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Differentially expressed genes in sporadic amyotrophic lateral sclerosis spinal cords – screening by molecular indexing and subsequent cDNA microarray analysis

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Abstract To analyze the genes related to the pathophysiology of sporadic amyotrophic lateral sclerosis (SALS) we performed gene profiling of SALS spinal cords using molecular indexing combined with cDNA microarray. Eighty-four fragments were cloned in the first screening procedure with molecular indexing. Subsequent quantitative microarray screening revealed 11 genes which were differentially expressed in SALS. Real-time RT-PCR verified that the expression level of the following six genes was altered in SALS: dorfin, metallothionein-3, 30 kDa TATA-binding protein-associated factor, neugrin, ubiquitin-like protein 5 and macrophage-inhibiting factor-related protein-8. These results indicated that genes associated with the ubiquitin–proteasome system, oxidative toxicity, transcription, neuronal differentiation and inflammation might be involved in the pathogenesis of SALS.

Key words: Amyotrophic lateral sclerosis; Gene expression profile; Molecular indexing; Dorfin; Neugrin

1. Introduction

Amyotrophic lateral sclerosis (ALS) is one of the most common neurodegenerative disorders, characterized by selective motor neuron degeneration in the spinal cord, brainstem and cortex. Approximately 10% of ALS is familial, and 10–20% of these familial ALS (FALS) cases are caused by missense mutations in the Cu/Zn superoxide dismutase (SOD1) gene [1], while others are considered to be sporadic ALS (SALS). Although the etiology of SALS is not known, a great number of studies to date have proposed possible mechanisms of the motor neuron degeneration in SALS. These include oxidative toxicity, glutamate receptor abnormality, ubiquitin–proteasome dysfunction, inflammatory and cytokine activation, dysfunction in neurotrophic factors, damage to mitochondria, cytoskeletal abnormalities and activation of the apoptosis pathway [2,3]. One of the most promising strategies to identify pathophysiologically related molecules in SALS would be the comparison of gene expression profiles of human SALS spinal cords with those of normal controls by the gene expression profiling technique. The spectrum of obtained genes differentially expressed in SALS tissues from normal control tissues would largely depend on the screening systems employed and tissue samples used, particularly in postmortem tissues. Used for human postmortem samples this approach could yield not otherwise obtainable information on the molecular pathophysiology of neuronal degenerations. The only report of large-scale gene profiling on SALS tissues to date, using membrane arrays, has documented that genes involved in oxidative toxicity, neuroinflammation, apoptosis, and lipid metabolism were up-regulated in the spinal cords of SALS patients [4]. We previously used microarray analysis to detect differentially expressed genes in FALS transgenic mice, showing that inflammation- and apoptosis-related genes were up-regulated in G93A-SOD1 transgenic mice [5]. These results indicated that molecules with similar functions were associated with the pathogenesis of both SALS and FALS; however, it is only able to explain the partial pathomechanism for SALS motor neuron degeneration. To identify genes differentially expressed in the SALS spinal cord, particularly novel or low-abundant genes, we adopted molecular indexing, a PCR-based screening procedure, for the primary screening and performed subsequent cDNA microarray analysis.

2. Materials and methods

2.1. Subjects

Postmortem spinal cords samples at segments L3–L5 were taken from eight SALS patients and eight non-neurological disease controls after obtaining their informed consent. Each SALS case was clinically and pathologically verified. Diagnostic criteria for ALS were based on the El Escorial criteria outlined by the World Federation of Neurology [6]. Bunina body inclusions and hyaline ubiquitinated inclusions were detected in the residual motor neurons in all SALS cases. One SALS case (ALS1) and a control (C1) were used for the molecular indexing. Five cases each (ALS1–5 and C1–5) were analyzed for the second screening, microarray analysis. For verification, real-time RT-PCR with TaqMan probe was performed on all cases (Table 1). Each autopsy was performed within 9 h post mortem, and spinal cord samples of lumbar segments (L4/L5) were snap frozen in liquid nitrogen, then stored at −80°C until use. Total RNA was isolated from anterior horn specimens, where motor neurons exist, using the Micro RNA Isolation Kit (Stratagene, La Jolla, CA, USA), and it was reverse transcribed by MMLV reverse transcriptase, Superscript II (Life Technologies, Grand Island, NY, USA).

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2.2. Molecular indexing and collection of screened cDNAs

SALS (ALS1) and control (C1) spinal cords were used for molecular indexing. Briefly, after synthesizing from total RNA, double-stranded cDNA was cut with one of three class IIS restriction enzymes, *Bsm*FI, *Bsm*AI or *Fok*I, and ligated to each of 64 biotinylated adapters cohesive to all possible overhangs. Ligated molecules were digested by the other class IIS restriction enzymes, and were recovered with streptavidin-coated paramagnetic beads. PCR was performed with the adapter primer and an anchored oligo-dT primer. The amplified fragments were separated by polyacrylamide gel electrophoresis. By carrying out the experiment with 64 adapters, three enzymes and three anchored oligo-dT primers, a total of 576 profiles of cDNAs were established. This procedure has been described in detail in previous reports [7,8].

The 576 profiles obtained from the SALS spinal cords were compared with those from the controls, and the cDNA fragments with an apparent difference in peak between the ALS spinal cord and control were chosen as candidate gene fragments. The standard used for this selection was that ratios between SALS and control were more than 5 or less than 0.2. The purified PCR products were inserted into pGEM T-Easy vector (Promega). The insert was sequenced using an autosequencer. The sequence was applied to the search for the information using BLAST of NCBI on the web.

2.3. Screening by custom-spotted cDNA microarray

The cDNA fragments obtained were amplified and spotted on glass arrays in duplicate (DNA Chip Research, Kanagawa, Japan). The samples of total RNA (5 μg) were reverse transcribed into first-strand cDNA using 20 U Superscript II and 1 μl of 0.5 mg/ml T7-oligo-dT primer (5′-TCT AGT CGA CGG CCA GTG AAT TGT AAT ACG ACT CAC TAT AGG GCG T_{21}-3′) at 42°C for 1 h in 20 μl of reaction [1×first-strand reaction buffer, 10 mM dithiothreitol (DTT), 0.25 mM dNTPs, 20 U RNasin (Promega, Madison, WI, USA)]. Next, 30 μl of 5×second-strand synthesis buffer, 3 μl of 10 mM dNTPs, 4 μl DNA polymerase I, 1 μl *Escherichia coli* RNase H, 1 μl *E. coli* DNA ligase and 92 μl of RNase-free H_2O were added, incubated at 16°C for 2 h, then incubated at 16°C for 10 min after the addition of 2 μl of T4 DNA polymerase. The double-stranded cDNA was extracted with 150 μl of phenol–chloroform to eliminate proteins and then purified using a Microcon-100 column (Millipore, Tokyo, Japan) to separate out the unincorporated nucleotides and salts. RNA amplification was performed using Ampliscribe T7 Transcription Kit (Epicentre Technologies, Madison, WI, USA) in a mixture of 8 μl double-stranded cDNA, 2 μl of 10×Ampliscribe T7 buffer, 1.5 μl each of 100 mM ATP, CTP, GTP and UTP, 2 μl 0.1 M DTT and 2 μl of T7 RNA polymerase, which were incubated at 42°C for 3 h. The amplified RNA was purified with RNeasy Mini Kits (Qiagen, Valencia, CA, USA). T7 RNA polymerase-amplified RNA (aRNA) from control mix and SALS was labeled with Cy3 and Cy5 fluorescence dyes, respectively, using the Atlas Glass Fluorescent Labeling Kit (Clontech, Palo Alto, CA, USA).

Cy3- and Cy5-labeled probes were hybridized to the custom-spotted arrays described above at 62°C for 16 h in a solution of 2×Denhardt's solution, 4×SSC and 0.2% SDS. After hybridization the arrays were washed in 2×SSC–0.1% SDS and 0.2×SSC for 20 min, respectively, and then rinsed with 0.2×SSC and 0.05×SSC. The slides were air-blown dried and prepared for scanning. The microarrays were scanned for fluorescence with GenePix (Axon, Union City, CA, USA).

2.4. Real-time RT-PCR

First-strand cDNA was synthesized from total RNA (1 μg) using 20 U Superscript II. Real-time RT-PCR was carried out using iCycler system (Bio-Rad Laboratories, Hercules, CA, USA) as described elsewhere [9]. All experiments were carried out in quadruplicate, and several negative controls were included. Fluorescence emission spectra were continuously monitored and analyzed with sequence detection software. For internal standard control, the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was quantified simultaneously.

Primers and probe sequences (forward primer, reverse primer, and TaqMan probe): [GAPDH] 5′-CCT GGA GAA ACC TGC CAA GTA T-3′, 5′-TGA AGT CGC AGG AGA CAA CCT-3′, 5′-CAT CAA GAA GGT GGT GAA GCA GGC ATC-3′; [clone 1] 5′-CCG TCA CTC AAA AGG TTG AA-3′, 5′-TGG CGT GTG AAG TGA CTT TT-3′, 5′-AGC ATG AAG AGA CCT TTG AGG AGA AAC TAG TG-3′; [clone 2] 5′-AAG AGC AAG GAC CGC AAG TA-3′, 5′-GGG TGG CTC AGG TGA AGT AG-3′, 5′-CTC AGC GAG TAT GGC ATC AAT GTG AAG AAG-3′; [clone 3] 5′-TAG GAG CCA TCC GAG ACA AC-3′, 5′-TAC CAT GGC ACT TCC TGA CA-3′, 5′-CAC TAG CTG GAG CCA GTA TAA CGG GGA-3′; [clone 4] 5′-GAA GCT GAA GCA GGA TCA AA-3′, 5′-AGG GAG CAG CTT TCA CCT AT-3′, 5′-AAG TCC TTA AGA AAG CTG GGC TTG CCC AC-3′; [clone 5] 5′-GCC TGT TCT GTC ACC ATC AA-3′, 5′-GCA GAG GCC ACA GGT TTA GA-3′, 5′-CCA TTC AGC AGT CCA TTG AAA GGC TCT TAG-3′; [clone 6] 5′-CCT GTT GGA GGA GCA GAA CT-3′, 5′-GAA GCC ACA GAC AGC ACA GA-3′, 5′-AGG GCC CTA ACT ACC TGA CGG CCT GT-3′; [clone 7] 5′-GTT GAC CGA GCT GGA GAA AG-3′, 5′-CCT GTA GAC GGC ATG GAA AT-3′, 5′-CAT CGA CGT CTA CCA CAA GTA CTC CCT GAT-3′; [clone 8] 5′-AAG TGC GAG GGA TGC AAA T-3′, 5′-ACA CAG TCC TTG GCA CAC TT-3′, 5′-ACC TCC TGC AAG AAG AGC TGC TGC TC-3′; [clone 9] 5′-TCC CTT GAT CCC ACA AGT TC-3′, 5′-ACA GGC ATA CAC CAC CAC AT-3′, 5′-AGG CAG CAT AGT GAG ACC CCC ATC TCT ATA-3′; [clone 10] 5′-AGC TGC AGA ACA AGG AGC AT-3′, 5′-GTG AAG CCC CAC TTC TTT GA-3′, 5′-AGT TCA AGT TTC CTG GCC GCC AGA AGA T-3′; [clone 11] 5′-ACT GGT ACC CGT TGG AAC AA-3′, 5′-GTT CAT CCC ATC GTG GAT TT-3′, 5′-TGG TAC ACG ATT TTT AAG GAC CAC GTG TCT-3′.

Table 1
Details of patients

Patient	Age at death (years)	Sex	Postmortem interval (hours)	Disease duration (months)
ALS1	79	F	6	36
ALS2	67	F	6	48
ALS3	47	F	6	17
ALS4	74	M	6	45
ALS5	65	M	7	56
ALS6	57	M	5	48
ALS7	59	F	3	16
ALS8	57	M	6	68
C1	57	M	2	
C2	72	M	4.5	
C3	78	M	4.5	
C4	52	F	6	
C5	75	M	3	
C6	65	F	6	
C7	48	M	6	
C8	42	M	9	
ALS Ave.	63.1 ± 10.3		5.6 ± 1.2	
Control Ave.	61.1 ± 13.4		5.1 ± 2.2	

ALS1–8 are SALS patients. C1–8 are controls without neurological diseases.

Table 2
Candidate fragments from molecular indexing

	Up-regulated in ALS	Down-regulated in ALS	Total
BLAST matched genes	35	23	58
EST	6	11	17
Genome data base	5	4	9
Total	46	38	84

Molecular indexing yielded 84 candidate genes differentially expressed in SALS spinal cord compared with control spinal cord.

2.5. Statistics

Because of the size and distribution of the samples, a non-parametric two-tailed test was chosen (Mann–Whitney *U*-test). Significant values were calculated using StatView software (Hulinks, Tokyo, Japan).

3. Results and discussion

3.1. Molecular indexing and subsequent cDNA microarray analysis

Molecular indexing is a modified version of differential display. Compared with commercially based cDNA microarrays, these PCR-based screening procedures have the advantage of being able to cover an unrestricted range of expressed genes and to detect mRNAs of low abundance [10]. On the other hand, only one or a very limited number of samples can be practicably screened. Compared with conventional differential displays, this technique displays genes with the least redundancy and is less sensitive to the quality of RNA, which is advantageous in handling clinical materials [7,8].

The 576 electropherograms obtained from the SALS spinal cords were compared to control spinal cords. Eighty-four fragments with obvious differences in intensity were obtained. Forty-six of the 84 fragments showed stronger expression in the SALS spinal cord. The other 38 were highly expressed in the control spinal cord. Among the 84 fragments, 58 matched known genes in BLAST searches, 17 were expressed sequence tags (ESTs) and nine matched neither known genes nor ESTs (Table 2).

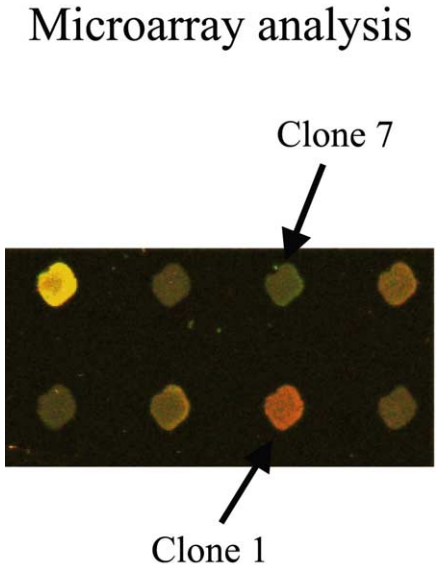

Fig. 1. Close-up image of microarray. Two different fluorescence dyes, Cy3 and Cy5, were on the array together. A stronger Cy3 signal (green) indicates down-regulation and a stronger Cy5 signal (red) indicates up-regulation in SALS. For example, clone 1 shows an orange color, implying down-regulation, whereas clone 7 with a green color is up-regulated in SALS compared to control.

For the second screening, we spotted the 84 fragments on glass arrays and hybridized fluorescent aRNA from the SALS and control spinal cords (ALS1–5 and C1–5) on the arrays. The aRNA samples of all the control cases were mixed and used for the comparison with each SALS case. The expression ratio was calculated by dividing the fluorescence intensity of gene elements in each SALS sample by the fluorescence intensity of gene elements in the control mix. Several genes markedly altered their expression levels in the spinal cords between SALS and control mix (Fig. 1). Genes whose expression was consistently different (ratio of more than 2.0 or less than 0.5, SALS/control) between each SALS case and control mix were selected as the second candidates. Four genes showed stronger signals (clones 1–4) and seven showed weaker signals (clones 5–11) in the SALS spinal cords (Table 3).

3.2. Verification of candidate genes by real-time RT-PCR

The expression of 11 candidate clones in the SALS and the control spinal cords was examined by real-time RT-PCR using TaqMan probes. Six clones were determined to have significantly different expression in the SALS spinal cords. Three clones (2, 3 and 4) were significantly up-regulated in SALS cases compared with controls ($P < 0.05$), and the other three clones (7, 8 and 11) showed significantly lower expression in SALS cases than in controls ($P < 0.05$). However, clones 1, 5, 6, 9 and 10 showed no significant difference (Fig. 2).

A similarity search revealed that clones 2, 7, 8 and 11 were identical to four known cDNAs, 30-kDa TATA-binding protein-associated factor (TAFII30), macrophage-inhibiting factor-related protein-8 (MRP8), metallothionein-3 (MT-3) and ubiquitin-like protein 5 (UBL5), respectively. Clones 3 and 4 proved to be identical to ESTs. We cloned these two unknown

Table 3
Gene expression on microarray analysis

Clone number	Expression ratio (ALS/control)
1	2.94 ± 1.33
2	2.67 ± 1.41
3	2.39 ± 1.21
4	2.12 ± 0.77
5	0.44 ± 0.19
6	0.28 ± 0.14
7	0.27 ± 0.21
8	0.36 ± 0.18
9	0.35 ± 0.16
10	0.33 ± 0.15
11	0.45 ± 0.25

Data are means ± S.D. of five different SALS case/control mixes.
Genes whose expression was consistently different (ratio of more than 2.0 or less than 0.5, SALS/control) between each SALS case and control mix were selected as the second candidates.

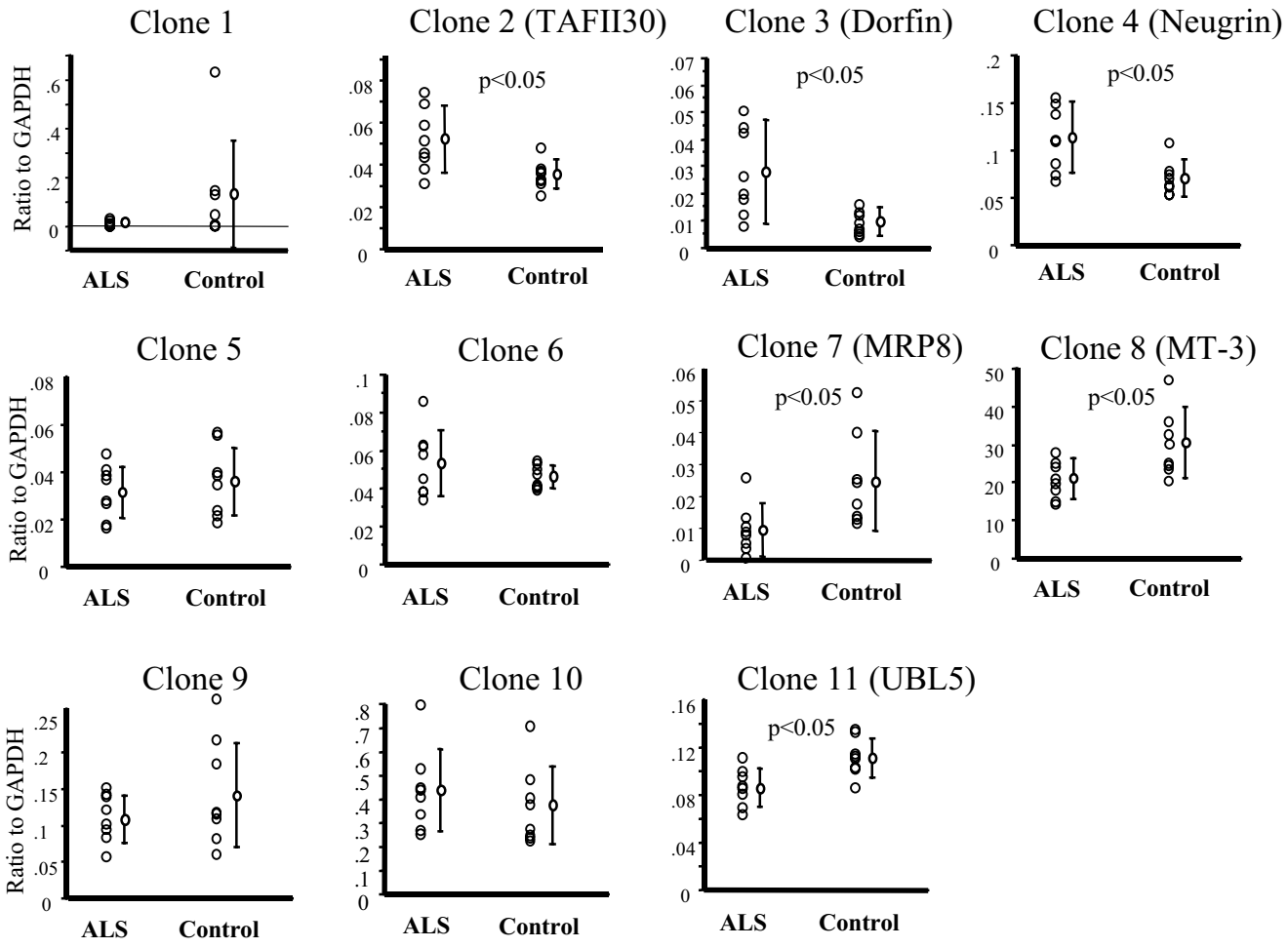

Fig. 2. Verification of SALS-specific gene expression by real-time RT-PCR. The expression level is shown as the weight ratio of the target gene to GAPDH. Quantification of standard templates and each sample was determined by four different experiments. Eight samples each of SALS and controls were examined. Values are the means ± S.D., $n = 8$; statistics were carried out by Mann–Whitney U-test. There were significant differences between SALS and control in clones 2, 3, 4, 7, 8 and 11 ($P < 0.05$). No significant difference was found for other clones.

genes using RACE methods and named them dorfin and neugrin in previous reports [11,12].

TAFII30 is one of the TATA-binding protein-associated factors required for transcription of a subset of genes [13]. An immunohistochemical study using SCA7 transgenic mice showed that TAFII30 is accumulated in nuclear inclusions in neurons. The trapping of this transcription initiation factor in nuclear inclusions may alter the activity of cellular transcription, and contribute to neuronal toxicity in SCA7 model mice [14]. Our result showed up-regulation of the TAFII30 gene in the SALS spinal cords, which suggests that this molecule may also be involved in SALS pathophysiology. More investigation is necessary to clarify whether up-regulation of this molecule has a pivotal role in SALS or only reflects some disruption of transcription function occurring in SALS.

Dorfin is a new RING finger-type ubiquitin ligase, containing two RING finger motifs and an IBR motif at its N-terminus. This gene is ubiquitously expressed in central nervous system tissues [11]. Our recent study showed that dorfin is localized in the ubiquitinated inclusions of FALS and SALS, bound and ubiquitinated various mutant forms of SOD1 in vitro, and protected from mutant SOD1-mediated neurotoxicity in mutant SOD1 culture cells [15]. These results indicate that dorfin has a crucial role in the pathomechanism of mutant SOD1-mediated FALS. In the present study we showed that the expression of the dorfin gene was significantly higher in the SALS than in the control spinal cords. Considering that it has a protective function in FALS and is localized in inclusions of SALS, dorfin may play some protective role in the pathogenesis of SALS as well. Since some authors consider that misfolded protein generally play a central role in various neurodegenerative disorders, such as Huntington disease, Alzheimer disease, Parkinson disease, familial spinocerebellar degeneration and ALS [16], dorfin may possibly work to eliminate proteins which are toxic to motor neurons in the SALS spinal cord.

The expression of neugrin increases with the process of neuronal differentiation. This gene is strongly expressed in the nervous system and mainly in neurons in the spinal cord [12]. These results indicate that up-regulation of this gene in the SALS spinal cord reflects gene expression changes in dying neurons, not in reactive proliferation of glial cells. However, the potential role of neugrin remains to be elucidated.

MRP8 is mainly expressed in microglial cells in neuronal tissues, and it is up-regulated with microglial activation, such as traumatic injury or viral infection [17,18]. MRP8 decreased in the SALS spinal cords in the present study. This is paradoxical because gliosis is one of the hallmarks in ALS path-

ology. However, it indicates that an inflammatory disturbance may influence the etiology.

MT-3 is a neuron-specific member of the zinc- and copper-binding metallothionein family with a role in metal homeostasis and in scavenging free radicals [19,20]. The elevation of MT-3 expression in G93A SOD1 transgenic mice was reported [21,22], and reduction of MT-3 promotes the onset of disease and death in G93A SOD1 transgenic mice [23]. In sporadic Alzheimer's disease brains, MT-3 is significantly down-regulated [19,24]. In the present study we demonstrated that MT-3 mRNA is decreased in the SALS spinal cords, but another report failed to show a significant difference in the MT-3 mRNA expression level in SALS [25]. The fact that MT-3 was up-regulated in FALS but down-regulated in SALS leads one to speculate that this molecule may work for protection in FALS but at the same time may have a causative role in the disease progression of SALS pathogenesis. Although the reduction of its expression might be only due to the loss of neurons in the SALS spinal cords, we did not investigate its expression level in single neurons.

UBL5 is one of the ubiquitin-like proteins. It may possibly organize another ubiquitin-like system, but its function remains unknown [26].

In summary, we identified six differentially expressed genes in SALS spinal cords, including two novel genes, using molecular indexing, subsequent microarray analysis, and real-time RT-PCR for verification. These molecules are involved in cellular mechanisms known to be associated with SALS pathophysiology, including oxidative toxicity, inflammation, transcription and the ubiquitin–proteasome system.

Thus, our screening method using molecular indexing for preliminary screening was useful to uncover unknown genes and genes with rather low expression levels that might have pivotal roles in the pathogenesis of SALS.

Extensive analysis of these genes is necessary to understand how they participate in the disease course and if they are key molecules to explore treatments for ALS.

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