice. Rajan et al. [22] created transgenic mouse (Tg) overexpressing TPM1k protein in a cardiac-specific manner. Anatomical analyses displayed no differences between Tg and NTg mice. However, a functional difference by echocardiography was found with increased end-systolic and end-diastolic left ventricular dimensions inTPM1k Tg mice. This resulted in the development of a dilated cardiomyopathy like syndrome in transgenic mice over-expressing TPM1k. Rajan et al. also showed an increased expression of TPM1k protein in the hearts of patients with chronic dilated cardiomyopathy (DCM) and in hearts from end stage heart failure patients. In a transgenic mouse model, the overexpression of TPM2a isoform to 57.8 % of total heart tropomyosin resulted in altered diastolic function with reduced rates of contraction and relaxation of isolated myocytes [23]. If the overexpression of TPM2a protein is over 75% of total sarcomeric tropomyosin, the mice die 10 to 14 days after birth and show severe cardiac abnormalities and thrombus formation [23]. In the mouse model for overexpressing of the TPM3a isoform, the mice developed tachycardia and reduced Ca^{+2} sensitivity [24]. Both TPM2 and TPM3 genes have been implicated in Nemaline myopathy in humans [15,18] whereas TPM1 has been implicated in various cardiomypathies [25].

Isoform diversity of the human *TPM4* gene

TPM4a, the cardiac specific sarcomeric isoform of the *TPM4* gene, has been known and studied for a long time in non-mammalian systems (Table 1) [26,27]. In chickens, TPM4a is the only sarcomeric tropomyosin isoform in adult hearts although TPM1a, TPM1k, and TPM2a along with TPM4a are expressed in embryonic chicken hearts [13]. In zebrafish, knocking out TPM4-1a results in a non-beating heart proving that it is essential for cardiac contractility [28].

In mammals, the exon and intron organization of *TPM* genes are very similar, if not identical, except for *TPM4*. In rodents, exons 1a and 2 are absent or truncated from the TPM4 gene; as a result, the high molecular weight isoforms encoding 284 amino acid residues such as TPM4α and TPM4β are not expressed as they are in non-mammalian species (Figure 1) [1]. It was speculated for a long time that the *TPM4* gene in humans, as in rodents, is also truncated [1,2]. Hence, it was assumed that the *TPM4* gene in humans also did not encode the high molecular weight protein with 284 amino acid residues. The expression of a high molecular weight TPM4 protein was reported first in human ovarian tumor tissues in 2004 (Accession # AK023385). This isoform, defined as TPM4β, contains exons 1a, 2, 3, 4, 5, 6, 7, 8, and 9d (Figure 1), which differs from the sarcomeric TPM4α isoform containing exon 9b instead of 9d. It is to be noted that exon 9a/b, but not 9d, only encodes the peptide that is essential for binding TPM with troponins [2,3], essential components of thin filament. Although the entire human genome sequence has been known for a while, the existence of the exon 9a in the *TPM4* gene was not reported until recently. To the best of our knowledge, the expression of the TPM4α isoform in humans has not been reported. Recently, two predicted sequences (derived from a genomic sequence) of the two different isoforms of the human TPM4 gene containing exon 9a have been reported in Gen Bank (on March 12, 2015). One of the predicted isoforms is TPM4α (as in avian and amphibians) containing exons 1a, 2, 3, 4, 5, 6b, 7, 8, 9a/b encoding 284 amino acids (Accession # XM_006722865). The other one may encode a low molecular weight novel protein with 248 amino acids consisting of exon 1b, 3, 4, 5, 6, 7, 8, 9a/b (Accession # XM_005260042.2). We have for the first time reported the expression of these two new transcripts, viz TPM4α and TPM4δ, by RT-PCR with the RNA from human adult heart, fetal heart and skeletal muscle. We confirmed the sequences of both the isoforms by cloning and sequencing of the amplified products. We also determined the relative expression of the two transcripts by qRT-PCR with RNA from human adult heart, fetal heart, and skeletal muscle using isoform specific primer-pairs (Figure 2). Our results show that the transcripts of these two isoforms are expressed in fetal heart, adult heart, and skeletal muscle. The expression level is somewhat higher in hearts compared to skeletal muscle (Figure 2). Also, the expression of TPM4a is significantly higher in fetal human hearts.

In order to demonstrate the expression of TPM4a protein in human hearts, we have performed 2D western blot analyses with CH1 monoclonal antibody specific for sarcomeric TPM isoforms. The gel-extracted proteins from various spots with positive signal were analysed by LS-MS/MS. The results indicate the expression of TPM4a (Figure 3)

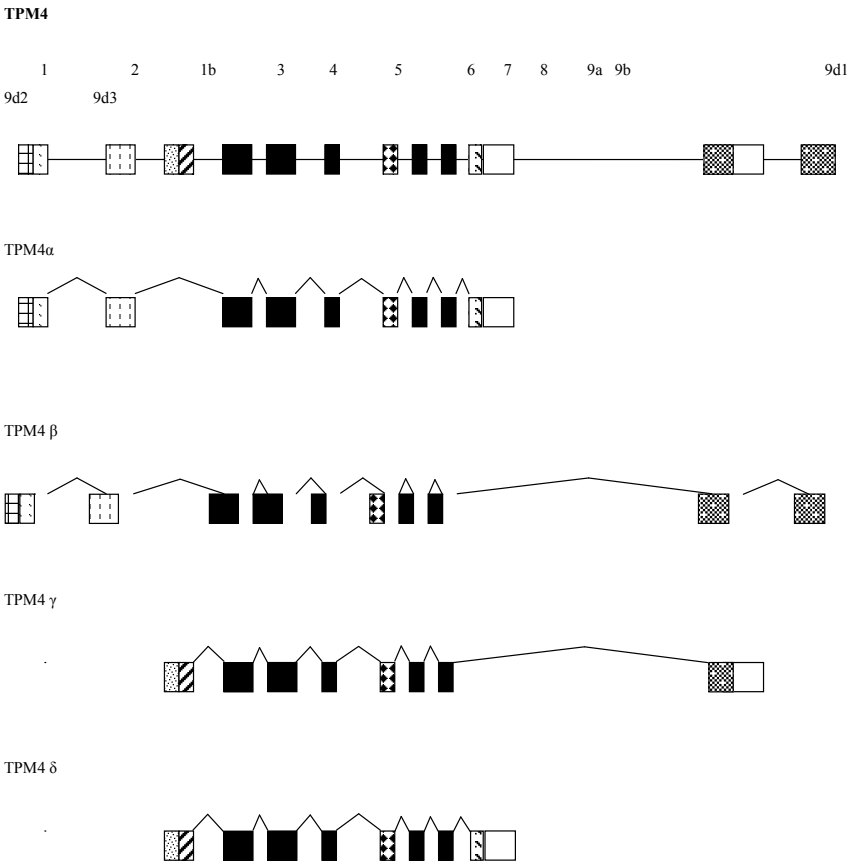

Figure 1: Alternative splicing pattern of the TPM4 gene in humans.

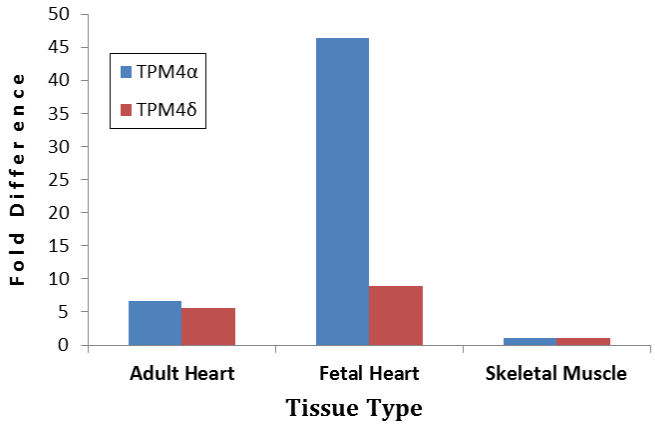

Figure 2: Relative expression of TPM4α and TPM4δ in human adult heart, fetal heart, and skeletal muscle.

TPM4α

```
  1  MEAIKKKMQM LKLDKENAID RAEQAEADKK AAEDKCKQVE EELTHLQKKL
 51 KGTEDELDKY SEDLKDAQEK LELTEKKASD AEGDVAALNR RIQLVEEELD
101 RAQERLATAL QKLEEAEKAA DESERGMKVI ENRAMKDEEK MEIQEMQLKE
151 AKHIAEEADR KYEEVARKLV ILEGELERAE ERAEVSELKC GDLEEELKNV
201 TNNLKSLEAA SEKYSEKEDK YEEEIKLLSD KLKEAETRAE FAERTVAKLE
251 KTIDDLEDEL YAQKLKYKAI SEELDHALND MTSL
```

Figure 3: Deduced amino acid sequences of human TPM4a and the dentification of amino acid sequences from the peptides extracted from CH1 antibody positive signals from 2D western blot with adult human heart extract {30]. Red color letters indicates the isolated peptides that have been sequenced by Mass spectrometry. Three amino acid residues in exons 1a, 8, and 9a are different in TPM4α compared to human TPM1a is marked as bold.

[29] and TPM2a protein in adult human heart extracts [29]. In addition, we have detected the expression of TPM1a, TPM1k, TPM2a, and TPM3a. However, we have failed to detect the expression of TPM4d, a ~28 kDa protein, by both 1D and 2D western blot analyses using CH1 monoclonal antibody [29].

TPM4 expression in breast cancer cell lines

Stress fibers are the major contractile structures fibroblasts, smooth muscle, endothelial and some cancer cell lines. TPM4g or Tpm4.2 is expressed in various human cells and tissues. It performs a supporting role in different motile and contractile events. Besides many other functions, it is involved in the formation of stress fibres in fibroblasts. TPM4g?expression has been found to be upregulated in some types of cancer cells. In the case of infiltrating ductal breast carcinoma, an elevated TPM4g expression has been implicated to the aggressiveness of the tumours [30].

Although TPM4b was first detected and characterized from human tumor cells, its expression pattern in breast tumour cell lines is not well documented. The monoclonal antibody LC-24 is specific for TPM4. It recognizes both TPM4b and TPM4g as these isoforms contain the peptide encoded by exon 9d. Western blot analyses using LC-24

antibody show that TPM4g but not TPM4b is expressed in human heart (Table 2, unpublished results). TPM4g is long been known to be involved in stress fibre formation in non-muscle cells. Again, stress fibers play important role(s) in cancer metastasis, which is a multistep process. Hence, we evaluated the expression of TPM4b and TPM4g in a few breast malignant epithelial tumor cell lines as well as normal breast cell lines by western blot analyses with the extracts of various cells using LC-24 monoclonal antibody. Our results show that the high molecular TPM4b protein in malignant breast epithelial cells in varying quantity for example it is lower in MCF7 and BT-474; however it is higher in normal MCF10 and in some malignant breast cell lines like MDA-MB-453 (Table 2). On the contrary, the expression level of low molecular weight TPM4g is relatively high in MCF7 or it is similar in some malignant breast cell lines like in normal MCF10 cell lines. Zhang et al. also reported the expression patterns of two TPM4 isoforms viz. TPM4b and TPM4g in MCF7 (T) and HMEC (N) breast cell lines [31]. They also reported the expression of both the transcripts in normal and malignant breast cell lines. The results in Figure 4 strongly suggest that the expression level of TPM4g is higher in malignant cells whereas high molecular weight TPM4b is higher in normal cells. The results of Zhang et al. are in good agreement with our findings that the level of expression of TPM4g is higher in malignant breast cell lines (Figure 4) [31].

TPM4g protein as a non-invasive biomarker in prenatal diagnosis of congenital heart defects (CHDS)

Chen et al. in 2016 have reported that a comprehensive maternal serum proteomics helped identify TPM4g as one of the four protein markers for congenital heart defect. The other three proteins are LMNA, FLNA, and ACTG1. This discovery of cytoskeletal protein changes in maternal serum not only could help in prenatal diagnosis of CHDs, but also may shed new light on CHD embryogenesis studies [32].

Breast Cell Line	TPM4 protein expression with LC-24 antibody*	
	TPM4β	TPM4γ
MCF7	-	+++
BT 474	-	++
HCC1187	++	++
MCF10A	++	++
Human Heart	-	+++
*Unpublished results		

Table 2: Western blot analysis of human adult heart and various breast cancer cell lines with LC-24 antibody.

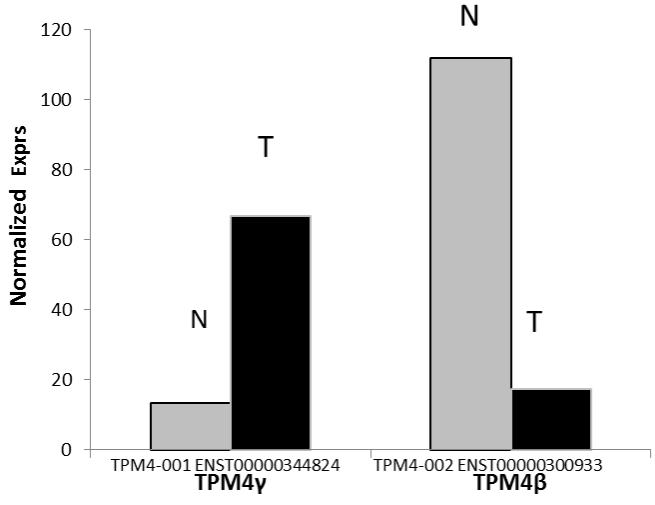

Figure 4: Mean normalized expression of TPM4b (ENST00000300933) and TPM4g (ENST00000344824) by qRT-PCR in non-oncogenic (HMEC designated as N) and oncogenic (MCF7 designated as T) [32].

Mutations in TPM4 cause Macrothombocytopenia in mice and humans

Gieger et al. in 2011 first reported that the common SNP rs8109288 in the first intron of the human TPM4 gene exerts an effect on the volume and count of platelets [33]. Pleines et al. isolated a mouse line with an ENU-induced missense mutation in TPM4 gene; mice carrying this mutation displayed macrothrombocytopenia in a dose-dependent manner. Interestingly, neither the life span nor the *in vitro* function of the mutant platelets was affected due to the deficiency of the TPM4. Also, there was no increase in propensity to bleeding [34]. Mutant megakaryocytes displayed altered morphology and the megakaryocytes numbers in the bone marrow were increased. Mutant megakaryocytes displayed altered morphology and markedly decreased Proplatelet formation *in vitro*. These authors then examined the functional requirements for TPM4 in human megakaryocytes by knocking down the expression using shRNA. They provided compelling evidence that TPM4 is a critical regulator of platelet production in humans. In fact, they actually found TPM4 mutated in three pedigrees with macrothrombocytopenia [34].

TPM4-ALK oncogenes in inflammatory myofibroblastic tumors

Chromosomal rearrangements involving TPM4 and ALK are known to cause some human inflammatory myofibroblastic tumors. ALK is the gene for a receptor tyrosine kinase, which is expressed normally in neural tissues. However, TPM4 and ALK fusion protein is overproduced in the myofibroblastic spindle cells, which in turn makes the inflammatory myofbriloblastic tumor. This may occur in many different parts of the body. Like TPM4-ALK, TPM3-ALK is an oncogene in inflammatory myofibroblastic tumors [35].

Concluding Remarks

The immediate future objective is to further confirmation of the TPM4a protein expression in adult human hearts. As the expression of TPM4a transcripts is higher in human fetal hearts one should also evaluate the protein expression in fetal hearts. The most important as well as the most difficult question yet to be addressed is whether minute expression of the sarcomeric TPM4a proteins plays any significant role in cardiac contractility and/or cardiac function. As TPM4a is not expressed in rodent, knock out or knock down experiment is not possible in rodent. However, one can create transgenic mouse over-expressing human TPM4a in a cardiac-specific manner as has been done for TPM1a, TPM1k, TPM2a and TPM3a (ref) and evaluate the effect of various level TPM4a expression on the cardiac contractility and physiological function as was done in case of TPM1k [22].

Equally important would be to understand the functional role of TPM4b and TPM4g on stress fiber formation in non-muscle cell and/or in cancer cells. One can create GFP. TPM4b or TPM4g fusion protein expressions construct(s), transfect non-muscle and/or breast cell line, and evaluate the dynamics of each isoform in non-muscle as well as in various malignant breast epithelial cell lines using Fluorescence Recovery after Photo bleaching (FRAP). One may also down regulate each TPM4 isoform separately using isoform specific siRNAor shRNA and evaluate the phenotype of the transfected cells or cell lines. However,

immunohistochemical analyses with an existing anti-TPM4 antibody like LC-24 would not be of much help as the antibody recognizes both isoforms. Of course, one can try to generate TPM4b or TPM4g specific antibodies.

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