Expression of cardiac α-actin spares extraocular muscles in skeletal muscle α-actin diseases – Quantification of striated α-actins by MRM-mass spectrometry

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ABSTRACT

As with many skeletal muscle diseases, the extraocular muscles (EOMs) are spared in skeletal muscle α-actin diseases, with no ophthalmoplegia even in severely affected patients. We hypothesised that the extraocular muscles sparing in these patients was due to significant expression of cardiac α-actin, the α-actin isoform expressed in heart and foetal skeletal muscle. We have shown by immunochemistry, Western blotting and a novel MRM-mass spectrometry technique, comparable levels of cardiac α-actin in the extraocular muscles of human, pig and sheep to those in the heart. The sparing of extraocular muscles in skeletal muscle α-actin disease is thus probably due to greater levels of cardiac α-actin, than the negligible amounts in skeletal muscles, diluting out the effects of the mutant skeletal muscle α-actin.

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1. Introduction

Many skeletal muscle diseases (including Duchenne, Becker, limb-girdle and most congenital muscular dystrophies and congenital myopathies) do not affect the extraocular muscles (EOMs), while other diseases (e.g. mitochondrial disorders and myasthenia gravis) do affect the extraocular muscles [1–3]. Mutations in the skeletal muscle α-actin gene (ACTA1) have been shown to cause several congenital myopathies (reviewed in [4]). More than 170 different mutations have been identified to date (http://www.hg md.cf.ac.uk/ac/index.php and NG Laing unpublished observations) in patients presenting with actin myopathy, nemaline myopathy and intranuclear rod myopathy e.g. [5–7], nemaline myopathy with cores [8], core myopathy [9] and congenital fibre type disproportion [10]. However, it has been observed that the EOMs are unaffected even in the most severely affected of these patients [2,10].

The structural, cellular, molecular and physiologic properties of the EOMs are highly divergent from all other skeletal muscle fibre types. Numerous central concepts to skeletal muscle physiology, such as fibre type classification schemes and the M-line/creatine kinase system, do not apply to the EOMs [11,12]. For example, EOMs have two non-twitch, multiply-innervated fibre types and express embryonic, neonatal, cardiac and tissue-specific isoforms of various proteins that are atypical of adult skeletal muscle [13–16]. It is thought that the unique properties of the EOMs evolved to adapt to the dynamic range of contractions required for eye movement control and that these adaptations determine the patterned differential involvement of EOMs in muscle disorders [17–19].

It has been proposed that the sparing of EOMs in Duchenne muscular dystrophy (DMD) is in part due to the retention of utrophin expression (the foetal homologue of dystrophin, the absent protein causing DMD [20]) in this muscle group [21]. This is one piece of evidence supporting the pursuit of up-regulation of utrophin in post-natal skeletal muscle as a possible treatment of the dystrophinopathies [22].

Ilkovski et al. [23] explored the finding that patients with skeletal muscle α-actin diseases are rarely affected by cardiomyopathy and that skeletal muscle α-actin diseases manifest only late during gestation. The reason they suggest for these findings is that cardiac α-actin, the foetal striated α-actin isoform in skeletal muscle

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(expressed from the *ACTC* gene), is normally expressed at significant levels in skeletal muscle *in utero* until the third trimester. Around birth the amount of cardiac α-actin then decreases to low levels in humans [23], after which it accounts for less than 5% of the striated α-actin present and instead skeletal muscle α-actin predominates [24]. In contrast, in the developing heart the skeletal muscle α-actin is the foetal isoform but unlike cardiac α-actin, it remains as much as 20% of the striated α-actin present in the human adult heart [24]. Ilkovski et al. argue that it is the expression levels of cardiac α-actin in skeletal muscle *in utero* and in the adult heart that prevent actin myopathies having effects *in utero* and in the heart.

Recently, we have shown that the expression level of cardiac α-actin in skeletal muscle after birth inversely correlates with disease severity in patients homozygous for null mutations in the *ACTA1* gene (which results in the absence of skeletal muscle α-actin protein) [25]. This finding highlights the ability of cardiac α-actin to functionally substitute at least in part for skeletal muscle α-actin in skeletal muscle and suggests that up-regulation of cardiac α-actin may be a potential therapeutic option for the treatment of the skeletal muscle α-actin-based congenital myopathies. Based upon this finding, and that the EOMs are known to express numerous isoforms of various proteins (including foetal isoforms), we hypothesised that the sparing of the EOMs in the skeletal muscle α-actin-based congenital myopathies might be due to expression of cardiac α-actin. Previously, levels of cardiac α-actin mRNA in human EOM, as determined by Affymetrix GeneChip-based transcriptome analysis, were shown to be 4-fold greater than in limb muscle [26]. As stated, cardiac α-actin makes up 5% of the striated α-actin present in skeletal muscle [24]. A cardiac α-actin level of 20% at the protein level might be insufficient to prevent disease and so measurement of cardiac α-actin protein levels was essential.

The high sequence homology [24] between cardiac and skeletal muscle α-actin (they differ at only four amino acid residues out of the 375 in the mature actin protein) does not allow these isoforms to be resolved in traditional SDS–PAGE analysis, as they are virtually the same molecular mass (cardiac α-actin = 41.840 kDa and skeletal muscle α-actin = 41.873 kDa) and have an identical isoelectric focusing point. In order to overcome this Achilles' heel of the actin research arena, we developed a novel Multiple Reaction Monitoring (MRM) mass spectrometry approach that can distinguish these isoforms and can be conducted relatively quickly and at high throughput if required, in contrast to previously described methods [24,27].

We examined the expression of the two striated α-actins in EOM from humans, sheep and pig by immunochemistry, Western blotting and MRM-mass spectroscopy in order to examine the relative contribution of each isoform in the EOM of large mammals.

2. Methods

2.1. Sample preparation

All animal procedures were performed in accordance with the guidelines and approved protocols of the University of Western Australia Animal Ethics Committee. The human extraocular (lateral and medial rectus muscle) samples were segments of EOM removed during corrective squint surgery. These and the skeletal muscle sample were obtained following ethics approval from Royal Perth Hospital and informed consent of the patients. The human heart sample, from a control subject, was a kind donation from Prof Cristobal dos Remedios (The University of Sydney). EOM, heart and skeletal muscle samples were also collected from a three-month old pig and a two-year old sheep. Muscle segments were dissected and snap-frozen in liquid nitrogen for Western blot analysis or

frozen in liquid nitrogen-cooled isopentane for immunostaining studies, and subsequently stored at −80 °C.

2.2. Antibodies

Monoclonal antibodies against α-actinin (clone EA-53), striated muscle α-actins (clone 5C5) and smooth muscle α-actin (clone 1A4; FITC-conjugated) as well as the secondary antibodies (horse-radish peroxidase (HRP)-conjugated anti-mouse IgG and HRP/FITC-conjugated goat anti-rabbit), were obtained from Sigma–Aldrich (Sydney, NSW, Australia). The cardiac α-actin specific antibody (clone Ac1-20.4.2) was obtained from Research Diagnostics Inc. (Concord, MA). The polyclonal skeletal muscle α-actin specific antibody has been previously described [28].

2.3. SDS–polyacrylamide gel electrophoresis (PAGE) and Western blot

Procedures were essentially as outlined in Ravenscroft et al. [29]. Briefly, total proteins were extracted from approximately 30, eight-micron cryostat sections (as per Cooper et al. [30]) and then quantified using the BCA™ protein assay kit (Pierce Biotechnology Inc., Rockford, IL). For all Western blots shown, samples were loaded to give an equal quantity of total protein (10 μg). Samples were electrophoresed through 4–15% Criterion gradient gels (Biorad Laboratories Pty. Ltd., Regents Park, NSW, Australia) before electroblotting onto polyvinylidene fluoride membrane in Towbin's transfer buffer (25 mM Tris, 192 mM glycine and 20% methanol) at 300 mA for 2 h at room temperature (RT).

After blocking, membranes were incubated for at least 1 h at RT or overnight at 4 °C in primary antibodies (anti-cardiac α-actin, 1:4000; anti-skeletal muscle α-actin, 1:20,000; anti-α-actinin, 1:500,000; anti-striated α-actin, 1:500,000). The membrane was then incubated in the appropriate HRP-conjugated secondary antibody at 1:15,000 for 1 h at RT. After washing, detection was performed using the ECL Plus Western Blotting Detection System™ (Amersham, GE Healthcare, Buckinghamshire, UK).

2.4. MRM-liquid chromatography-mass spectrometry

Samples were prepared and run on 4–15% gradient SDS–PAGE gels and then Coomassie stained. Gel bands surrounding the area where actin proteins would be present (e.g. around approximately 35–50 kDa) were excised from the gel, cut into 4 pieces and destained with 50 μL of 25 mM ammonium bicarbonate in 50% acetonitrile (Merck, Kilsyth, Vic., Australia) for 45 min at 37 °C. This was repeated until all stain was removed with the same number of destaining steps applied to all samples. After removing the liquid, the gel pieces were dried and resuspended in 20 μL of 10 mM dithiothreitol for 1 h at 60 °C. Then 20 μL of 55 mM iodoacetamide was added to the pieces and they were subsequently incubated in the dark at RT for 1 h. Liquid was then removed, the gel pieces dried and 10 μL of a 12.5 μg/mL solution of trypsin (Roche Products Pty Ltd., Dee Why, NSW, Australia) in 25 mM ammonium bicarbonate was added with incubation for 16 h at 37 °C. To each sample was added 10 μL of acetontrile with 1% formic acid and the solution was kept at RT for 20 min. The solution was removed to a fresh vial and a further 10 μL of acetontrile with 1% formic acid was added to the gel pieces with incubation at RT for 20 min in a second extraction. Both extractions were then combined. The combined extractions were then dried in preparation for mass spectrometry.

Dried samples prepared as above were resuspended in 50 μL of 2% acetontrile (0.05% trifluoroacetic acid), with 10 μL injected into an Ultimate 3000 nano-HPLC system (Dionex, Bannockburn, IL). The peptides were eluted with a gradient of 10–25% acetontrile (0.1% formic acid) over 45 min through a Dionex C18PepMap100
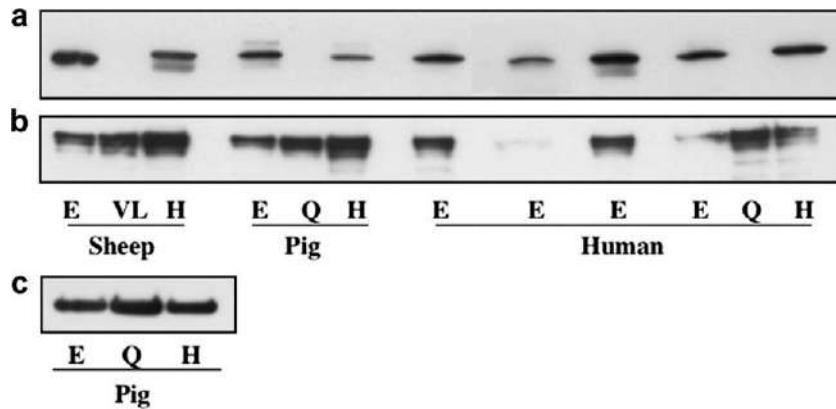
Fig. 1. Western blot analysis for cardiac α-actin (a) in extraocular (E), quadriceps (Q), vastus lateralis (VL) and heart (H) muscle samples from sheep, pig and humans. Each of the four human extraocular samples is from a different patient. Samples were loaded to give 5 μg of total protein and a sarcomeric α-actinin antibody was used as an indicator of sarcomeric protein content across samples (b). An antibody specific for skeletal muscle α-actin (c) shows that the pig heart sample expresses levels of skeletal muscle α-actin similar to that seen in the pig EOM sample.
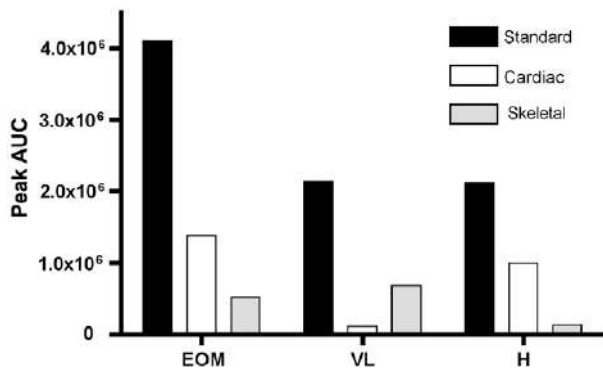

Fig. 2. MRM-mass spectrometry. The values of the area under the curve (AUC) for the sheep samples (EOM, vastus lateralis and heart) for each of the transitions of interest: the transition of the common striated α-actin N-terminal (standard), the cardiac α-actin specific b2 daughter ion (cardiac) and the skeletal muscle α-actin specific b2 daughter ion (skeletal).

Table 1
The contribution of cardiac α-actin to the striated α-actin expressed as a percentage for each of the samples, as determined by MRM-mass spectrometry

Sample	% Cardiac α-actin
Human EOM	65 ± 3.4 ($n = 5$)
Human quadriceps	11
Human heart	70
Sheep EOM	73
Sheep vastus lateralis	14
Sheep heart	88
Pig EOM	67
Pig quadriceps	12
Pig heart	48

The percentage of cardiac α-actin expression was determined by calculating the ratio of the AUC of the skeletal muscle α-actin specific b2 daughter ion (Sk) to the transition of the common striated α-actin N-terminal (Std) and the ratio of the AuC of the cardiac α-actin specific b2 daughter ion (Cr) to the Std. All samples were from single samples run in triplicate to produce an average result except for the human EOM samples where samples from 5 separate patients were analysed.

column (3 μm, 100 Å, 75 micron ID × 15 cm) at 300 nL/min into the mass spectrometer via a PicoTip emitter (FS360-75-15-N-5-C15, New Objective, Woburn, MA). A blank 30 min run including gradient and equilibration steps was also performed between samples to minimise any potential carry over of one sample to the next. Unless stated otherwise, all reagents were obtained from Sigma–Aldrich (Sydney, NSW, Australia).

A 4000 Q TRAP triple quadrupole linear ion trap mass spectrometer (Applied Biosystems, Foster City, CA) was interfaced with a nanospray source. Source temperature was set at 150 °C and source voltage was set at 2650 V. MRM transitions and collision energies (CE) for the acetylated (Ac), carbamidomethyl modified (∗) peptides were as follows:

Transition 982.9/1048.5 Ac-DED(DE)ETTALVC*DNGSGLVK, 54 V (common striated actin N-terminal transition to y10 ion),

Transition 982.9/287.1 Ac-DEDETTALVC*DNGSGLVK, 65 V (skeletal muscle α-actin specific b2 daughter ion),

Transition 982.9/273.1 Ac-DDEETTALVC*DNGSGLVK, 65 V (cardiac α-actin specific b2 daughter ion).

The declustering potential (DP) was set at 50 V, exit potential (EP) to 10 V, collision cell exit potential (CXP) to 10 V, the resolution for Q1 and Q3 to low, and the time for all transitions was kept at 120 ms. For quantitative measurement, the area under the curve (AUC) was calculated for all transitions using MultiQuant software (version 1.0, Applied Biosystems, Foster City, CA).

Analysis was conducted by determining the ratio of the AUC for the specific b2 daughter ion transitions of cardiac α-actin and skeletal muscle α-actin relative to the common N-terminal transition for each sample. The percentage of cardiac α-actin was then calculated by dividing the ratio of the cardiac α-actin specific transition to the common peptide transition by the sum of the ratios between the two striated α-actins (e.g. each of the cardiac and skeletal muscle α-actin specific daughter ion transitions) to the common peptide transition.

2.5. Immunohistochemistry

Eight-micron thick cryostat sections were blocked in 10% foetal calf serum (FCS) and 1% bovine serum albumin (BSA) in phosphate buffered saline (PBS) for 20 min. The anti-cardiac α-actin antibody (1:20) was labelled with the Zenon® Alexa Fluor® 594 labelling kit (Invitrogen, Mount Waverley, Vic., Australia) before being incubated on the sections overnight at 4 °C. The anti-skeletal muscle α-actin polyclonal antibody (1:100) was also incubated overnight before detection with a FITC-conjugated goat anti-rabbit secondary (1:1000). Stained sections were mounted using Hydromount (National Diagnostics, Atlanta, GA) and visualised using a fluorescent microscope (Olympus IX-71 inverted) and digital camera (Olympus DP-71, Mount Waverley, Vic., Australia).

3. Results

Western blot analysis showed that human, sheep and pig EOMs express high levels of cardiac α-actin and that these levels are similar to that found in heart tissue, when samples were loaded to give equal total protein (Fig. 1).

MRM-mass spectrometry was performed on human, sheep and pig muscle samples and the area under the peaks of the specific transitions for cardiac and skeletal muscle α-actin peptides as well as a transition for the common parent peptide were determined. A representative graph of these is displayed in Fig. 2. The percentages of cardiac α-actin detected in all the samples analysed by MRM-mass spectrometry are displayed in Table 1. It was found that approximately $65 \pm 3\%$ ($n = 5$ individual EOM samples) of the striated α-actin expressed in human EOMs is the cardiac α-actin isoform. Similarly it was found that cardiac α-actin accounts for 67% and 73% of the α-actin pool in pig and sheep EOMs, respectively. The quadriceps and vastus lateralis muscles express low levels of cardiac α-actin, with approximately 85–90% of the striated α-actin being the skeletal muscle α-actin isoform. Sheep and human ventricular muscle were found to express 88% and 70% of cardiac α-actin respectively. Interestingly, the pig ventricle sample expressed high levels of skeletal muscle α-actin (52%). Following this observation, Western blotting was performed using a skeletal muscle α-actin specific antibody. It was confirmed that the pig ventricle sample expressed relatively high levels of skeletal muscle α-actin, similar to that seen in the EOM sample (Fig. 1c).

Immunochemistry conducted on human, sheep and pig EOMs demonstrated that 100% of the EOM fibres were positive for cardiac α-actin (Fig. 3). Furthermore, 100% of human and pig EOM fibres expressed skeletal muscle α-actin while only a proportion of the sheep EOM fibres were immunopositive for skeletal muscle α-actin (Fig. 3). Expression of smooth muscle α-actin, which is the predominant isoform during the earliest development of cardiac and skeletal muscle [31], was confined to the blood vessels within the EOMs of all of the species, as determined by immunochemistry (data not shown).

4. Discussion

Sparing of the EOMs has been documented in a number of muscle disorders, including the skeletal muscle α-actin-based congenital myopathies. We have demonstrated for the first time, by Western blotting, immunochemistry and MRM-mass spectrometry that human, pig and sheep EOMs express high levels of cardiac α-actin, in addition to skeletal muscle α-actin. Indeed EOMs express more cardiac α-actin (between 64% and 73% of the total striated α-actin present) than skeletal muscle α-actin, which is the opposite of what is seen with these two isoforms in skeletal muscle (where cardiac α-actin is between 11% and 14% of the total striated α-actin present). The cardiac α-actin levels in EOMs are more similar to those seen in cardiac muscle (cardiac α-actin in sheep heart = 88% and in human heart = 70% of the total striated α-actin present). Our results using a novel MRM-mass spectrometry technique are not completely in concordance with those reported in Vandekerckhove et al. These authors showed that 82% of the striated α-actin pool was the cardiac α-actin isoform in adult human and pig heart [24]. In the present study we show that the pig heart sample contained only 48% cardiac α-actin, but we were able to support this unexpected mass spectrometry finding by demonstrating a higher level of skeletal muscle α-actin protein than anticipated in the pig heart (Fig. 1c), similar to that in the EOM sample.

Given that cardiac α-actin accounts for approximately 65% of the striated α-actin protein expressed in the EOM, similar to the
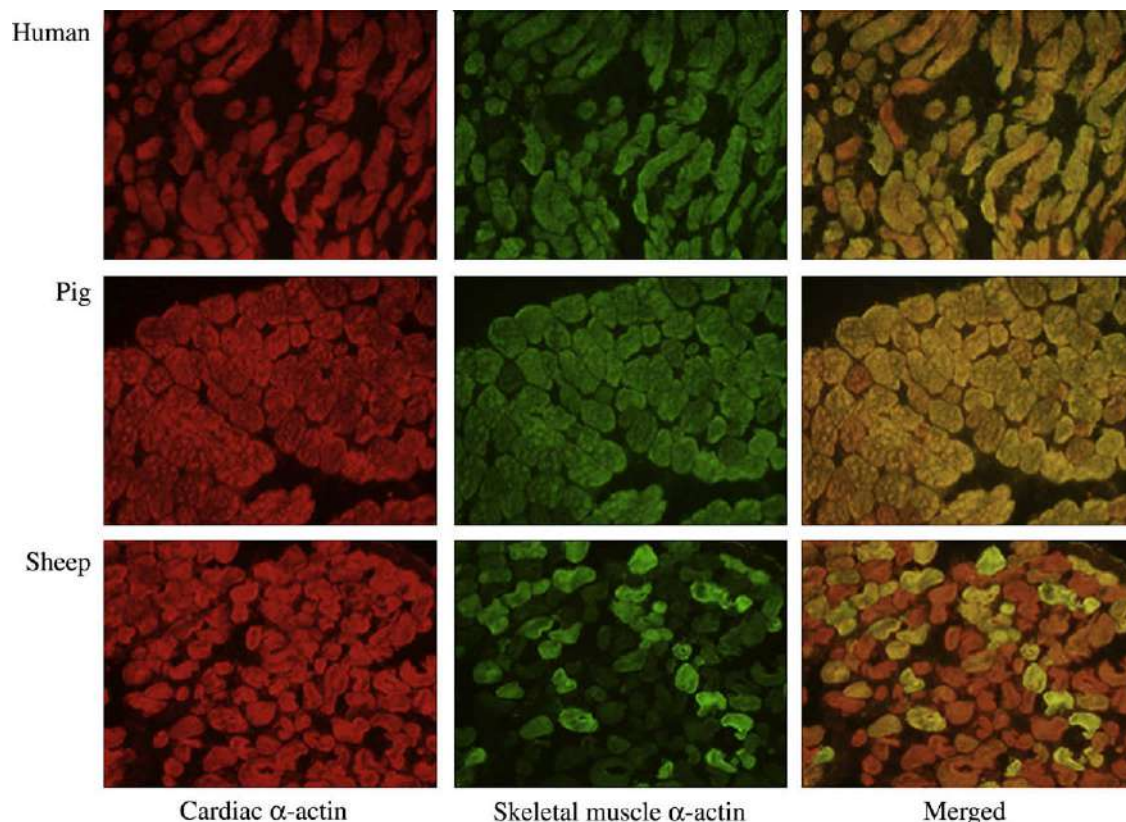

Fig. 3. Human, pig and sheep extraocular muscle co-immunolabelled using specific antibodies to cardiac α-actin (red) and skeletal muscle α-actin (green). In the merged images, fibres appearing yellow contain both cardiac and skeletal muscle α-actin.

levels in the heart, the sparing of the EOMs even in severe skeletal muscle α-actin-based congenital myopathies can be argued to be due to the dilution of the mutant skeletal muscle α-actin protein by the presence of cardiac α-actin as was argued by Ilkovski et al. [23], for the heart and skeletal muscle in utero.

While the human, pig and sheep EOM samples we had available for study showed consistent cardiac α-actin expression, our preliminary investigations of mouse and rat EOMs indicated variable levels of cardiac α-actin expression in different regions. These may be layer-specific, in similarity to previous studies of other structural proteins [32–34]. While cardiac α-actin is expressed in all human, sheep and pig EOM fibres examined, it is conceivable that layer and/or muscle-specific differences in cardiac α-actin expression may also be present in EOM of these species.

An ever-present stumbling block in the study of sarcomeric α-actin expression levels has been the lack of a suitable method by which to directly quantify and compare the expression levels of the cardiac and skeletal muscle α-actin isoforms. We have shown that this can be accomplished using mass spectrometry and MRM detection to measure the levels of isoform-specific peptides. Previous techniques used to distinguish between the cardiac and skeletal muscle α-actin isoforms have required laborious purification of α-actin or a highly specialised mass spectrometer (7 Tesla Fourier Transform ion cyclotron resonance mass spectrometer) [24,27]. The MRM-mass spectrometry technique described herein is able to accurately distinguish between the 99% identical cardiac and skeletal muscle α-actin isoforms, whereas other mass spectrometry studies have been unsuccessful discriminating less homologous protein isoforms [35].

Alterations in the skeletal muscle/cardiac α-actin ratio are an important predictive indicator of the extent of cardiovascular disease, which is an area of intensive research both in animal models [36–39] and human patients [40]. The ability to accurately quantify the levels of these isoforms with extremely high sequence homology using MRM-mass spectrometry should be important to this research area.

5. Conclusions

We have shown that unlike human skeletal muscles, human EOMs express higher levels of cardiac α-actin than skeletal muscle α-actin, and hypothesise that this is responsible for the preferential sparing of this muscle group in the skeletal muscle α-actin-based congenital myopathies. In addition, the MRM-mass spectrometry method used to quantify the relative amounts of the two striated muscle α-actin isoforms makes it possible to characterise changes in the ratio of these almost identical proteins in diseased states in a higher throughput manner than with previously described techniques. This could prove to be an important tool not only in skeletal muscle disease analysis but also in cardiovascular disease diagnosis and research.

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