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Missplicing of Troponin T transcripts in Myotonic dystrophy type 2 human biopsies

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Abstract

An RNA-mediated toxic gain-of-function has been indicated as the pathogenic mechanism underlying Myotonic dystrophies type 1 and type 2 (DM1 and DM2), two dominantly inherited myopathies with complex phenotypes and involvement of multiple organs. A number of gene transcripts are aberrantly spliced in DM1 and DM2 leading to the expression of foetal isoforms during the adult life. In particular, mutated mRNAs derived from the Troponin T (TnT) genes in striated muscles have been reported. The aim of this work is to demonstrate the abnormal expression of foetal TnT isoforms in adult DM2 human biopsies. PCR-amplified products have been analysed by agarose-gel electrophoresis, and amplicons abnormally migrating, with respect to the controls, have been sequenced. We show here that at least two isoforms derived from the alternative splicing within the hypervariable region of the fast TNNT3 gene still contain the foetal exon. The persistence of the foetal exon has been found both within a full-length isoform not previously described in adults and in association with a shorter sequence derived from alternative splicing in the hypervariable region.
Key Words: myotonic dystrophy type 2, troponin T isoforms, alternative splicing, foetal exon

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Muscle contraction is initiated by the release of Ca^{2+} from the cellular stores and its binding to the trimeric troponin (Tn) complex. When Ca^{2+} concentration is increased up to 1 mM, a conformational change is produced in the troponin complex that is transmitted to tropomyosin (Tm) dimers located along F-actin in the groove of the thin filaments. The altered conformation leads to an increased binding affinity, and then stronger interactions, between myosin heads and actin filaments with subsequent muscle contraction and force generation.

The Tn complex is formed by the protein subunits TnT, TnI and TnC. TnC binds Ca^{2+} and undergoes a conformational change which exposes binding sites for the inhibitory component TnI, thus removing its inhibitory effect on actin-myosin interaction. By means of TnT, the Tn complex binds to the tropomyosin (Tm) permitting its displacement from the actin and, in turn, the formation of bridges between myosin and actin. Finally, the myosin head discloses its actin-dependent ATPase activity which is the basis for the sliding of filaments and hence muscle contraction.

This highly ordered sequence of molecular events makes the fine regulation of muscle contraction to occur. Furthermore, TnI, TnT and Tm are encoded by different genes in different muscle fibre types and each gene may undergo alternative splicing leading to huge muscle plasticity. In humans all skeletal muscles are mixed in terms of fibre type composition and each muscle has a very wide range of specific isoforms, accordingly. It must be also considered that the complete set of proteins is subjected to development-driven variations and that even subtle alterations of this great diversity may be the basis for pathological phenotypes (see [11,18,28] for review).

Myotonic Dystrophy (DM) is a dominantly inherited myopathy characterized by a wide range of symptoms and by a variable expressivity. Two types of the disease are known (DM1 and DM2) both belonging to the group of tandem repeat expansion disorders. Despite their overlapping phenotype, they can be distinguished on clinical features and chromosomal localization. DM1 is caused by the expansion of a CTG repeat in the 3' UTR of a gene coding for the Myotonic dystrophy protein kinase (DMPK). DM2 is caused by the amplification of a CCTG repeat within the first intron of the gene encoding the RNA binding protein ZFN9. The new definition of spliceopathy has been

Table 1. Human slow skeletal muscle TnT isoforms – Differences in length, molecular mass and isoelectric point

SP Identifier	Exons							Length (AA)	Mr (kDa)	pI
	4	5	6	7	8	11a	11b			
P13805-1	+	+	+	+	+	+	+	278	32.9	5.86
P13805-2	+	-	+	+	+	+	-	251	30.1	6.14
P13805-3	+	+	+	+	+	+	-	262	31.2	5.64

Three human slow skeletal muscle TnT isoforms are retrieved from the SwissProt (SP) database. In muscle tissue only two isoforms (P13805-2 and -3) are expressed (see ref. [19] and references therein). Transcripts from the longest isoform were never found in tissue extracts and probably derive from a minor clone very poorly represented in vivo. The ratio between transcripts of lower and higher Mr with respect to the normal control was found to decrease or increase in DM1 and DM2, respectively (see ref. [19] and Table 2). Note that the presence of exon 5, corresponding to the peptide APEEPEPVAEPE, confers a more acidic pI other than a higher Mr to the protein.

introduced to explain the pathogenic mechanism shared by the two conditions [17]. In fact, dysregulation of alternative splicing of many RNA transcripts (insulin receptor, muscle-type chloride channel, fast troponin T, among other) may give a good explanation of the complex clinical appearance of these two genetically determined myopathies.

It has been demonstrated that the expression of the immature form of the insulin receptor (the insulin-independent isoform IR-A) is increased as a consequence of the incorrect alternative splicing in DM1 [20], DM2 [19,21] and congenital form of DM1 [4]. It has also been shown by us and others [19,26] that DM2 muscle biopsies presented a higher degree of alternative splicing dysregulation for fast TnT transcripts (derived from TNNT3 gene) as compared with both DM1 and normal control muscles.

We have reinvestigated this aspect and here we report that at least five different transcripts were present after the total RNA extraction from adult DM2 biopsies and that two of them contained the foetal exon.

Materials and Methods

Patients and skeletal muscle biopsies

After written consent, DM2 muscle samples were obtained from three vastus lateralis, two triceps brachii and one biceps brachii. The size of CCTG expansions ranged from 70 to 2,500. Normal control muscles (vastus lateralis) were obtained from 6 subjects deemed free of neuromuscular disorders. In control samples, the percentage of type 2 fibres (determined by routine ATPase) varied from 60 to 85% of the total. All muscle biopsies were frozen in isopentane chilled in liquid nitrogen, immediately after surgery and stored at -80°C until use.

Table 2. Increased expression of the alternative RNA splicing transcript lacking TNNT1 exon 5 in DM2 muscle biopsies

	Number of cases	TNNT1 (-ex5 / +ex5)	TNNT1 (Δ %)	t TEST (p value)
Control	6	0.38 ± 0.14		
DM2 (all muscles)	6	0.44 ± 0.16	+ 33	0.080
DM2 (arm muscles only)	3	0.56 ± 0.06	+ 70	0.0019

Reported data are arbitrary densitometric units expressed as mean values ± SD. TNNT1 values refer to the ratio between lower (lacking exon 5) and upper (having exon 5) silver-stained fragments, as measured after RT-PCR and PAGE, using primers for the exon 5 as previously reported [19]. Data on DM2 biopsies are from the typical experiment represented in Fig. 1. Statistical significance ($p<0.05$) was calculated using an unpaired Student's t test (one tail). A very significant difference of the ratio was found in the subset of brachial muscles.

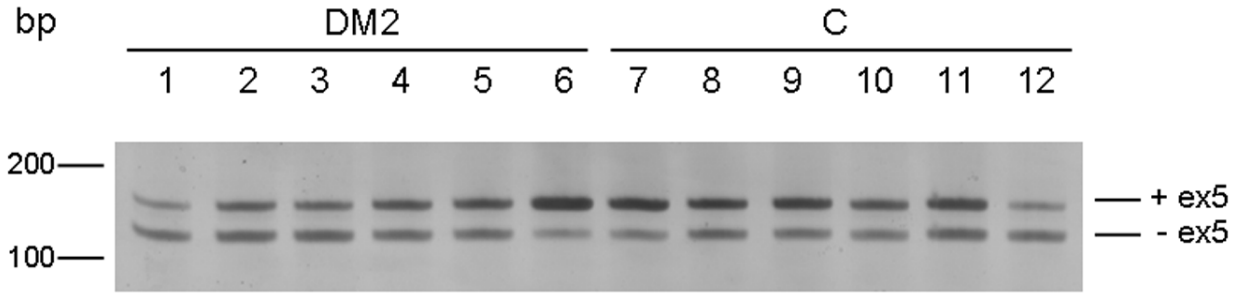

Fig. 1 *RT-PCR analysis of slow TnT expression in muscle biopsies from DM2 (lanes 1 to 6) and normal control (C; lanes 7 to 12). DNA fragments were resolved by polyacrylamide gel electrophoresis and stained with silver staining. RT-PCR amplified products were obtained from TNNT1 cDNA using specific primers flanking the exon 5, as previously described [19]. Only the range between 100 and 200 bp is shown. Lane 1: biceps brachii; lanes 2-3: triceps brachii; lanes 4-12: vastus lateralis.*

Preparation of RNA and reverse transcriptase-polymerase chain reaction on muscle biopsies

Total RNA was extracted from 5–10 mg of frozen muscle biopsies using the SV Total RNA Isolation Kit (Promega). RNA was eluted in 50 µl of RNase-free water, quantified on a spectrophotometer (Biophotometer Plus, Eppendorf) and stored at -80°C until use.

Synthesis of the first strand of cDNA was completed using Superscript II Reverse Transcriptase (Invitrogen, Carlsbad, CA, U.S.A.) with 400 ng of total RNA in a final volume of 20 µl, as described in the manufacturer's instructions. PCR reactions were performed with 0.4-1 µl cDNA as a template in 25 µl under standard conditions. α-tubulin was used as an internal control for cDNA quantification and normalization of the amplified products.

The RT-PCR analysis for TNNT3 variable region (exons 4 to 8) was carried out as previously described [19].

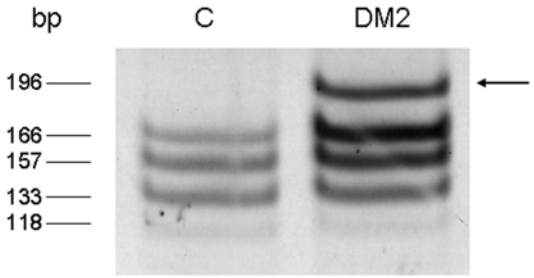

Fig. 2 *RT-PCR analysis of fast TnT expression in muscle biopsies from DM2 and normal control (C). DNA fragments were resolved by polyacrylamide gel electrophoresis and stained with silver staining. RT-PCR amplified products were obtained from TNNT3 cDNA using specific primers spanning exons from 2 to 11, as previously described [12, 19]. The length of the five detected bands were calculated from a curve derived from a DNA ladder (bp = base pairs).*

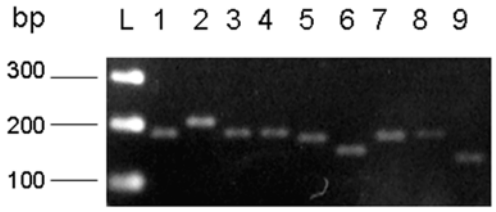

Fig. 3 *Batch purification of DNA fragments from low-melt agarose gel, cloning and sequencing of PCR products. After horizontal electrophoresis on a 3% low-melt agarose gel the bands were visualized with ethidium bromide (0.5 µg/ml) and those of higher Mr (about 196 bp) that were detectable in DM2 but not in the control muscles were sliced out, solubilized and extracted by using a gel extraction kit following the manufacturer's protocol. The fragments were eluted in a Tris-buffer and cloned in the suitable vector. After transformation in chemical competent E. coli strain, ten recombinant clones were picked and analysed by restriction digestion and horizontal electrophoresis on a 3% agarose gel. The clones were sequenced by automated cycle sequencing followed by capillary electrophoresis.*

Table 3. Cross-reference for the clones sequenced in Figure 3.

Clone no.	Insert length	Foetal exon	NM no.	NP no.	SP|P45387 SV no.
2	190	+	this work	this work	1
5	157	+	006757.3	006748.1	2
1	166	-	this work	this work	3
6	133	-	001042780.2	001036245.1	4
9	118	-	this work	this work	5

The clones were grouped according to their insert length which varied from 118 to 190 bp. Clones 1, 3, 4, 7 and 8 were of the same length and are reported here as clone 1. Accession numbers for DNA (NM) and protein (NP) sequences in the NCBI databases are given. SV = splice variants of fast skeletal TnT protein in the SwissProt (SP) database.

Purification, cloning and sequencing of PCR products

Amplified purified products were obtained by using QIAEX II gel extraction kit (Qiagen, Manchester, U.K.). After horizontal electrophoresis on a 3% agarose gel (Low Melt 4, AppliChem, St. Louis, MO, U.S.A.) the bands were visualized with ethidium bromide (0.5 μg/ml) and those of higher MW (about 196 bp) that were detectable in DM2 but not in the control muscles were sliced out, solubilized and extracted following the manufacturer's protocol. The fragments were eluted in a Tris-buffer and cloned in the TA-TOPO vector (Invitrogen). After transformation in chemical competent E. coli strain Top10 (Invitrogen) ten recombinant clones were picked and analysed by restriction digestion and horizontal electrophoresis on a 3% agarose gel.
The clones were sequenced by automated cycle sequencing followed by capillary electrophoresis (Applied Biosystems, BMR Genomics sequencing service).

SYBR green experiments

Quantification was performed in a 96-well IQ5 Thermal Cycler (Bio-Rad, Hercules, CA, U.S.A.). The reaction mix consists of 10 μl of 2x iQ SYBR Green Supermix (Bio-Rad), 0.3 pmol/ μl primers, 2 ng of cDNA and DNase/RNase-free water up to 20 μl. The PCR parameters were initial denaturation at 95 °C for 30 s followed by 40 cycles of 10 s at 95 °C and 30 s at the corresponding annealing temperature (55-59 °C) for acquisition of fluorescence signal.
The RT-PCR for TNNT3 mutually exclusive exons (16 and 17) was carried out with the following primers: TGAAACGCCAGAAATATGAC (forward) and GGTCCCAGCCTTCTTGCT (reverse). A melting curve was generated by the iQ5 software following the end of the final cycle for each sample, by continuous monitoring the SYBR Green fluorescence decrease throughout the temperature ramp from 65 °C to 99 °C in 0.5 s increments.

Results and Discussion

The heterotrimeric complex of troponins plays a crucial role in the contraction of striated muscles. In particular, TnT has direct interactions with the other components of the thin filament regulatory system. Crystallography and protein binding studies on TnT have identified two specific tropomyosin-binding sites in the middle conserved region, and one binding site for TnI and TnC, respectively, in the C-terminal segment [9,23,27]. The diversity of three TnT isoform genes is strictly related to the muscle fibre-type specificity and resides almost exclusively in the N-terminal region, giving the structural basis for functional and adaptive features in different types of muscles as well as in different stages of development. Importantly, the middle and the C-terminal regions of the three isoforms, where the main sites of interaction with other thin filament components are located, are the most conserved.
Instead, the N-terminal region is much more variable from one isoform to another and this variability is mainly attained through alternative splicing. This is

Table 4. Human fast skeletal muscle TnT isoforms – Differences in length, molecular mass and isoelectric point.

SP identifier	Exons								AA	Mr (kDa)	pI
	4	5	6	7	8	F	16	17			
P45378-1	+	+	+	+	+	+	+	-	269	31.8	5.71-5.75
	+	+	+	+	+	+	-	+			
P45378-2	+	+	+	-	+	+	+	-	258	30.6	6.07-6.19
	+	+	+	-	+	+	-	+			
P45378-3	+	+	+	+	+	-	+	-	261	30.8	6.67-7.02
	+	+	+	+	+	-	-	+			
P45378-4	+	+	+	-	+	-	+	-	250	29.7	8.40-8.69
	+	+	+	-	+	-	-	+			
P45378-5	+	+	+	-	-	-	+	-	245	29.1	9.01-9.12
	+	+	+	-	-	-	-	+			

Five human fast skeletal muscle TnT isoforms are retrieved from the SwissProt (SP) database. Only two isoforms (P45378-2 and -4) have been reported to be expressed in adult muscle tissue. Transcripts from the longest isoform were never found in adult tissue extracts. The calculated pI values are given as a range depending on the presence of either exon 16 or exon 17. Note that the presence of F-exon, corresponding to the peptide DTAEEDAE, confers a slightly higher Mr and a much more acidic pI to the protein: compare, for instance, SV-2 and SV-4.

particularly true for fast TnT since in its N-terminal region five exons (from 4 to 8) undergo alternative splicing as it occurs during the foetal development for the foetal exon (F-exon). Other two exons, 16 and 17, are alternatively spliced in a mutual fashion. As regards slow TnT, only two alternative spliced exons have been described [5,8,19] leading to three possible combinations, two of which actually detected in the muscular tissue [19].

To expand our understanding of the pathological consequences of the spliceopathy in DM1 and DM2, it is of interest to study how the splicing alterations manifest themselves in the skeletal TnT mRNAs expression. We describe here some experimental observations on TNNT1 and TNNT3 transcripts obtained from human muscle biopsies.

TNNT1 – Slow skeletal muscle TnT isoforms derived from the TNNT1 gene are summarized in Table 1. Our previous RT-PCR analysis of slow TnT transcripts [19] showed that only two of these products were actually expressed in human skeletal muscles. They differ for the presence or absence of the exon 5, which encodes for a very acidic peptide, and are expressed both in normal and in DM muscles. However, the relative amount of the less acidic low-molecular-mass (Mr) to the more acidic high-Mr PCR transcripts varied from about 1/3 in normal control muscles at about 1/4 in DM1 muscles. By contrast, we found that, in DM2 muscles, the same ratio was increased, particularly in biopsies taken from the arm muscles (Fig. 1 and Table 2). Variation of the internal ratio between the two isoforms has been reported also for other pathological conditions as Charcot-Marie-Tooth disease, a neurological disorder causing damage to peripheral nerves [13]. The internal ratio of the two expressed isoforms seems to undergo changes, as a consequence of neuromuscular diseases. It is of particular interest that the divergent up-regulation and down-regulation of the two transcripts in DM1 and DM2 may provide a biochemical marker for the differential diagnosis.

TNNT3 – We have recently demonstrated that DM2 muscles exhibit a higher degree of alternative splicing dysregulation for fast TnT transcripts when compared to both DM1 muscles and normal controls [19]. In order to understand if this circumstantial evidence may be due to the prevalence of some specific TnT transcripts, we reinvestigated this aspect by examining in more details the apparently anomalous electrophoretic bands obtained by RT-PCR analysis of DM2 muscle cDNA. In particular, the comparison of the amplified transcripts obtained by using primers specifically designed for the N-terminal variable region [12,19] showed a different pattern in the range of 100-200 bp. The heavier band, approximately 196 bp, was absent in the normal control muscles, but clearly evident in the majority of DM2 biopsies (see arrow in Fig. 2). Since the persistence of foetal exons has been described as one of the molecular features of DM1 pathogenesis for both cardiac and fast skeletal TnT, we explored the possibility that the supernumerary transcript might contain the foetal exon (F-exon) also in DM2 muscles. To this purpose, PCR fragments were extracted from the gel and subjected to digestion with the endonuclease restriction enzyme Mvn I that recognizes the sequence CGCG contained within the F-exon. Despite the correct result obtained in the same experimental conditions with a positive control (MYH1) containing the restriction site recognized by

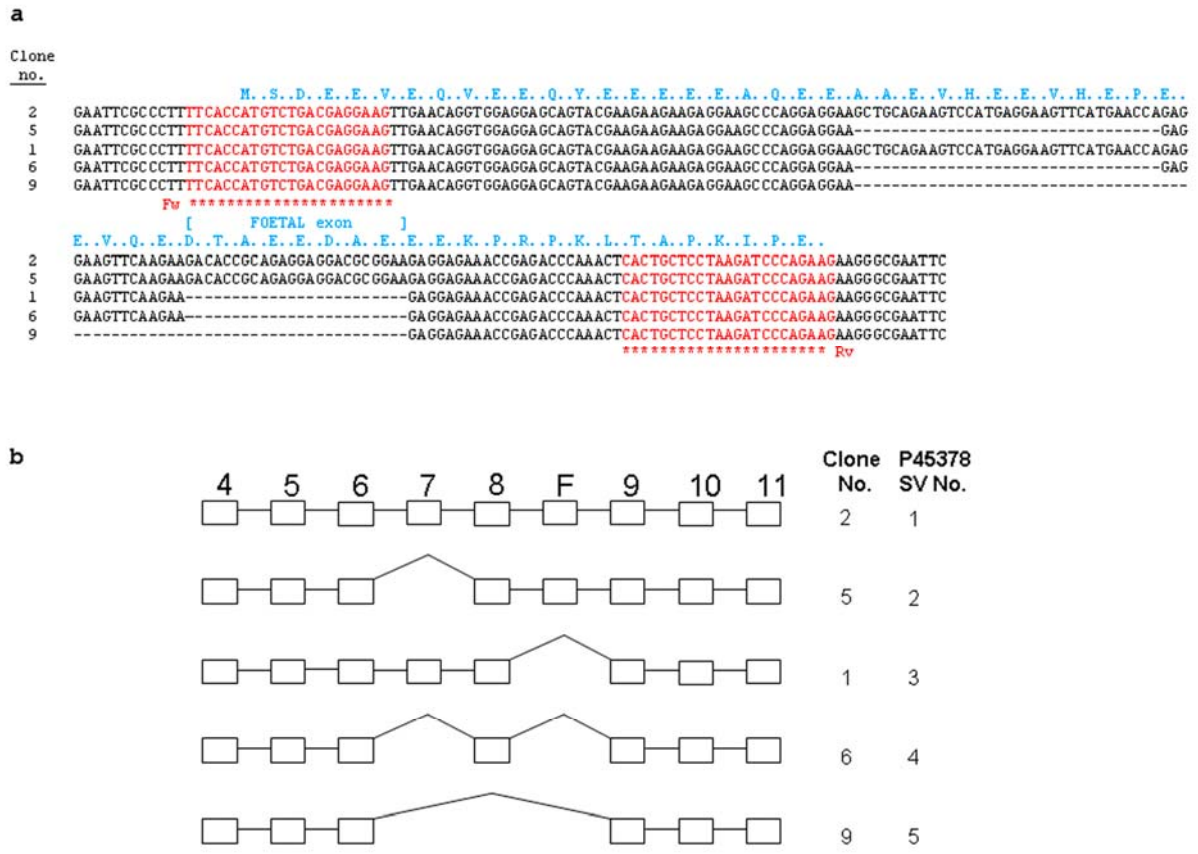

Fig. 4 *Nucleotide sequences and alternative splicing patterns of TnT transcripts. a) nucleotide sequences from the clones listed in Table 3. The two lines of asterisks indicate the forward (Fw) and reverse (Rv) primers used for the RT-PCR. The deduced amino acid sequences of the amplicons are also shown. Clones 2 and 5 contain the foetal exon sequence [DTAEEDAE]; b) alternative splicing patterns in the N-terminal variable region of TnT transcripts in DM2 skeletal muscle biopsies. Exons 4, 5, 6, 9, 10 and 11 were expressed in all sequenced clones. Alternative splicing prominently involved exons 7 and 8. Two of five isoforms showed retention of the foetal exon in the adult life.*

Mvn I, we could not cleave the foetal exon. This negative result may be due either to the absence of the foetal exon or to the inhibition of Mvn I by 5'-methylcytosine. Thus, to discriminate between these two possibilities, we decided to clone the heavier PCR fragment. After a PCR carried out as described before, the samples were pooled to get an adequate amount of fragment. This preparation was run on a low melting agarose gel and the higher band extracted. The PCR product was ligated in a TA/TOPO vector and transformed in E. coli strain TOP10. After selection with Xgal, 10 clones were picked and run on a 2% agarose. The results (Fig. 3) clearly show that the clones could be arranged in at least five groups, according to the relative mobility of their inserts. The clones were then sequenced, aligned (Fig. 4a) and numbered in the same order of the putative splicing variants (SV) of TNNT3, as reported in the UniProtKB/SwissProt database. The longest inserts, designated as clone n. 2 and clone n.1, contained the F-exon, indicating its retention in several patients. A summary of the cDNA sequences shown in Fig. 4 is reported in Table 3. The presence of the F-exon in two of the investigated sequences definitely demonstrated that the development-regulated alternative splicing may undergo aberrant processing not only in DM1, but also in DM2 muscles.

It seems that in the case of TNNT1 the expression of the two isoforms is quantitatively regulated and that the DM affects the balance between higher and lower Mr products (Fig. 1 and Table 1). Instead, in the case of TNNT3 a combination of many isoforms, including those normally absent in the adult life, are expressed as a consequence of the disease. In particular, the hypervariable region in the N-terminal tract may account for the wide range of TnT isoform variety. In normal conditions and throughout physiological development, a shift from large to small and from

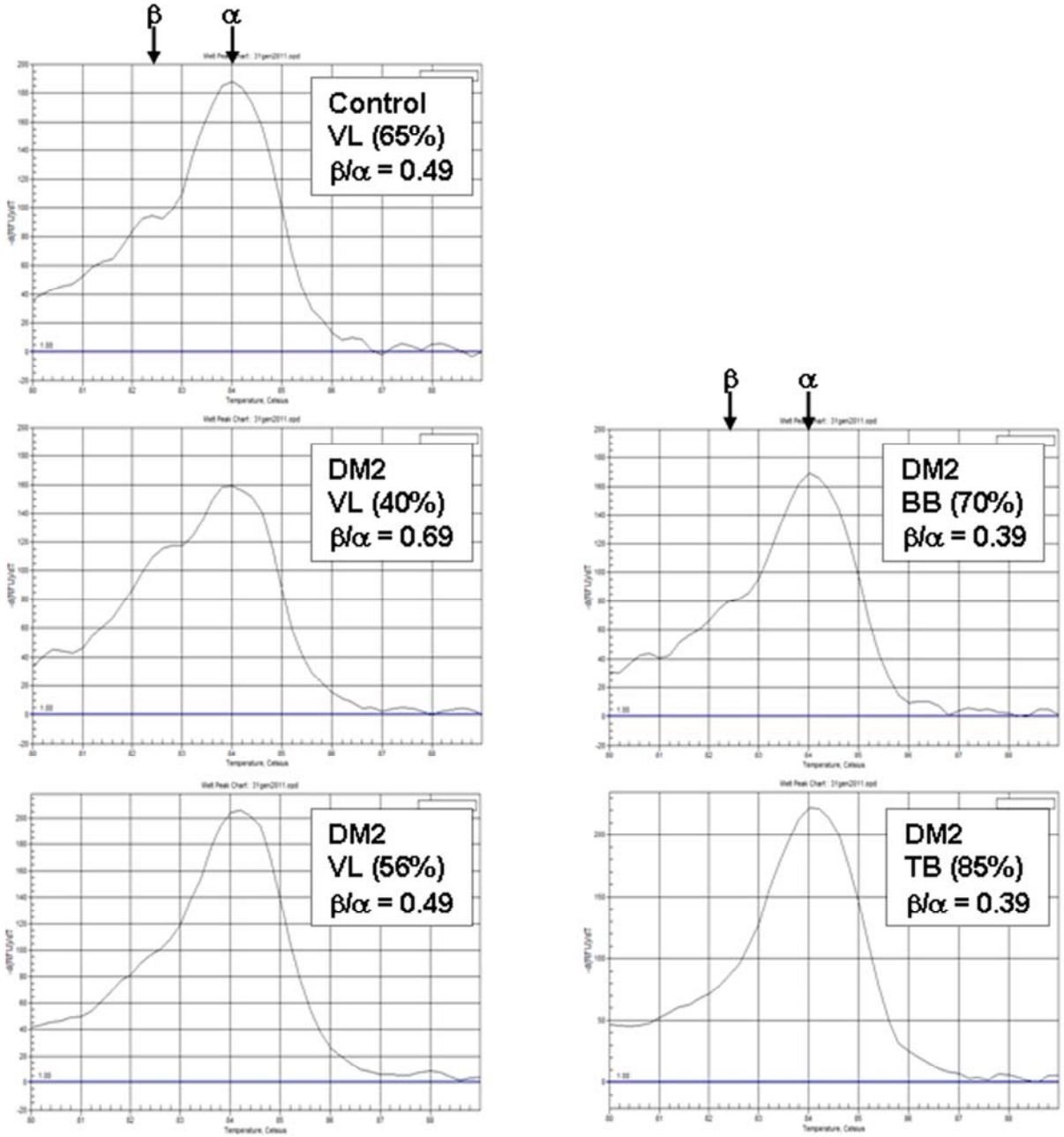

Fig. 5 *Melting curve of TNNT3 RT-PCR fragments corresponding to the mutually exclusive exons 16 (α) and 17 (β). The temperature-dependent dissociation of the two DNA strands were followed using the DNA-intercalating fluorophore SYBR green. Negative first derivative (-d(RFU)/dT) of melting curve to pin-point the dissociation temperature of the two amplicons is shown. The β exon was discernible as a shoulder corresponding to 82.5 °C only in the control muscle and in the DM2 muscles showing a prevalence of slow fibres. The predominant α exon peaks at higher temperature (84.0 °C) as expected from its higher content of CG to AT ratio. VL = vastus lateralis; TB = triceps brachii, BB = biceps brachii. The percentage values refer to the content of fast twitch muscle fibres.*

acidic to basic isoforms takes place [28]. The F-exon is a very acidic segment (6 of its 9 aminoacids being acidic) and its only removal, not considering other changes, formally contributes to a shift of 0.7 pH units. It has been demonstrated that the F-exon exerts an inhibitory effect on the biological activity of fast TnT by changing the Ca^{2+}-sensitivity of the troponin complex [3]. The physiological exclusion of the F-exon at the birth allows the TnT to bind correctly to the other complex components. However, the presence of the two mutually exclusive exons, 16 or α (adult-specific) and 17 or β (foetal/neonatal and adult), leads

to another kind of biological activity control [3]. In fact, α–exon confers a greater Ca^{2+}-sensitivity to reconstituted thin filaments, compared with those containing β–exon [6]. The presence of the F-exon also has a strong inhibitory effect on binary interactions between TnT and other troponins, whereas the balance between α- and β- exons seems to have a modulatory effect [3]. In this context, the combinatorial expression of isoforms differing in the variable region allows the troponin complex to undergo a huge plasticity.

In view of the fact that TNNT3 misregulated alternative splicing was a frequent event in DM2 muscles [19], we investigated if the altered splicing might also involve the two mutually exclusive exons 16 and 17. Preliminary melting curve data from qPCR analysis (Fig. 5) showed that β- and α- isoforms have a slightly different melting temperature in the given experimental conditions and that the corresponding relative fluorescence unit (RFU) values were in a ratio of about 1:2 in the control muscle. In contrast, the β/α ratio appeared to be inversely related to the fast fibre content of muscles, independently of the normal or pathological conditions. This was particularly evident in the brachial muscles, suggesting that β-isoform is prevalently expressed in slow fibres.

It has been established long time ago that the alternative splicing pattern is determined by the muscle type as well as the differentiation state [2] and that trans-acting regulatory factors are required for the correct splicing of the alternative exons during myogenesis. Therefore, it is very likely that the modulation of alternative splicing of the mutually exclusive exons and the combinatorial ones determines the specific distribution of fast TnT isoforms in different adult fast skeletal muscles, allowing isoform transitions in both physiological (as in course of the development or throughout muscle regeneration) and pathological conditions (as in case of denervation / reinnervation and myopathies).

It was also shown that the extreme variability in the expression of the exons 4-8 dictates the steric conformation of TnT, its Ca^{2+}-sensitivity and, finally, changes in pCa/tension relationship and MgATPase activity of myosin [15,24]. The other region of isoform diversity, depending on the alternative splicing of α- and β- exons, resides in the very C-terminal sequence where the binding sites with the other components of the troponin complex are located. In this context, the observation that reduced values of pH may influence the interaction between TnC, TnI and TnT in the heart of transgenic mouse expressing fast skeletal muscle TnT is particularly interesting [14].

In other models, as those based on the reconstitution of thin filaments holding fast TnT isoforms with different N-terminal structure, altered actomyosin ATPase activation has been found [1,7,16,25]. Moreover, the structure of the variable N-terminus region is able to modulate the conformation of the TnT molecule and its binding with the other components of the Troponin complex [10]. In addition, the presence of relatively basic isoforms of fast TnT lowers the tolerance of cardiac muscle to the acidic environmental pH [14].

Many other observations can be found in the literature on the relevance of TnT variable region.

Slow skeletal muscle has a higher tolerance to fatigue than the fast skeletal muscle and the N-terminal variable region of its TnT is more acidic than that of the fast counterpart [29; see also table 4]. This greater fatigue resistance may be due to its higher tolerance towards inhibitory effects of fatigue-dependent intracellular pH decrease [14].

Similar to slow TnT, cardiac TnT is more acidic than the adult fast TnT and exhibits a wider similarity with slow rather than fast TnT sequence. This is not surprising since slow and cardiac muscles have to sustain prolonged or continuous work, respectively. Experimental data suggest that embryonic cardiac muscle, expressing the foetal exon 5, is less affected from acidosis than the adult one [22].

Fast skeletal muscle TnT expressed in transgenic mouse cardiac muscle exhibits a greater pH sensitivity than wild type cardiac TnT [14]. Moreover, the binding affinity of basic TnT isoforms toward thin filaments proteins is more affected by pH lowering than that of acidic TnT [15].

Overall, the possibility arises from these studies that charge variation of TnT isoforms may represent a physiological mechanism for opposing the pH-dependent damage of thin filaments.

We have shown here that in DM2 patients the missplicing of RNA transcripts from TNNT3 determines the expression of several isoforms including those containing the foetal exon. Hence, the expressed isoforms could be more acidic than those normally synthesized and consequently more resistant to the pH lowering. Our previous data indicating that the missplicing of TNNT3 transcripts were more frequent in DM2 than in DM1 [19] may appear contradictory since DM2 is characterized by a rather specific type 2 fibre atrophy and by a milder clinical phenotype, compared to DM1.

However, the epiphenomenic expression of more acidic TnT isoforms, in both slow and fast fibres may be beneficial for DM2 muscles, thus explaining, at least in part, the milder physical conditions of this disease as opposing to the more severe DM1.

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