Different atrophy-hypertrophy transcription pathways in muscles affected by severe and mild spinal muscular atrophy

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Abstract

Background: Spinal muscular atrophy (SMA) is a neurodegenerative disorder associated with mutations of the survival motor neuron gene SMN and is characterized by muscle weakness and atrophy caused by degeneration of spinal motor neurons. SMN has a role in neurons but its deficiency may have a direct effect on muscle tissue.

Methods: We applied microarray and quantitative real-time PCR to study at transcriptional level the effects of a defective SMN gene in skeletal muscles affected by the two forms of SMA: the most severe type I and the mild type III.

Results: The two forms of SMA generated distinct expression signatures: the SMA III muscle transcriptome is close to that found under normal conditions, whereas in SMA I there is strong alteration of gene expression. Genes implicated in signal transduction were up-regulated in SMA III whereas those of energy metabolism and muscle contraction were consistently down-regulated in SMA I. The expression pattern of gene networks involved in atrophy signaling was completed by qRT-PCR, showing that specific pathways are involved, namely IGF/PI3K/Akt, TNF-α/p38 MAPK and Ras/ERK pathways.

Conclusion: Our study suggests a different picture of atrophy pathways in each of the two forms of SMA. In particular, p38 may be the regulator of protein synthesis in SMA I. The SMA III profile appears as the result of the concurrent presence of atrophic and hypertrophic fibers. This more favorable condition might be due to the over-expression of MTOR that, given its role in the activation of protein synthesis, could lead to compensatory hypertrophy in SMA III muscle fibers.

Background

Spinal muscular atrophy (SMA) is a neurodegenerative disorder with progressive paralysis caused by the loss of motor neurons. Mutations of both alleles of the telomeric survival motor neuron (SMN) gene SMN1 are correlated with the development of SMA [1]. The SMA phenotype can be influenced by the variable copy number of the paralogous centromeric gene SMN2 [2-4] which, lacking
SMN is a ubiquitously expressed protein complex implicated in a variety of processes, including the formation and function of neuromuscular junctions [7,8]. Deficiency of the SMN protein may have a specific effect within the motor neuron, connected to RNA metabolism or transcription, which impairs the biogenesis of axons. SMN may be important in the muscle cell itself, and its lack might lead to faulty signaling from skeletal muscle [9-11]. The effect of SMN gene mutations in the degeneration of muscle fibers, independent of motor neurons, is supported by results obtained in mice with a deletion of SMN exon 7 restricted to skeletal muscle [10]. In Drosophila melanogaster a sarcomeric SMN protein has been demonstrated, implicating a muscle-specific function and underlining the importance of this tissue in modulating the severity of SMA phenotype [11].

The main pathological trait of SMA muscles is atrophy, albeit with a variable severity. Many studies have identified elements of the signaling cascades leading to muscle atrophy [12-16]. We compared the expression signatures of human muscles affected by the two extreme forms of SMA (I and III) to understand which genes, other than SMN, are involved in muscle-specific SMA pathways and to understand the mechanisms leading to and sustaining atrophy in different forms of SMA.

**Methods**

**Characterization of patients with SMA and SMA samples**

For this study we analyzed muscle biopsies and genomic DNA from peripheral blood of four patients with SMA I and five patients with SMA III from the Neuromuscular Bank of the University of Padova. The Bank has been approved by the Ethical Committee of the University of Padova in compliance with the Helsinki Declaration. The clinical traits of the patients are summarized in Additional file 1. Atrophy and hypertrophy values of muscle biopsies were obtained by comparing the diameters of random selections of SMA muscle fibers with normal muscle of similar age (see Additional file 2, Extended Methods for details of the methodology).

**Genomic analysis of patients with SMA**

Genomic DNA was isolated from whole blood by the salting-out procedure [17]. To identify the presence of SMN1 gene deletions in exon 7 and 8, PCR amplifications and restriction enzyme digestions were carried out according to the method proposed by Van der Steege et al [18]. Deletions of exon 5 in the NAIP gene were also checked by performing multiplex PCR as proposed by Roy et al [19]. To identify the number of the SMN2 gene copies we employed a fluorescence-based competitive PCR assay previously described by Scheffer et al [20], with modifications. Primer sequences and experimental conditions used in multiplexed PCR tests are reported in Additional file 2.

**Microarray experiments**

We used a microarray platform containing 4670 muscle-specific 3′-cDNA fragments that is deposited and described in the GEO database (GPL2011) [21]. Patient and control biopsies were taken from quadriceps femoralis muscles. Total RNA was purified by the TRIzol protocol and quality checked by Agilent Bioanalyzer 2100. One microgram of each RNA sample was linearly amplified using the Amino Allyl MessageAmp II aRNA Amplification Kit (Ambion) and labeled with fluorescent amino allyl-dUTP (Cy3 or Cy5, GE Healthcare). Patient and control cDNAs, labeled with different dyes, were competitively hybridized to microarray platforms. Each microarray experiment was done in duplicate, inverting the labeling dye. Microarrays were read in a Perkin-Elmer LITE confocal laser scanner, and images were analyzed with QuantArray Software (GSI Lumonics, Ottawa, Canada). Statistical analysis of data was performed as detailed in Additional file 2. Expression datasets are compiled according to the standards proposed by the Microarray Gene Expression Data Society and are available at the GEO database with ID series GSE8359.

**Real-time quantitative PCR**

Real-time quantitative PCR (qRT-PCR) was carried out using the SYBR Green chemistry with GeneAmp 5700 Sequence Detection System (Applied Biosystems). The sequences of gene-specific primers and experimental conditions are reported in Additional file 2.

**Results**

**Genomic characterization of patients with SMA**

We demonstrated a diagnostic deletion of exon 7 and 8 of the SMN1 gene in all patients with SMA I and III. For the SMN2 gene we found two copies in all patients with SMA I and in a single patient with SMA III, and three or four copies in the remaining four patients with SMA III. We also studied the NAIP gene, since deletions in this gene have been associated with SMA [19]. No variation in the NAIP gene structure was found in any of the SMA genomes. Table 1 summarizes the results of our genomic studies.

**Expression profiling**

RNA from each SMA muscle sample was competitively hybridized with age-matched muscle control samples to microarrays. The differential gene expression profiles were subjected to hierarchical clustering, resulting in the patients with SMA being classified in two different groups corresponding to the clinical categorization (Figure 1). This suggests a different gene profile for each muscle atrophic condition (SMA I and III). We determined the
concordance between patient classification resulting from microarrays and clinical data with prediction analysis for microarrays (PAM) [22]. The cross-validation test showed that each patient is assigned to the correct clinical class by expression signature. In particular, the transcription level of only 22 significant genes appears to be sufficient to discriminate between SMA I and III profiles, with a misclassification error of 0 (Additional file 3).

The expression data were then analyzed with significance analysis of microarrays for the identification of differentially expressed genes. We only analyzed transcripts with a false discovery rate equal to 0, and with values that showed ratios >0.7 or <-0.7. To study in detail the specific alterations in gene expression caused by the atrophic condition in different SMA muscles we analyzed four different groups of expression data: a) 33 genes that are differentially expressed in patients with SMA I in comparison with controls (10 up- and 23 down-regulated, Additional file 4); b) 10 genes differentially expressed in SMA III in comparison with controls (seven up- and three down-regulated, Additional file 5); c) 46 genes differentially expressed between SMA I and SMA III (15 up- and 31 down-regulated, Additional file 6); d) 19 genes differentially expressed in both SMA I and SMA III in comparison with normal controls (12 up- and seven down-regulated, Additional file 7). These genes have been classified with FatiGO into a number of functional categories: cellular metabolism, muscle contraction, signal transduction, transport, RNA metabolism, molecular recognition system, immune/defense response, cell cycle and others. The similarities in the functional classification of the four groups of differentially expressed transcripts suggest that there should be few metabolic mechanisms involved in the pathological status of SMA muscles.

The analysis of differentially expressed genes in patients with SMA III (Additional file 5) shows a reduced number of expression changes compared to SMA I. The expression data in SMA III samples show a reduced number of differentially expressed genes. This suggests that the disease process in SMA III may involve fewer metabolic pathways compared to SMA I.

### Table 1: Genotype of SMA patients.

| Biopsy code | SMA type | Sex | Age at biopsy (years) | SMN1 Deletion | SMN2 copy number | NAIP deletion |
|-------------|----------|-----|-----------------------|---------------|-----------------|--------------|
| SMA I samples |          |     |                       |               |                 |              |
| A           | IA       | M   | 0.2                   | 7.8 ex.       | 2               | NO           |
| B           | IB       | F   | 0.3                   | 7 ex.         | 2               | NO           |
| C           | IB       | M   | 0.5                   | 7.8 ex.       | 2               | NO           |
| D           | IB       | F   | 0.9                   | 7.8 ex.       | 2               | NO           |
| SMA I control |         |     |                       |               |                 |              |
| Cont. 1     | NO       | M   | 0.6                   | NO            | -               | NO           |
| SMA III samples |       |     |                       |               |                 |              |
| E           | 3A       | M   | 7                     | 7 ex.         | 3               | NO           |
| F           | 3A       | F   | 7                     | 7.8 ex.       | 2               | NO           |
| G           | 3A       | F   | 8                     | 7.8 ex.       | 3               | NO           |
| H           | 3A       | F   | 7                     | 7.8 ex.       | 4               | NO           |
| I           | 3A       | F   | 11                    | 7.8 ex.       | 4               | NO           |

SMA III control

| SMA III control |     |     |     |               |                 |              |
|-----------------|-----|-----|-----|---------------|-----------------|--------------|
| Cont. 2         | NO  | M   | 8   | NO            | -               | NO           |

High-molecular weight genomic DNA was obtained from all patients with SMA type I and III enrolled for this study. This was used to control for the presence of SMN1 and NAIP gene deletions and SMN2 copy number. Genomic DNA from age-matched unaffected donors was used to perform parallel control analyses.
of altered genes, mainly involved in signal transduction. This suggests proximity of SMA III to the normal muscle condition. Also, the comparison of expression profiles of SMA muscle biopsies using the complete linkage cluster method and the Euclidean distance measure. Patients with SMA are clearly divided into two groups corresponding to the clinical classification: cluster 1 for the more severe type I and cluster 2 for the milder type III.

Finally, Additional file 7 lists deregulated genes that are shared by the two classes of SMA. As can be seen, these are the same genes listed in Additional file 5. Therefore patients with SMA I and III seem to share all those genes whose expression differentiates SMA III muscles from normal controls.

Many transcripts involved in glucose and glycogen metabolism were found to be down-regulated, mainly in SMA I (Additional file 4). mRNAs encoding several enzymes catalyzing different steps of glycolysis, the tricarboxylic cycle and oxidative phosphorylation were reduced. The impairment of cell energy systems seems to be distinctive of the severe disease. Genes implicated in muscle contraction were consistently deregulated, mainly in SMA I samples. Among this class we found genes that codify proteins of the Z disc such as nebulin (up-regulated), desmin and titin (down-regulated) and titin-associated such as MYBP-C (down-regulated). At the same time, some genes regulated by MYBP-C such as MYH 1 and 7 showed a reduced expression. The analysis also showed a significant under-expression of CKM.

qRT-PCR was applied to validate the microarray results. The following transcripts were studied: *Glutathione peroxidase 4* (GPX4) and *Muscle creatine kinase* (CKM), that resulted respectively in over and under-expression in SMA I: Complement factor H (CFH), that resulted in over-expression in both SMA classes. The results confirm those obtained with microarray, and also revealed the under-expression of the CKM transcript in SMA III. This is probably due to the higher sensitivity of qRT-PCR technology with respect to microarray [23].

**Molecular pathways of atrophy in SMA I and SMA III muscles**

In SMA muscles we monitored the principal pathways involved in atrophic conditions, namely the IGF/P3K/Akt, TNF-α/p38 MAPK and Ras/ERK pathways. The results are summarized in Table 2 and indicate a different picture for SMA I and III. The analysis of transcriptional profiles showed an under-expression of the eukaryotic translation initiation factor 4E binding protein 3 gene (eIF4EBP3) only in SMA I, while in other studies the homologous gene eIF4EBP was indicated as over-expressed [24]. To study this discrepancy, we decided to probe by qRT-PCR the mRNA levels of some of the principal members of IGF/P3K/Akt pathway, namely IGF-1R, FRAP1 (MTOR), FOXO, eIF4EBP1 and FBOX32 (Atrogin-1). The patients with SMA I showed over-expression of IGF-1R and FOXO, and under-expression of FBOX32 and eIF4EBP1, confirming the microarray data. Conversely, in SMA III samples there is a general under-expression of this group of transcripts, with the exception of MTOR. Therefore, MTOR over-expression might result in the more favorable SMA III phenotype due to its positive signal for protein synthesis activation.

Many studies have demonstrated the important role played by *tumor necrosis factor alpha* (TNF-α) in regulating muscle size through apoptosis [25]. The MAPK14 (p38) gene belongs to TNF-α/p38 MAPK pathway and regulates MAP kinase interacting serine/threonine kinase (MNK1/2). MNK2 is down-regulated in patients with SMA I, like IL-32, a positive regulator of p38 and TNF-α. Finally TXN, a negative regulator of MEK5 (ASK1) that is upstream of p38, is even more up-regulated in SMA I samples. Considering all these data and the notion that MNK1/2 phosphorylates eIF4E [26], we monitored the TNF-α/p38 MAPK pathway looking for TRAF2 and MAPK14 (p38). SMA I muscles showed an under-expression of p38 that may be the protein synthesis regulator. In SMA III there was instead a general up-regulation of these genes.

Finally, we investigated the Ras/Raf/MEK/ERK pathway, testing the expression of two members of this pathway (RRAS2 and ERK) because ERK, like p38, regulates the MNK1/2 activity. This analysis showed that muscles from both groups of SMA resulted in the same expression pro-
file for these factors: RAS is down-regulated and ERK is up-regulated.

**Correlation of altered molecular pathways with SMA muscle histopathology**

The different expression data obtained from the SMA I and III biopsies (Figure 2), and above all the over-expression of MTOR, led us to investigate for the possible presence of hypertrophic fibers in SMA III muscle specimens. Stained sections of the patient biopsies were analyzed and the diameter of muscle fibers was measured in order to calculate atrophy and hypertrophy rate. The result of this investigation (Figure 3) shows that, as expected, both SMA I and III muscles have an increased rate of atrophy in comparison with normal muscle of the same type. The atrophy factor of SMA I is 509 and of SMA III is 1116, the control varying from 0 to 150. On the other hand, fiber measurements show an increased hypertrophy phenotype only for SMA III muscles: the average factor of hypertrophy is indeed 680 when the control varies from 0 to 150.

**Discussion**

We studied the differences in the expression signatures of muscles affected by the mild (III) and severe (I) SMA phenotype. All patients with SMA presented an SMN1 deletion, and the SMN2 copy number was variable and not correlated with the phenotype severity (see Table 1).

Table 2: Real-time quantitative PCR analysis.

| Gene              | SMA I Expression value | SMA III Expression value |
|-------------------|------------------------|--------------------------|
| IGF-1R            | +3.2                   | -2.9                     |
| FRAP1 (MTOR)      | 1                      | +2.8                     |
| FOXO3A            | +1.3                   | -1.6                     |
| elf4EBP1          | -1.4                   | -1.7                     |
| FBOX32 (Atrogin-1)| -1.6                   | -1.7                     |
| TRAF2             | 1                      | +4.2                     |
| MAPK14 (p38)      | -1.25                  | +1.6                     |
| RRAS2             | -2                     | -2.5                     |
| MAPK1 (ERK)       | +1.2                   | +2.4                     |

Expression ratios of selected intracellular signaling molecules of atrophy and hypertrophy pathways tested in affected SMA I and III muscles versus control muscles (p < 0.05).

Therefore, the phenotype of these patients is probably affected by SMN protein level. Deficiency of the SMN protein could lead to this neuromuscular pathology, having a specific role in RNA metabolism of motor neurons or triggering a primary involvement of skeletal muscle which may contribute to motor defects [10]. We investigated these muscle-specific signals by studying the gene expression signatures of SMA muscles. The microarray data outcome gives a different gene expression profile for each of the two forms of SMA. In SMA III muscles the low number of genes presenting an altered expression level suggests a proximity to the normal condition, while in SMA I muscles there is a more complex picture. The hierarchical clustering separates the gene profiles into two groups that correspond to the clinical classification, identifying a shortlist of 22 discriminant transcripts out of 4670 measured by the microarray.

We have compared our results with the list of ‘atrogenes’ proposed by Goldberg and colleagues as a set of genes that are found commonly deregulated in various types of muscle atrophy in mouse and rat [24,27]. The goal was to understand if the muscular atrophy of patients with SMA involves the same set of genes or a specific transcriptional program. There is a different picture for the two SMA phenotypes. The great majority of genes deregulated in SMA I belonging to the functional classes of energy metabolism and muscle contraction are found among the ‘atrogenes’ and show a similar expression alteration. With SMA III muscles, this concurrence is found for the genes included in the signal transduction class. This finding indicates once more that SMA I and III muscles are in different phases with distinct transcriptional patterns.

Our study show a complex down-regulation of sarcomeric genes with a reduced metabolic activity more pronounced in the severe form of SMA. mRNAs encoding several enzymes of glycolysis, the tricarboxylic acid cycle and oxidative phosphorylation are reduced (Additional file 4). These changes in gene expression would be expected to suppress the muscle capacity for utilizing glucose and to generally reduce energy production in this tissue. The impairment of the energetic system seems to be distinctive of the more severe SMA I. Indeed, in SMA III muscles we found only two under-expressed transcripts: isocitrate dehydrogenase 2 (IDH2) and adenylsuccinate synthase 1 (ADSSL1), enzymes which function downstream of glycolysis. It might be that in the milder form of SMA only the final phases of glucose metabolism are impaired, whereas in the more severe form the impairment is extended also to glycolysis. Genes implicated in muscle contraction were also found consistently deregulated, including some codifying for structural proteins (nebulin, desmin, MYH 1 and 7) as well as for regulative proteins (myosin-binding protein C), confirming the data obtained by others [28].
Both the microarray and qRT-PCR experiments showed the involvement of some molecular pathways that have been already associated with the establishment and maintenance of atrophy in skeletal muscle, namely the IGF/PI3K/Akt pathway and those that regulate MNK1–2 (TNF-α/p38 MAPK and Ras/ERK). Figure 2 summarizes these molecular pathways as determined in the SMA I and III muscles. Akt-1 activity on FOXO and MTOR leads respectively to inhibition or activation of eukaryotic translation initiation factor 4E binding protein (eIF4EBP), determining in turn the depression or the enhancement respectively of cap-dependent protein synthesis [29].

The analysis of these factors, as well as of FBOX32 (Atrogin-1), gives interesting results, indicating two different conditions for SMA I and III. In SMA I muscle there is a 'prolonged' atrophic pathway where FOXO is over-expressed and potentially able to sustain atrophy. It should be pointed out, however, that our study is done at the transcriptional level and it is known that the FOXO protein needs not only to be up-regulated but also dephosphorylated to enter the myonuclei and activate atrogenes. In contrast, probably by a survival mechanism, there is under-expression of atrogenes downstream of FOXO such as Atrogin-1 and eIF4EBP. This is in agreement with expression profiles obtained for denervated mouse or rat muscles because the expression of these genes in patients with SMA I correlates closely with the profiles of animal muscle in advanced stage of atrophy [30,31]. The inhibition of cap-dependent protein synthesis is probably due to MNK activity regulated by p38 (down-regulated) rather than by ERK. The microarray data corroborate this hypothesis since they show over-expression of thioredoxin that inhibits ASK1, which is an upstream positive regulator of p38 [32], and under-expression of IL32 that can also activate p38 [33]. Moreover, it was found that the low amount of active MNK resulted in a selective increase of the cap-independent protein synthesis [34]. Therefore, in SMA muscle the under-expression of MNK might favor the switching of protein synthesis from cap-dependent to cap-independent.

The situation of atrophy molecular pathways in SMA III muscle is very different. IGF-1R, FOXO, Atrogin-1 and eIF4EBP1 are all down-regulated while MTOR is up-regulated. These results suggest a possible activation of protein synthesis through the mRNA binding capacity of eIF4 without the inhibiting influence of eIF4EBP. MTOR activation might therefore prevent a general atrophy degeneration, typical of SMA I patients, inducing a compensative
Panel 1. Fiber size measurements in SMA I and III muscle sections, compared with normal age-matched muscle specimens.

Figure 3

Panel 1. Fiber size measurements in SMA I and III muscle sections, compared with normal age-matched muscle specimens. Portions of patient biopsies used for microarray experiments were sectioned and stained with haematoxylin and eosin. Diameters of sample fibers were obtained and atrophy/hypertrophy values were calculated by the methods described in the Additional file 2. Panel 2. Cross-sections of biopsies of quadriceps muscle of patients with SMA I (A) and SMA III (B) photographed at the same enlargement. Muscle from a patient with SMA I shows generalized fiber atrophy and few scattered hypertrophic fibres (asterisks; microscope magnification ×200). Muscle from a patient with SMA III shows groups of atrophic and normotrophic fibers (on the upper-right) and groups of large hypertrophied fibers (asterisks; microscope magnification ×200).
skeletal muscle hypertrophy [35]. Indeed, the analysis of the diameter of SMA III fibers shows the presence of a consistent number of hypertrophic fibers that are not detected in SMA I muscle. The histological analysis of SMA I and III muscle has also evidenced atrophy. It is probable therefore that the transcriptome of SMA III muscle is not very different from normal muscle, because it is actually the sum of two opposite transcriptional profiles. Our microarray and qRT-PCR analyses have been carried out with whole muscle biopsies and therefore we have measured the co-existing atrophy and hypertrophy transcriptional profiles with the mutual cancellation of some specific signals, with the exception of MTOR.

Conclusion
We have studied the transcriptional signature of skeletal muscle tissue affected by spinal muscular atrophy with the significant analysis of very rare human muscle biopsies of the severe SMA type I. Other authors have used this approach to study the transcriptional changes in mouse or rat muscle undergoing atrophy following different physiopathological conditions, but this is the first study focused on human muscle tissues affected by a genetically determined atrophic condition. Our work indicates that SMA I and III muscles are in different phases: the ‘prolonged’ atrophic condition typical of the SMA I muscle and the co-existence of atrophy and hypertrophy in SMA III muscle. In SMA I muscle there is an extended atrophic pathway with FOXO over-expression, the under-expression of downstream FOXO atrogenes and p38 as a probable protein synthesis regulator. Conversely, SMA III muscle appears to have a complex transcriptome arising from co-existence of atrophic and hypertrophic signals. This more favorable condition might be caused by the action of MTOR, whose over-expression could lead to a compensative hypertrophy.

Competing interests
These or similar data have not been submitted, in whole or in part, for publication elsewhere. The authors have no competing interests to disclose with regard to the manuscript submitted for review.

Authors’ contributions
CM conducted experiments concerning gene expression studies by microarrays and qRT-PCR tests, analysis of expression data and interpretations of results, and contributed to the writing of manuscript draft. MF performed the histopathological characterization of muscle specimens, and helped in the interpretation of expression data. AV conducted the genomic analysis on patients’ DNA and interpreted the results. MLM participated in the design of the genomic study of patients’ DNA. PL contributed to the interpretation of expression data. CA organized the recruitment of patients, collection of muscle biopsies and clinical histopathological characterization of patients, and contributed to the draft of the manuscript. GL conceived and coordinated the entire study, contributed in the design of experiments and interpretations of results and wrote the manuscript. All authors have read and approved the final manuscript.

Additional material

Additional File 1
Clinical traits. Clinical traits of SMA patients.
Click here for file
[http://www.biomedcentral.com/content/supplementary/1741-7015-7-14-S1.doc]

Additional File 2
Extended methods. This file contains methods for: i) calculation of atrophy and hypertrophy values in children muscle biopsies, ii) SMN2 copy-number assay, iii) statistical analysis of expression data, iv) qRT-PCR.
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[http://www.biomedcentral.com/content/supplementary/1741-7015-7-14-S2.doc]

Additional File 3
Additional Table S2. This table contains the list of 22 significant transcripts whose differential expression levels allow for a good class prediction with SMA I and III transcriptional profiles. Data have been obtained by PAM analysis.
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Additional File 4
Additional Table S3. This table contains the list of differentially expressed genes in SMA I muscles in comparison to normal age-matched muscle control.
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Additional File 5
Additional Table S4. This table contains the list of differentially expressed genes in SMA III muscles in comparison to normal age-matched muscle control.
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Additional File 6
Additional Table S5. This table contains the list of differentially expressed genes between SMA I and SMA III muscles.
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Additional File 7
Additional Table S6. This table contains the list of genes with an altered expression values in both SMA I and SMA III muscles, in comparison to normal controls.
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