Gene Expressions Specifically Detected in Motor Neurons (Dynactin 1, Early Growth Response 3, Acetyl-CoA Transporter, Death Receptor 5, and Cyclin C) Differentially Correlate to Pathologic Markers in Sporadic Amyotrophic Lateral Sclerosis

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Abstract
In a differential gene expression profile, we showed previously that dynactin 1 (DCTN1), early growth response 3 (EGR3), acetyl-CoA transporter (ACATN), death receptor 5 (DR5), and cyclin C (CCNC) were prominently up- or downregulated in motor neurons of sporadic amyotrophic lateral sclerosis (ALS). In the present study, we examined the correlation between the expression levels of these genes and the levels of pathologic markers for motor neuron degeneration (i.e. cytoplasmic accumulation of phosphorylated neurofilament H [pNF-H] and ubiquitylated protein) and the numbers of residual motor neurons in 20 autopsies of patients with sporadic ALS. DCTN1 and EGR3 were widely downregulated, and the changes in gene expression were correlated to the number of residual motor neurons. In particular, DCTN1 was markedly downregulated in most residual motor neurons before the accumulation of pNF-H. ACATN, DR5, and CCNC were upregulated in subpopulations of residual motor neurons, and their expression levels were well correlated with the levels of pNF-H accumulation and the number of residual motor neurons. The expressions of DCTN1, EGR3, ACATN, and DR5 were all markedly altered before ubiquitylated protein accumulation. DCTN1 downregulation appears to be an early event before the appearance of neurodegeneration markers, whereas upregulations of DR5 and CCNC are relatively later phenomena associated with pathologic markers and leading to neuronal death. The sequence of motor neuron-specific gene expression changes in sporadic ALS can be beneficial information in developing appropriate therapeutic strategies for neurodegeneration.

Key Words: Amyotrophic lateral sclerosis (ALS), Axonal transport, Cell death, Dynactin 1, Motor neuron.

INTRODUCTION
Amyotrophic lateral sclerosis (ALS) is a devastating neurodegenerative disease characterized by loss of motor neurons in the spinal cord, brainstem, and motor cortex (1), causing weakness of the limbs, abnormalities of speech, and difficulties in swallowing. The weakness ultimately progresses to respiratory impairment and half of the patients die within 3 years of the onset of symptoms, largely due to respiratory failure. About 5% to 10% of all patients with ALS show familial traits, and 20% to 30% of patients with familial ALS have a mutation in the copper/zinc superoxide dismutase 1 gene (SOD1). However, in more than 90% of patients with ALS, the disease is sporadic and does not show any familial traits. The presence of Bunina bodies in the remaining spinal motor neurons is a hallmark of cases of sporadic ALS (2, 3). There is at present no obvious consensus understanding of the pathogenic mechanism or an effective therapeutic approach for sporadic ALS, although several hypotheses, including oxidative stress, glutamate excitotoxicity, impaired axonal transport, neurofilament disintegration, mitochondrial dysfunction, neurotrophic deprivation, and proteasomal dysfunction have been proposed as causal mechanisms of motor neuron degeneration (4–11). In contrast, wide-ranging research activities have been initiated for a subgroup of patients with familial ALS, those with mutant SOD1, including a search for the pathogenic mechanisms of mutant SOD1-induced motor neuron death and the examination of therapeutic perspectives using a transgenic rodent model for mutant SOD1 familial ALS (12–14).

One effective approach to begin the uncovering of the pathogenic mechanism of sporadic ALS is a description of
the gene expression profile of motor neurons. Motor neuron-specific gene expression profiling would eventually lead us to a profound understanding of the pathophysiology of motor neuron degeneration in sporadic ALS. We have successfully created such a motor neuron-specific gene expression profile in patients with sporadic ALS by using microarray technology combined with laser-captured microdissection, the results of which were further verified by in situ hybridization and quantitative wide-ranging research activities (15). The genes with differential expressions in these profiles were particularly related to axonal transport, transcription, energy production, cell death, and protection from cell death. Gene expressions of dynactin 1 (DCTN1) and early growth response 3 (EGR3), related to cytoskeleton/axonal transport and transcription, respectively, were markedly decreased in motor neurons, whereas cell death-associated genes such as death receptor 5 (DR5), cyclin C (CCNC), and acetyl-CoA transporter (ACATN) were greatly upregulated. It is, however, uncertain how these gene expression alterations correlate with motor neuron degeneration and death and whether they play a role in the pathogenesis of sporadic ALS. A description of the molecular events underlying motor neuron degeneration in sporadic ALS, even particular short aspects of a long sequence of the degeneration process, would provide a beneficial therapeutic avenue for sporadic ALS by enabling development of a disease model simulating these molecular events.

In this study we further characterized the gene expression profiles of DCTN1, EGR3, ACATN, DR5, and CCNC in individual motor neurons and compared their expression levels with those of known motor neuron neurodegeneration markers, cytoplasmic accumulations of phosphorylated neurofilament H (pNF-H), ubiquitylated proteins, and with neuronal loss (5, 16–18). We found that changes in expression levels of these genes differentially reflect the motor neuron degeneration process, and, in particular, DCTN1 is extensively downregulated before the appearance of these degeneration markers.

MATERIALS AND METHODS

Tissues From Patients with Amyotrophic Lateral Sclerosis and Control Patients

Specimens of lumbar spinal cord (L4–L5 segments) from 20 patients with sporadic ALS (11 male and 9 female) and 8 neurologically normal patients (4 male and 4 female) as controls were obtained at autopsy. The diagnosis of ALS was confirmed by El Escorial diagnostic criteria defined by the World Federation of Neurology and by histopathologic findings, particularly the presence of Bunina bodies (2, 3). All patients with ALS had sporadic ALS and showed no hereditary traits. Patients with a SOD1 mutation were excluded. The collection of tissues and their use for this study were approved by the ethics committee of Nagoya University Graduate School of Medicine. The ages for patients with ALS and control patients were 63.3 ± 11.5 (mean ± SD) (range 43–80) and 65.4 ± 12.7 (42–79) years, respectively, and the ALS illness duration was 2.9 ± 0.87 (1.2–4.3) years. The postmortem intervals to autopsy for patients with ALS and control patients were 7.3 ± 3.9 (3–15) and 8.6 ± 3.2 (4–13) hours, respectively. There were no significant differences in either age or postmortem interval between the ALS and control groups. Among 20 patients, severe bulbar symptoms were seen in 14 cases, severe upper limb wasting in 17 cases, and severe lower limb wasting in 13 cases in the advanced stage. The upper motor neuron signs were seen in 13 patients, whereas others showed predominantly lower motor neuron signs. Most of the patients with ALS developed respiratory dysfunction in various degrees, with eventually resulted in respiratory failure in all patients, which was the cause of death. The cause of death in the control patients was pneumonia, cancer, stroke, or acute heart attack. Tissues were immediately frozen in liquid nitrogen and stored at −80°C until use. Parts of the lumbar spinal cord were fixed in 10% buffered formalin solution and processed for paraffin sections. The sections were stained with hematoxylin and eosin and Klüver-Barrera techniques and further histologic assessments were performed.

Selection of Genes Examined Based on Our Previous Microarray Analysis in Laser-Captured Motor Neurons of Patients with Sporadic Amyotrophic Lateral Sclerosis

By using microarray technology combined with laser-captured microdissection, gene expression profiles of degenerating spinal motor neurons isolated from autopsied patients with sporadic ALS were previously reported (15). Three percent of genes examined were downregulated, and 1% were upregulated. We selected 5 genes (DCTN1 associated with cytoskeleton/axonal transport, EGR3 as a transcription factor, and ACATN, DR5 and CCNC as cell death-associated genes), which were most prominently down- or upregulated (15). These changes in gene expression were confirmed by cluster analyses of hierarchical clustering, self-organizing maps, and principal component analyses after logarithmic transformation, as motor neuron-specific gene expression changes distinctive from the spinal ventral horn as a whole, and their alterations were further quantitatively verified by real-time wide-ranging research activities and in situ hybridization. In addition, these 5 genes were chosen for the present study because they cover a wide range and represent different aspects of the functional hierarchy.

In Situ Hybridization

Frozen, 10-μm-thick spinal cord sections were prepared and immediately fixed in 4% paraformaldehyde. The sections were then treated with 0.1% diethylpyrocarbonate twice for 15 minutes and prehybridized at 45°C for 1 hour. Digoxigenin-labeled cRNA probes were generated from linearized plasmids for the genes of interest using SP6 or T7 polymerase (Roche Diagnostics, Basel, Switzerland). Gene names, GenBank accession numbers, probe positions (nucleotide [nt] number), and probe sizes (base pairs [bp]) were as follows: acetyl-CoA transporter (ACATN), D88152, nt 397–741, 345 bp; dynactin 1 (DCTN1), NM_004082, nt 2392–2774, 383 bp; death receptor 5 (DR5), NM_004082, nt 682–1070, 389 bp; and early growth response 3 (EGR3), NM_004430, nt 1433–1794, 362 bp. After prehybridization
the sections were hybridized with digoxigenin-labeled cRNA probes overnight at 45°C. The washed sections were incubated with alkaline phosphatase-conjugated, anti-digoxigenin antibody (Roche Diagnostics). The signal was visualized with nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (Roche Diagnostics). No hybridization signal was observed with the sense probe for the expression of each gene in spinal motor neurons.

Immunohistochemistry

Frozen, 10-μm-thick spinal cord sections were prepared and immediately fixed in 4% paraformaldehyde. The sections were then blocked with 2% bovine serum albumin (Sigma) in Tris-buffered saline at room temperature for 20 minutes and incubated with either a monoclonal antibody against the phosphorylated epitope domain of neurofilament H (anti-SMI 31, 1:1000; Sternberger Monoclonals Inc., Lutherville, MD), anti-cyclin C antibody (1:200; Santa Cruz Biotechnology, Santa Cruz, CA), or anti-ubiquitin (1:1000; Santa Cruz Biotechnology) overnight at 4°C. Subsequent procedures were carried out using the EnVision+Kit/HRP (DAB) (DAKO, Glostrup, Denmark) according to the manufacturer’s protocol.

Quantitative Assessment of Gene Expression Levels, Population of Residual Motor Neurons, and Cytoplasmic Accumulation of Phosphorylated Neurofilament H and Ubiquitylated Proteins

To assess gene and protein expression levels in spinal motor neurons, signal intensities of in situ hybridization and immunohistochemistry, respectively, were quantified using a CCD image analyzer (Zeiss Axiovert S100TV) as described previously (19, 20). Images of individual motor neurons on transverse sections of spinal cord with signals for ACATN, EGR3, ACATN, DR5, or CCNC and pNF-H were captured at the desired magnification and stored with image software (Adobe Photoshop). Grey-scale levels in 65,536 gradations of the images were quantitatively analyzed with image analysis software (Image Gauge version 4.0, Fujifilm, Tokyo, Japan). Signal intensities were expressed as individual intracellular cytoplasmic signal levels (arbitrary absorbance units/mm²) of motor neuron for each gene of interest by subtracting the mean background levels of 3 regions of interest in each section. We also assessed motor neurons harboring ubiquitylated proteins on the anti-ubiquitin-stained sections. Motor neurons with dot-like accumulations, skein-like accumulations, or large inclusions of cytoplasmic ubiquitylated proteins were designated as positive for ubiquitylated protein accumulation.

To count the number of remaining spinal motor neurons, 10-μm-thick serial sections were prepared from the lumbar segment of spinal cords and every 10th section was stained with the Klüver-Barrera technique. The ventral spinal horn was designated as the grey matter ventral to the line through the central spinal canal perpendicular to the ventral spinal sulcus, and the residual motor neuron population was defined as the number of relatively large-sized ventral horn cells of 24.8 μm or more in diameter with distinct nucleoli on 10 sections, as described previously (21). Our previous study demonstrated the neuron loss predominantly in the relatively large neurons in ALS (21).

The frequency of motor neurons showing changes in gene expression levels was assessed in at least 10 transverse sections from each of 20 patients with ALS and 8 control patients. The number of motor neurons with gene expression levels more than ±2 SDs from the control levels was expressed as a percentage of motor neurons. Ten to > 100 motor neurons were examined for each individual case.

To investigate the correlation between gene expression levels and pathologic markers in an individual motor neuron we used consecutive transverse spinal cord sections. Ten sets of consecutive sections for each gene of interest were prepared from each patient and the correlations of gene expression levels with pNF-H accumulation levels and positive or negative ubiquitylated protein accumulations were assessed on individual motor neurons. This assessment was performed for 8 representative patients with ALS and 8 control patients whose sections were available for examination.

Statistical Analyses

Simple correlation tests were performed to assess the correlation of gene expression levels of DCTN1, EGR3, ACATN, and DR5 and protein expression of CCNC, with the degree of pNF-H accumulation in individual motor neurons. This test was also applied to assess the correlation of the gene expression changes with the numbers of residual motor neurons. Mann-Whitney U tests were used to compare gene expression levels among the motor neurons that were either positive or negative for ubiquitylated proteins in patients with ALS and in control patients. Significance levels were set to p < 0.05.

RESULTS

Differential Frequencies of Gene Expression Changes in Residual Motor Neurons: DCTN1 Is Highly Downregulated

Among the genes examined, DCTN1 and EGR3 were downregulated in the vast majority of spinal motor neuron populations in most patients (Fig. 1A, B). In 15 of 20 patients, all of the residual motor neurons had reduced DCTN1 expression compared with controls (Fig. 1B). Ten of the 20 patients showed downregulation of EGR3 in all residual neurons (Fig. 1B). ACATN, by contrast, was upregulated in all residual motor neurons in only 8 of 20 patients (Fig. 1A, B). DR5 was upregulated in subpopulations of motor neurons, whereas only 4 patients had upregulated gene expression in all of the residual neurons (Fig. 1A, B). Nuclear accumulation of CCNC protein assessed by immunohistochemistry was observed in only a small percentage of motor neurons in most patients (Fig. 1A, B). There were no patients with CCNC nuclear accumulation in all of the residual neurons (Fig. 1B). Cytoplasmic accumulation of pNF-H was seen in more than half of the residual motor neurons in most of the patients (Fig. 1A). Quantitative assessment showed...
that in 9 of the 20 patients, all of the residual motor neurons were positive for pNF-H (Fig. 1B). Thus, the frequency of residual motor neurons with gene up- or downregulation was markedly different depending on the individual gene. This gene-dependent differential gene expression among the residual motor neurons is clearly demonstrated in Figure 1C. Two consecutive transverse sections were subjected to in situ hybridization with different gene probes. DCTN1

**FIGURE 1.** In situ hybridization (ISH) and immunohistochemistry (IHC) in spinal motor neurons. (A) Representative ISHs are shown for dynactin 1 (*DCTN1*), early growth response 3 (*EGR3*), acetyl-CoA transporter (*ACATN*), and death receptor 5 (*DR5*). The antisense probe detects positive signals for the expression of each gene in spinal motor neurons for patients with amyotrophic lateral sclerosis (ALS) and/or control patients, but the sense probe does not (15). RNase treatment before the hybridization abolished the hybridization signals. IHC was performed for cyclin C (*CCNC*) and phosphorylated neurofilament H (pNF-H). The nuclear staining of CCNC was prominent in ALS motor neurons. Lipofuscin granules are seen as yellowish granules. (B) Percentage of motor neurons with gene expression or protein accumulation changes, relative to control levels, among the residual motor neurons in 20 patients with ALS. The quantitative analyses of gene expression and frequency assessments are described in the Materials and Methods section. (C) ISH of 2 genes in consecutive sections demonstrates the relationship between up- and downregulated genes in individual motor neurons. DCTN1 was downregulated to a great extent in all the remaining motor neurons in ALS, whereas ACATN and DR5 were upregulated in only a subpopulation of motor neurons. Arrows denote motor neurons with gene expression changes, and asterisks denote those without changes compared with controls. Scale bars = 25 μm.
expression was reduced in all the neurons in each panel, whereas some of the residual motor neurons showed unchanged ACATN and DR5 gene expression (Fig. 1C). Moreover, DCTN1 expression was preserved in neurons other than motor neurons such as those in the dorsal nucleus of Clarke and the intermediolateral nucleus in spinal cords, Purkinje cells of the cerebellum and cortical neurons in the occipital cortex in patients with ALS as well as control patients (data not shown). These findings indicate that, among the genes examined, downregulation of DCTN1 was specific and extensive in the spinal motor neurons, whereas cell death-related genes such as ACATN and DR5, and the protein CCNC were upregulated in only subpopulations of motor neurons.

Gene Expression Changes Are Differentially Correlated With the Population of Residual Motor Neuron: DCTN1 is Markedly Downregulated Even in Patients With Relatively Well-Preserved Motor Neuron Populations

When the numbers of motor neurons with given gene expression or protein accumulation changes were compared with the numbers of residual motor neurons in the 20 patients, changes in DR5, pNF-H, EGR3, ACATN and DCTN1 were correlated with the residual motor neuron population (r = 0.49–0.83, p < 0.05 to 0.0001, Fig. 2). Among them, DCTN1 expression was most prominently downregulated, even in patients with relatively well-preserved motor neurons, suggesting that DCTN1 downregulation may be occurring even in early stages of the disease. The changes in expression of EGR3, ACATN, and DR5 in patients with well-preserved motor neuron populations were relatively mild compared with that of DCTN1 (Fig. 2). The change in DR5 expression was less correlated with the residual motor neuron population than that of other genes.

Gene Expression Changes are Differentially Correlated With the Extent of Motoneuronal Cytoplasmic Phosphorylated Neurofilament H Accumulation: DCTN1 and EGR3 Are Markedly Downregulated Before Phosphorylated Neurofilament H Accumulation

The correlation of gene expression and pNF-H accumulation, a marker of neuronal degeneration, in individual motor
neurons was assessed on consecutive sections (Fig. 3A, B). DCTN1 and EGR3 downregulations were both marked and independent of the degree of cytoplasmic pNF-H accumulation (Fig. 3B), implying that these genes were widely downregulated even in motor neurons with little or no pNF-H accumulation. By contrast, levels of ACATN, DR5, and CCNC ranged from just above the control levels to much higher levels and were well correlated with the degree of cytoplasmic pNF-H accumulation (r = 0.48–0.60, p < 0.001 to 0.0001, Fig. 3B).

These observations indicate that downregulation of DCTN1 and EGR3 occurs before the appearance of the neurodegeneration marker pNF-H and thus is a relatively early event in the neurodegeneration process. In contrast, the changes in ACATN and DR5 expression were milder than those in DCTN1 and EGR3, but proportional to pNF-H accumulation, suggesting that their upregulation is a relatively late event, occurring after the appearance of the neurodegeneration marker pNF-H.

Gene Expression Changes Occur Before Appearance of Motoneuronal Cytoplasmic Accumulation of Ubiquitylated Proteins: DCTN1, EGR3, ACATN and DR5 Are Changed Even in the Motor Neurons Without Ubiquitylated Protein Accumulation

The correlation of gene expression with cytoplasmic accumulation of ubiquitylated protein in individual motor neurons was assessed on consecutive sections (Fig. 4A, B). In patients with ALS, DCTN1 and EGR3 were markedly downregulated, and ACATN and DR5 were upregulated in motor neurons both with and without cytoplasmic accumulation of ubiquitylated proteins (Fig. 4B). However, the degree of downregulation or upregulation was significantly greater in the motor neurons with ubiquitylated protein accumulation compared with those without (Fig. 4B), suggesting that cytoplasmic accumulation of ubiquitylated proteins may be partially correlated to expression changes of DCTN1, EGR3, and ACATN. However, because the expression of these genes changed markedly even in the motor neurons without ubiquitylated proteins, it would imply that cytoplasmic ubiquitylated protein accumulation is a rather late event in the process of motor neuron degeneration.

We further examined the correlation between these 4 gene expression levels and subgroups of motor neurons with 3 different types of ubiquitylated protein accumulation (i.e. dot-like accumulations, skein-like accumulations, and large round inclusions of ubiquitylated proteins), and found that the expression levels of all 4 genes were changed before all types of ubiquitylated protein accumulation (data not shown).

DISCUSSION

We demonstrated that DCTN1, EGR3, ACATN, CCNC, and DR5 are differentially expressed in the residual motor neurons in sporadic ALS. Furthermore, the expression levels of these genes are differentially correlated with the levels of pathologic markers, the numbers of residual motor neurons, and the degrees of cytoplasmic accumulation of pNF-H and ubiquitylated protein, which are considered to reflect degeneration processes of motor neurons in sporadic ALS (5, 16–18, 22). These 5 genes were selected from those showing the most marked and specific altered expression levels among 4,845 genes that we had previously assessed in
FIGURE 3. Gene expression and cytoplasmic phosphorylated neurofilament H (pNF-H) accumulation. (A) Gene expressions and pNF-H accumulation in identical motor neurons. Representative in situ hybridization (ISH) for DCTN1, EGR3, ACATN, and DR5, and immunohistochemistry (IHC) for CCNC are shown compared with pNF-H staining (IHC) on consecutive spinal cord sections from patients with amyotrophic lateral sclerosis (ALS) and control patients. The accumulation of cytoplasmic pNF-H was prominent in ALS motor neurons. Arrows denote motor neurons with gene expression or protein accumulation changes compared with control patients, and asterisks denote those with unchanged levels. Scale bars = 25 μm. (B) Expression levels of genes were compared with the level of pNF-H accumulation in individual motor neurons. Expression levels of ACATN, DR5, and CCNC were correlated with the accumulation of pNF-H, whereas those of DCTN1 and EGR3 were not. Consecutive transverse spinal cord sections were assessed from 8 representative patients with ALS. The control values for gene and protein expression levels and the accumulation of pNF-H are shown as means ± SD for 8 control cases. AU, arbitrary absorbance units; NS, not significant.
FIGURE 4. Gene expression and cytoplasmic accumulation of ubiquitylated proteins. (A) Representative DCTN1 in situ hybridization (ISH) and ubiquitin (UB) immunohistochemistry (IHC) in identical motor neurons of consecutive sections are shown in patients with amyotrophic lateral sclerosis (ALS) and control patients. Scale bars = 25 μm. (B) Expression levels of genes were compared among the motor neurons that were either positive (n = 56) or negative (n = 175) for ubiquitylated proteins in 8 representative patients with ALS and those (n = 209) in 8 control patients. Expression levels of DCTN1, EGR3, and ACATN were significantly different in ubiquitin-positive compared to ubiquitin-negative neurons in ALS, whereas that of DR5 was not. The values are shown as means ± SE. AU, arbitrary absorbance units.
a differential gene expression profile in isolated, laser-captured motor neurons from patients with sporadic ALS (15). Thus, we consider these gene expression levels to reflect most significantly the molecular events of neurodegeneration processes in motor neurons.

We selected 3 pathologic features as markers for neurodegeneration: the residual population of spinal motor neurons, cytoplasmic accumulation of pNF-H, and cytoplasmic accumulation of ubiquitylated proteins. Cytoplasmic accumulation of pNF-H has been demonstrated to occur in ALS motor neurons, even when they have normal morphologic appearances, and is thought to be a consequence of impaired axonal transport (5, 23–25). Hence, it is considered to be a histologic marker indicating neuronal degeneration and dysfunction before neuronal death (5, 16, 17). Hence, the accumulation of pNF-H is a rather early event in the motor neuron degeneration process. The presence of ubiquitylated proteins in the motor neuron cytoplasm has also been identified as a histopathologic marker of motor neuron degeneration (18). Ubiquitylated inclusions are thought to be aggregated, modified, and misfolded proteins that are ubiquitylated by motor neuron ubiquitin ligase (9). Although ubiquitylated, round inclusions are considered to occur in rather advanced stages of degeneration, it is not known whether dot-like and skein-like small faint ubiquitylated accumulations occur in the early stages of neurodegeneration.

The striking observation was that DCTN1 expression was the most widely and most strongly downregulated among the genes examined in the residual motor neuron population, and was also severely downregulated even in the patients with large populations of motor neurons and in the motor neurons without pNF-H accumulations. The dramatic change in DCTN1 in ALS seems to be specific for motor neurons because DCTN1 expression was preserved in neurons in the dorsal nucleus of Clarke and the intermediolateral nucleus in the spinal cord, Purkinje cells of the cerebellum, and cortical neurons in the occipital cortex in patients with ALS. These observations suggest that DCTN1 downregulation is the specific molecular event that occurs before the appearance of these pathologic markers, and is, therefore, a rather early event in the molecular sequences of neurodegeneration, at least among the events related to the genes examined. DCTN1 codes for a protein that is a component of the retrograde transport protein complex with dynein (26, 27) and has been identified as a causative gene for human lower motor neuron disease (28, 29). Furthermore, it has been suggested that polymorphic amino acid substitution is a modifying factor accelerating pathogenesis and progression of sporadic ALS (30). A mouse model overexpressing dynamitin, which eventually results in late-onset progressive motor neuron degenerative disease, demonstrates the involvement of the dynactin-dynein complex (23). Two dominant point mutations in dynein cause progressive motor neuron degeneration in mice (31). These findings suggest that retrograde axonal transport involving the dynactin-dynein complex is strongly associated with motor neuron dysfunction and eventual motor neuron degeneration (32). By taking into account these findings, our present results strongly suggest that the downregulation of DCTN1 in motor neurons may play a significant role in this process and may lead to the subsequent sequences of motor neuron degeneration in sporadic ALS. This hypothesis should be tested by further study on another cohort of patients with ALS and by in vitro and in vivo experiments.

Another interesting observation was that ACATN, DR5, and the CCNC protein were upregulated in subpopulations of residual motor neurons and that their upregulation was well correlated to the accumulation of pNF-H and the degree of motor neuron loss. ACATN functions as a cofactor for acetylation of gangliosides and has been demonstrated to suppress proapoptotic activity of GD3 ganglioside (33–36). In the Drosophila model, knockout of ACATN leads to a lethal phenotype owing to brain damage (Y. Hirabayashi, personal communication, 2007). DR5 is another cell death-related receptor as a member of the tumor necrosis factor (TNF) receptor family (TNFR10b) (37). CCNC is a cell cycle regulator protein and increases in CCNC expression are associated with its nuclear translocation, as was also demonstrated in this study (38). The aberrant activation of cell cycle regulators has been proposed as a pathway inducing motor neuron death in ALS (39, 40). Moreover, upregulated DR5 was colocalized in motor neurons with CCNC nuclear translocation and also in those with downregulated TNFR-associated factor 6 (TRAF6) in our study (data not shown). The downregulated TRAF6, which is associated with nuclear factor-κB activation for cell survival, may not be able to sequester the overexpressed DR5 signaling, leading to a pathway of cell death (41). Taken together, expression of these genes is involved in the cell death-related pathway. Upregulation of these genes occurs in subpopulations of motor neurons in parallel to or after the emergence of histopathologic markers such as pNF-H accumulation and motor neuron loss, suggesting that they occur in a relatively late phase of neurodegeneration, especially compared with DCTN1 downregulation. The observation that active motor neuron degeneration processes for cell death that are probably mediated via cell death-related gene expression, such as ACATN, DR5 and CCNC upregulation, occur in subpopulations of the remaining motor neurons with sustained DCTN1 downregulation is consistent with our previous results that motor neurons in the remaining motor neuron pool randomly enter into the active degeneration process even up to the terminal stage in sporadic ALS (42).

The appearance of ubiquitylated protein accumulations or ubiquitylated inclusions is one of the hallmarks of motor neuron degeneration in sporadic ALS (18). In this study, however, the expression levels of DCTN1, EGR3, ACATN, and DR5 were significantly altered before the appearance of ubiquitylated protein accumulations. Because the morphologic features of cytoplasmic ubiquitylated protein accumulations vary considerably, ranging from fine dot-like or skein-like accumulations to large inclusions, the simple assessment of ubiquitin-positive or negative materials may not be sufficient to identify neurodegeneration. However, even when we assessed ubiquitylated accumulation in a more precise manner, the expressions of these 4 genes were markedly altered independent of the appearance...
of ubiquitylated protein accumulations. These findings suggest that appearance of ubiquitylated protein accumulation is a later pathologic event, occurring after the expressions of a number of genes are already altered. Alternatively, we may speculate that ubiquitylated protein accumulation is a secondary consequence of the series of molecular events accompanied by the alterations of a wide-range of gene expressions.

The present study also demonstrates that microarray analyses on laser-captured motor neurons followed by histopathologic analyses on tissues from large numbers of patients can provide significant information about molecular events in motor neuron degeneration and dysfunction in patients with sporadic ALS. The most serious problem in developing effective therapy for sporadic ALS is the lack of animal or cell models that properly reflect the motor neuron degeneration processes of sporadic ALS or even certain aspects of them. This is not a longitudinal and chronologic analysis of degeneration process in identical motor neurons, and it is not clear whether the changes seen in the present study represent the primary causes or secondary effects in the disease process because of the inherent problem of studying human disease using autopsy materials. However, we may be able to speculate that these results of human studies reflect the molecular sequence of motor neuron degeneration of ALS. Our present approach would provide an avenue for developing new molecular-targeted therapies for sporadic ALS by creating animal or cell models mimicking the molecular events seen in human patients.

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