Table 2
Classification of differentially regulated 207 genes in LF muscles compared to normal muscles

Category	Number of genes with increased expression	Number of genes with decreased expression
Apoptosis	3	0
Catabolism	9	2
Channel/receptor	5	0
Chaperone	0	1
Cytoskeleton	10	3
ECM/membrane	15	0
Energy metabolism	14	8
Enzyme	6	1
Immune response	5	1
Lipid metabolism	3	0
Sarcomere	10	3
Signal transduction	18	7
Transcription	10	8
Translation	4	3
Transport	3	1
Others	13	5
Unknown	22	14
Total	150	57

Total 207 genes were differentially expressed in LF muscles compared to control using ANOVA–Welch *t*-test (statistically significant at $P < 0.01$). Gene categories are from LocusLink (www.ncbi.nlm.nih.gov/LocusLink).

3.2. Specifically dysregulated genes in LF muscles

We performed hierarchical clustering of the 29 genes using Pearson correlation. By experimental clustering, specimens were largely classified into three groups, which corresponded to NRF, LF and control as shown in the top dendrogram (Fig. 2).

Table 3
Classification of differentially regulated 423 genes in LF muscles compared to NRF muscles

Category	Number of genes with increased expression	Number of genes with decreased expression
Apoptosis	3	2
Catabolism	19	11
Channel/receptor	7	8
Chaperone	4	3
Cytoskeleton	8	3
ECM/membrane	8	5
Energy metabolism	16	12
Enzyme	5	4
Immune response	3	1
Lipid metabolism	3	2
Sarcomere	2	7
Signal transduction	30	28
Transcription	42	16
Translation	14	7
Transport	0	9
Others	32	13
Unknown	56	40
Total	252	171

Total 423 genes were differentially expressed in LF muscles compared to NRF muscles using ANOVA–Welch *t*-test (statistically significant at $P < 0.01$). Gene categories are from LocusLink (www.ncbi.nlm.nih.gov/LocusLink).

By hierarchical gene clustering, these 29 genes were divided into four branches (branches A–D) (Fig. 2 and Table 4). The genes in branch A were less downregulated in NRF and LF muscles than in the controls, albeit more downregulation was observed in the LF muscles. Branch A was predominantly comprised of transcription-related and RNA-binding protein genes, such as *HNRPC*, *DDX1* and *RERE*, and a gene (*TMOD4*) that encodes an actin regulatory protein, tropomodulin 4. Only two genes, including sperm-associated antigen 9 (*SPAG9*), were found in branch B. Genes in this branch were only mildly downregulated in LF muscles and nearly normally expressed in NRF muscles. The genes in branch C were upregulated in LF muscles but mildly downregulated or normal expressed in NRF muscles. Genes encoding signal-mediating molecules comprised the bulk of this group. Interestingly, of the nine genes in branch C, the expression of *GSN* gene, which encodes gelsolin, an actin depolymerizing or stabilizing plus end protein, was greatly increased about 11-fold in LF muscles compared to NRF muscles (Table 4). The genes in branch D were highly upregulated in NRF muscles and, to a lesser extent, in LF muscles, and primarily included genes that encode sarcomeric proteins such as *TNNI1*, *PDLIM3* and *ACTC*. Based on our previously reported data (Noguchi et al., 2003), the expression of five of the nine genes in branch D was also altered in DMD (Table 4).

3.3. Changes in the expression of actin-associated genes in LF muscles

Of the 29 selected genes, we further focused on the upregulated genes *GSN*, *TNNI1* and *PDLIM3*, which encode

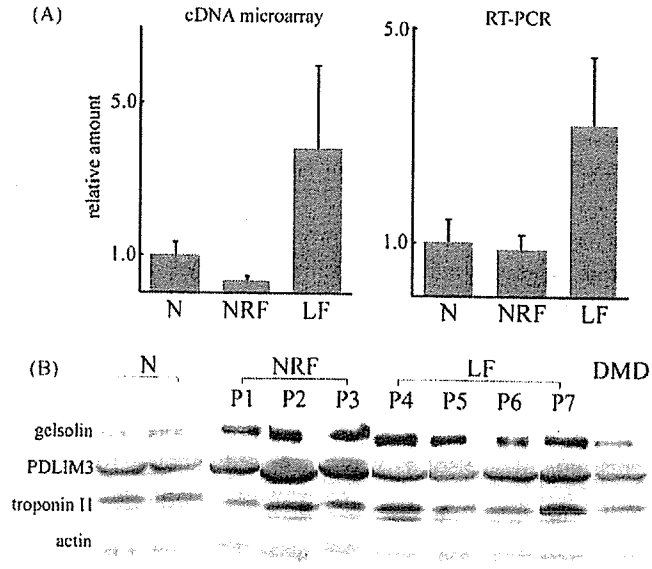

Fig. 3. Validation of cDNA microarray data by quantitative RT-PCR and Western blot analysis. Measurements of mRNA expression of *GSN* using cDNA array analysis or quantitative RT-PCR analysis (A). Western blot analysis of gelsolin, PDLIM3 and troponin I1 using skeletal muscle homogenates from seven LGMD2A patients, two controls, and one DMD patient (B). Note that compared to controls, the expression of these three proteins increases in both NRF and LF patients. The actin band was stained with Coomasie brilliant blue as a control.

Table 4
Specifically regulated 29 genes in patients with lobulated fibers

Accession	Gene	Function	LF/N	LF/NR	DMD	LGMD2B
(A)						
NM_022083	Clorf24	Unknown	−5.7	1.8		
NM_013353	TMOD4	Muscle structure	−4.6	2.6	Down[a]	
NM_005389	PCMT1	Chaperone	−2.8	1.6		
NM_004939	DDX1	Transcription	−2.7	1.5		
NM_031314	HNRPC	Transcription	−2.5	1.4		
NM_012102	RERE	Transcription	−2.1	1.2		
NM_005594	NACA	Others	−2.0	4.3		
NM_014320	HEBP2	Others	−1.8	1.4		
NM_001010853	ACY1L2	Catabolism	−1.1	1.2		
(B)						
NM_003971	SPAG9	Signal transduction	−1.4	−1.2		
NM_017875	FLJ20551	Others	−1.3	−1.2		
(C)						
NM_000177	GSN	Cytoskeletal protein	3.6	10.7	Up[a]	Up[b]
NM_006435	IFITM2	Immune response	3.0	2.0		
NM_032906	MGC14156	Signal transduction	2.0	1.6		
NM_001353	AKR1C1	Catabolism	1.6	2.6	Down[a]	
NM_152726	EFHA1	Signal transduction	1.6	1.6		
NM_001985	ETFB	Lipid metabolism	1.4	1.9	Down[a]	
NM_024863	TCEAL4	Translation	1.3	1.6		
NM_000671	ADH5	Redox	1.2	1.3		
AI026701	INPP5E	Signal transduction	1.2	1.1		
(D)						
NM_005159	ACTC	Muscle structure	14.4	−3.8	Up[a,c,d]	
NM_005368	MB	Energy metabolism	5.7	−2.1		
NM_024085	APG9L1	Catabolism	5.2	−2.1		
NM_014476	PDLIM3	Muscle structure	4.2	−2.1	Down[a]	
NM_000034	ALDOA	Glycolysis	3.9	−3.6	Down[a]	
NM_001404	EEF1G	Translation	3.7	−2.4	Down[a]	
NM_003281	TNNI1	Muscle structure	2.7	−1.9	Down[a]	
NM_170783	ZNRD1	Transcription	2.1	−5.0		
NM_005410	SEPP1	Extracellular matrix	1.5	−1.4		

LF/N represents the fold change in expression in muscles with LF to that in controls. LF/NR represents the fold change in expression in muscles with LF to that with NRF. (−) Denotes down-regulation.
[a]Genes differentially regulated in DMD patients according to Noguchi et al. (2003, 2005).
[b]Genes differentially regulated in LGMD2B patients according to Campanaro et al. (2002).
[c]Genes differentially regulated in DMD patients according to Chen et al. (2000).
[d]Genes differentially regulated in DMD patients according to Haslett et al. (2002).

cytoskeletal and sarcomeric proteins, because myofibrillar structure is greatly altered in LF muscles. We confirmed the upregulation of the *GSN* gene by quantitative RT-PCR (Fig. 3A) and the increase in the expression of its product, gelsolin, by Western blotting (Fig. 3B). Quantitative RT-PCR analysis showed four-fold upregulation of this gene in LF muscles as compared with NRF muscles (Fig. 3A). Moreover, protein expression of gelsolin was increased about 10-fold in LGMD2A muscles when compared with the control. However, there was no difference between LF muscles and NRF muscles (Fig. 3B). Immunohistochemical analysis of LF muscles showed that gelsolin was distributed in the subsarcolemmal regions of LFs (Fig. 4, patient 6, asterisks), and in connective tissue, although it was undetectable in control muscle fibers. In addition, in NRF muscles, gelsolin was diffusely present in the cytoplasm of small regenerating fibers and in the subsarcolemmal region of necrotic fibers and mononuclear cells (Fig. 4).

We also confirmed the upregulation of PDLIM3 and troponin I1 proteins by Western blot analysis both in NRF and LF muscles (Fig. 3B). Immunohistochemistry showed that the sarcomere of LFs and non-LFs was strongly stained with PDLIM3. In contrast, in NRF muscles, immunoreactivity was only mildly increased in apparently unaffected muscle fibers (Fig. 4). Troponin I1 expression was increased in the sarcomere of the small LFs. Fiber typing by anti-myosin heavy chain antibody showed that most of these small muscle fibers were slow-type fibers (data not shown, Chae et al., 2001). In NRF muscles, however, signals were undetectable in either necrotic or regenerating fibers (Fig. 4).

4. Discussion

In our analysis, we have clearly shown that gene expression in LGMD2A can be classified on the basis of differences in muscle pathology (S1), as with other muscular dystrophies

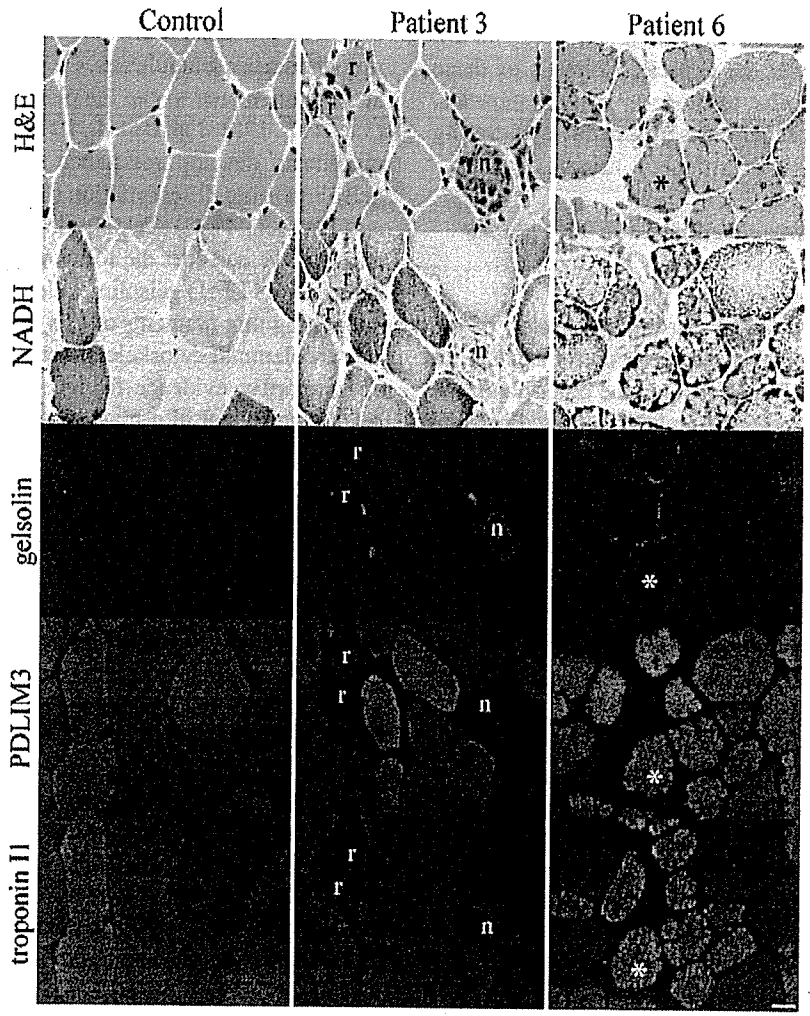

Fig. 4. Immunohistochemical analyses of gelsolin, PDLIM3 and troponin I1 reveal abnormal staining in NRF and LF muscles. Gelsolin, PDLIM3 and troponin I1were stained in control, NRF muscle (Patient 3) and LF muscle (Patient 6). Gelsolin localizes in the cytoplasmic region of regenerating fiber (r) and peripheral region of necrotic fiber (n), and the subsarcolemmal region of LFs (asterisk) and connective tissues. PDLIM3 showed a striation pattern in the control; however, it was not detectable in regenerating (r) or necrotic fibers (n) in NRF muscles (Patient 3). In LF muscle, PDLIM3 is abundantly distributed in the sarcomeric region of LFs (asterisks, Patient 6). Troponin I1 displayed diffuse sarcomeric staining in slow-type fibers in control and NRF muscles (Patient 3), and it localized strongly to the sarcomeric region of many slow-type LFs (white asterisk). Bar: 20 μm.

(Noguchi et al., 2003; Taniguchi et al., 2006). This suggests that the pathological changes seen in LGMD2A muscles might well be caused by molecular events initiated by these differences in gene expression. Since LF is characteristically observed in the later stage of LGMD2A, we therefore divided LGMD2A patients into two groups on the basis of pathological features in order to uncover the characteristic gene expression profiles of LGMD2A.

4.1. Outline of expression profiles in LF muscles

In LF muscles, several genes related to signal transduction and transcription were differentially expressed. These genes encode signaling mediators and transcriptional factors implicated in cell growth, calcium signaling and apoptosis. Interestingly, our results suggested that the genes for cell growth and calcium signaling were upregulated; in contrast, genes involved in apoptosis were rather suppressed in LF muscles. These data suggested that apoptosis signaling might be inactivated at least in LF or the later stage of LGMD2A muscles. In fact, the ratio of TUNEL-positive myonuclei to normal nuclei was less than 0.014% in our series (Table 1).

Genes coding for extracellular matrix (ECM)/membrane-related, cytoskeletal or sarcomeric proteins were also upregulated in LF muscles. These molecular changes could reflect the structural features of LF muscles. For example, upregulation of ECM/membrane-related genes might be associated with interstitial fibrosis observed in LF muscles; likewise, upregulation of cytoskeletal or sarcomeric-related genes might be associated with structural changes of the intramyofibril network in LFs.

The expression changes of some genes in the NRF muscles of LGMD2A patients were similar to those seen in Duchenne muscular dystrophy (DMD) muscles. In our previous study, in muscles from DMD patients, upregulated genes were mostly those related to immune response, sarcomeric, ECM and signal transduction, whereas, downregulated genes were associated with energy metabolism, transcription/translation,

signal transduction and the proteasome (Noguchi et al., 2003). In the present study, however the expression levels of these genes in LF stage were similar to those in control muscles. These results confirm the absence of necrotic and regenerating processes in LF muscles.

4.2. The 29 specifically dysregulated genes in LF muscles

4.2.1. Overview of the 29 genes in LF muscles

As shown in Table 4, we found that the expression of the genes in branch A was comparable between control and LF but was greatly decreased in NRF muscles. On the other hand, the expression of genes in branch D was greatly increased in NRF muscles compared to LF muscles. From these results, we considered that the expression of genes in branches A and D might be closely related to necrosis and regeneration in NRF. In contrast, the expression of genes in branches B and C underwent more extensive downregulation or upregulation in LF than in NRF. The genes in these branches may play specific roles in LF formation, although we were unable to get any conclusive information about, or identify common properties between the genes in these groups.

4.2.2. Apoptosis-associated genes among the 29 genes

Among the 29 genes, genes related to cytoskeletal, sarcomeric, signal transduction and catabolism proteins were upregulated, while genes related to transcription were downregulated in LF muscles. One of the downregulated genes was *SPAG9*, which encodes a scaffold protein that binds to several signaling molecules or transcription factors (Lee et al., 2002). Interestingly, SPAG9 interacts with NF-κB1/p105 but not with NF-κB2/p100, and expression of RNAi for *SPAG9* results in decreased NF-κB activity in response to treatment with tumor necrosis factor-α (Bouwmeester et al., 2004). NF-κB signaling pathway was reported to be downregulated in muscles from LGMD2A patients and calpain 3 knockout mice (Baghdiguian et al., 1999; Richard et al., 2000). The down-regulation of *SPAG9* observed in this study may partly reflect perturbation of the NF-κB signaling pathway as reported by Baghdiguian. Although, as earlier mentioned, other apoptotic signals seemed to be suppressed in LF, a more extensive analysis of the NF-κB pathway in LGMD2A patients is still needed to clarify the role of SPAG9 and other related genes in apoptosis.

4.2.3. Actin-associated genes among the 29 genes

Since the presence of LF is a characteristic structural change observed in LGMD2A muscles, changes in the expression of genes encoding intracellular structural proteins and their regulators should be important. In this study, we focused on upregulated genes for actin-associated proteins in LF muscles, such as gelsolin, troponin I1 and PDLIM3.

4.2.3.1. Gelsolin. A comparison of the present data with our previous data (Noguchi et al., 2003) shows that gelsolin gene expression is more upregulated in LF than in DMD muscles. We found that gelsolin was distributed diffusely in the cytoplasm of regenerating fibers, in contrast to its subsarcolemmal distribution in LFs, which are characterized by deranged myofibrils and mitochondria accumulation. Gelsolin mainly functions to sever actin filament and to cap the plus ends of the severed filaments (Yin et al., 1981; Lamb et al., 1993). In cultured myogenic cells, myofibrils develop resistance to this severing function of gelsolin during differentiation (Huckriede et al., 1988; Gonsior and Hinssen, 1995). Taking all the available information into account, we consider the following roles for gelsolin during its activation in LF:1) gelsolin could scavenge the disrupted actin-containing thin filaments of LFs and 2) gelsolin could sever the subsarcolemmal cytoskeletal actin network. Although we have no supportive evidence for any of these possibilities, the upregulation of gelsolin may be associated with actin derangement in LFs.

4.2.3.2. TroponinI1. Troponin I is a subunit of the actin filament-associated troponin–tropomyosin complex involved in the regulation of skeletal and cardiac muscle contraction (Gordon et al., 2000). In our study, troponin I1, which is a slow-twitch isoform of troponin I family, was highly upregulated in LGMD2A muscles with NRF, and to a lesser extent, in those with LF (Table 4). Other subunit genes in the troponin complex, such as *TNNI2*, *TNNC1*, *TNNC2* and *TNNT3* were also strongly upregulated in LGMD2A muscles with NRF and weakly in those with LF (S1). Expression of each troponin subunit gene seems to be similarly regulated during the progression of muscle pathology in LGMD2A. In LF muscles, troponin I1 immunoreactivity was observed strongly in the small slow-type LFs. Considering the predominance of slow-twitch fibers in LF muscles, the increase in troponin I1 mRNA expression in LF muscles might be due to the increased numbers of slow fibers in LF muscles (Chae et al., 2001). Interestingly, troponin I1 was not observed in the subsarcolemmal region and did not co-localize with gelsolin in LFs. From this result, the intensive expression of troponin I1 might not be directly related to the derangement of myofibrils in subsarcolemmal regions in LFs.

4.2.3.3. PDLIM3. PDLIM3 is known to bind the spectrin-like repeats of α-actinin via its PDZ domain and localize to the Z-lines (Xia et al., 1997; Jo et al., 2001). It has been reported that overexpression of PDLIM3 results in the increased ability of α-actinin to cross-link actin filaments (Pashmforoush et al., 2001). In our study, PDLIM3 showed stronger immunoreactivity in LFs, but did not colocalize with gelsolin accumulation. The upregulation of PDLIM3 observed in LF muscles might have some roles in stabilizing Z-line of myofibril structures.

4.3. Conclusion

Upregulation of the genes associated with actin filament observed in LF muscles is consistent with the markedly disorganized myofibrils in these muscles. Although, further analyses will be needed to explain relationship between calpain 3 and associated molecules whose functions remain unknown, our study shows that gene expression profiling of LGMD2A is a powerful tool that can provide clues to the pathomechanism of this disease.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.neures.2006.12.010.

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