Table 1
RNA editing efficiency of single motor neurons in SBMA

Case	Age at death (year)	Sex	No. of CAG repeats[a]	Postmortem delay (h)	GluR2(+) MN[b]	MN with 100% editing efficiency (% of GluR2(+) MN)
SBMA, case 1	71	M	48	2.5	12	12 (100)
SBMA, case 2	78	M	42	2.5	16	16 (100)
SBMA, case 3	60	M	44	1	16	16 (100)

[a] Number of CAG repeats in the androgen receptor gene.
[b] Motor neurons in which GluR2 RT-PCR amplifying product was detected.

repeat expansion in the androgen receptor gene has been demonstrated in SBMA (La Spada et al., 1991), and pharmacological castration is therapeutically effective in animal models (Katsuno et al., 2002, 2003), the death cascade responsible for SBMA is likely different from sporadic ALS. In this paper, an investigation is carried out into whether or not the dying mechanism underlying sporadic ALS is the same as ALS1 and SBMA by determining the editing status of the GluR2 Q/R site in single motor neurons.

2. Materials and methods

The animals used in this study were SOD1^{G93A} and SOD1^{H46R} transgenic male rats (Nagai et al., 2001) (n = 3 each) that had exhibited progressive neuromuscular weakness with their littermates as the control (n = 3 each) (Table 2). The first sign of disease in these rats was weakness of their hindlimbs, mostly exhibited by the dragging of one limb. Onset of motor neuron disease was scored as the first observation of abnormal gait or evidence of limb weakness. The mean age of onset of clinical weakness for the SOD1^{G93A} and SOD1^{H46R} lines was 122.9 ± 14.1 and 144.7 ± 6.4 days, respectively. As the disease progressed, the rats exhibited marked muscle wasting in their hindlimbs, and then in the forelimbs. The mean duration after the clinical expression of the disease in the SOD1^{G93A} and SOD1^{H46R} lines was 8.3 ± 0.7 and 24.2 ± 2.9 days, respectively (Nagai et al., 2001). The rats were killed 3 days and 2 weeks after the onset for the SOD1^{G93A} and SOD1^{H46R} lines, respectively, and we examined their fifth lumbar cord. Animals were handled according to Institutional Animal Care and Use Committee approved protocols that are in line with the Guideline for Animal Care and Use by the National Institute of Health. Spinal cords were isolated after deep pentobarbiturate anesthesia. In addition, spinal cords were obtained at autopsy from three genetically confirmed patients with SBMA (Table 1). Written informed consent was obtained from all subjects prior to death or from their relatives, and the Ethics Committees of Graduate School of Medicine, the University of Nagoya and the University of Tokyo approved the experimental procedures used. Spinal cords were rapidly frozen on dry ice and maintained at −80 8C until use.

Table 2
RNA editing efficiency of single motor neurons in mutated human SOD1 transgenic rats

Case (n)	GluR2(+) MN[a]	MN with 100% editing efficiency (% of GluR2(+) MN)
SOD1^{G93A}-1	13	13 (100)
SOD1^{G93A}-2	21	21 (100)
SOD1^{G93A}-3	21	21 (100)
SOD1^{H46R}-1	19	19 (100)
SOD1^{H46R}-2	23	23 (100)
SOD1^{H46R}-3	20	20 (100)
SOD1^{G93A}, littermates (3)	22	22 (100)
SOD1^{H46R}, littermates (3)	20	20 (100)

[a] Motor neurons in which GluR2 RT-PCR amplifying product was detected.

Single motor neurons were isolated and collected into respective single test tubes that contained 200 ml of TRIZOL Reagent (Invitrogen Corp., Carlsbad, CA, USA) using a laser microdissection system as previously described (Kawahara et al., 2003b, 2004) (LMD, Leica Microsystems Ltd., Germany) (Fig. 1a). After extracting total RNA from single neuron tissue, we analyzed the RNA editing efficiency at the GluR2 Q/R site by means of RT-PCR coupled with digestion of the PCR amplified products with a restriction enzyme Bbv-1 (New England BioLabs, Beverly, MA, USA) (Takuma et al., 1999; Kawahara et al., 2003a, 2004), and the editing efficiency was calculated by quantitatively analyzing the digests with a 2100 Bioanalyser (Agilent Technologies, Palo Alto, CA, USA), as previously described (Kawahara et al., 2003a). Briefly, after gel purification using Zymoclean Gel DNA Recovery Kit according to the manufacturer's protocol (Zymo Research, Orange, CA, USA), PCR products were quantified using a 2100 Bioanalyser. An aliquot (0.5 ng) was then incubated at 37 8C for 12 h with 10 × restriction buffer and 2 U of BbvI in a total volume of 20 ml and inactivated at 65 8C for 30 min. The PCR products had one intrinsic BbvI recognition sites, whereas the products originating from unedited GluR2 mRNA had an additional recognition site. Thus, restriction digestion of the PCR products originating from edited rat (278 bp) and human (182 bp) GluR2 mRNA should produce two bands (human GluR2 in parenthesis) at 219 (116) and 59 (66) bp, whereas those originating from unedited GluR2 mRNA should produce three bands at 140 (81), 79 (35), and 59 (66) bp. As the 59 (66) bp band would originate from both edited and unedited mRNA, but the 219 (116) bp band would originate from only edited mRNA, we quantified the molarity of the 219 (116) and 59 (66) bp bands using the 2100 Bioanalyser and calculated the editing efficiency as the ratio of the former to the latter for each sample.

The following primers were used for PCR for rat and human GluR2 (amplified product lengths are also indicated): for rat GluR2 (278 bp): rF (5^0-AGCAGATTTAGCCCCTACGAG-3^0) and rR (5^0-CAGCACTTTCGATGGGAGACAC-3^0). for human GluR2, the first PCR (187 bp): hG2F1 (5^0-TCTGGTTTTCCTTGGGTGCC-3^0) and hG2R1 (5^0-AGATCCTCAGCACTTTCG-3^0); for the nested PCR (182 bp): hG2F2 (5^0-GGTTTTCCTTGGGTGCCTTTAT-3^0) and hG2R2 (5^0-ATCCTCAGCACTTTCGATGG-3^0). We confirmed that these primer pairs were situated in two distinct exons with an intron between them and did not amplify products originating from other GluR subunits (data not shown). PCR amplification for rat GluR2 was initiated with a denaturation step that was carried out at 95 8C for 2 min, followed by 40 cycles of 95 8C for 30 s, 62 8C for 30 s, and 72 8C for 1 min. PCR amplification for human GluR2 began with a 1 min denaturation step at 95 8C, followed by 35 cycles of denaturation at 95 8C for 10 s, annealing at 64 8C for 30 s and extension at 68 8C for 60 s. Nested PCR was conducted on 2 ml of the first PCR product under the same conditions with the exception of the annealing temperature (66 8C).

3. Results

The number of motor neurons was severely decreased in the spinal cord of SBMA patients, and we analyzed 44 neurons dissected from three cases (12 from case 1, 16 from cases 2 and 3). Restriction digestion of the PCR products yielded only 116 and 66 bp fragments but no 81 or 35 bp fragments as seen in ALS motor neurons in all the SBMA motor neurons examined. Likewise, restriction digestion of the PCR products from motor

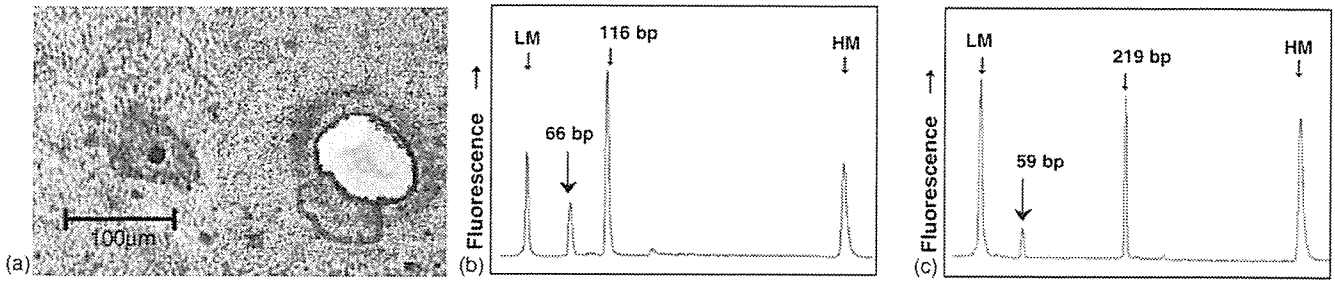

Fig. 1. (a) A single motor neuron from an SBMA patient before (left) and after (right) the dissection with a laser-microdissector. (b and c) An example of electropherogram by a 2100 Bioanalyser. Samples are the Bbv-1-digest of PCR product from tissues of a single motor neuron from an SBMA patient (b) and from a mutated human $SOD1^{G93A}$ transgenic mouse (c). LM: lower marker (15 bp), HM: higher marker (600 bp).

neurons of mutated human SOD1 transgenic rats yielded only 219 and 59 bp fragments (Fig. 1). Therefore, the values of RNA editing efficiency at the Q/R site of GluR2 were 100% in 44 motor neurons from three SBMA cases (Table 1), 55 single motor neurons from three $SOD1^{G93A}$ transgenic rats, 62 neurons from three $SOD1^{H46R}$ transgenic rats, as well as in 42 neurons from three littermate rats of each group (Table 2). The consistent finding that the GluR2 Q/R site is 100% edited in motor neurons of SBMA patients and transgenic rats for mutated human SOD1 is in marked contrast to the finding in ALS motor neurons that the editing efficiency widely varied among neurons ranging from 0% to 100% (Kawahara et al., 2004).

4. Discussion

Compared to the significant underediting reported for the GluR2 Q/R site in motor neurons of sporadic ALS (Kawahara et al., 2004), GluR2 mRNA in all the examined motor neurons of the mutated human SOD1 transgenic rats with two different mutation sites and SBMA patients was completely edited at the Q/R site. We have confirmed that postmortem delay hardly influenced the editing efficiency at the GluR2 Q/R site (Kawahara et al., 2003b), hence the significant difference in the postmortem delay between the SBMA patients in this study and ALS patients in the previous report (Kawahara et al., 2004) would not have affected these results. We examined the motor neurons in the spinal cord segment corresponding to the hindlimb of mutated human SOD1 transgenic rats after their hindlimbs had become weak, indicating that the motor neurons examined were already pathologically affected. Likewise, we found that only a small number of motor neurons remained in the spinal cord of SBMA patients. Thus our results indicate that GluR2 RNA editing was complete in the dying motor neurons in both the mutated human SOD1 transgenic rats and SBMA patients, implying that the neuronal death mechanism is not due to the underediting of GluR2 mRNA seen in sporadic ALS. Since the pathogenic mechanism underlying ALS1 is considered to be the same as in mutant human SOD1 transgenic animals, motor neurons in affected ALS1 patients would be expected to have only edited GluR2 mRNA. Indeed, an association study of the SOD1 gene in a considerable number of patients with sporadic ALS reported no significant association with mutations of the SOD1 gene (Jackson et al., 1997). Due to the lack of appropriate animal model for sporadic ALS, mutant human SOD1 transgenic animals have been used as a model for ALS in general, particularly in studies searching for therapeutically effective drugs. However, it should be kept in mind that mutated human SOD1 transgenic animals are merely a suggestive model for sporadic ALS and a gain of toxic function in mutated SOD1 kills motor neurons via mechanisms other than the demise of RNA editing. There are likely multiple different death pathways in motor neurons, and motor neurons in sporadic ALS, ALS1 and SBMA die by different death cascades.

Acknowledgements

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Progression and prognosis in pure autonomic failure (PAF): comparison with multiple system atrophy

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PAPER

Progression and prognosis in pure autonomic failure (PAF): comparison with multiple system atrophy

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Objective: To clarify the progression of autonomic symptoms and functional deterioration in pure autonomic failure (PAF), particularly in comparison with multiple system atrophy (MSA).

Methods: The investigation involved eight patients with PAF (M/F=7/1; mean age at onset, 57 years) and 22 with probable MSA matched for age at onset (M/F=14/8; onset 56 years). Subjects were followed up for neurological symptoms, activities of daily living, and autonomic function for more than seven years. Autonomic functional tests were carried out.

Results: In PAF, fainting or sudomotor dysfunction occurred first, followed by constipation and syncope. Urinary dysfunction developed late, and respiratory dysfunction was not evident. This clinical course contrasted sharply with that in MSA, where early urinary dysfunction usually proceeded to sudomotor dysfunction or orthostatic hypotension ($p=0.004$), followed by respiratory dysfunction ($p=0.0004$). Results of pharmacological tests also distinguished PAF from MSA. Progression and prognosis in patients with PAF did not worsen, unlike the steady progressive autonomic dysfunction in MSA (p, 0.0001, p, 0.0001, $p=0.0009$, and $p=0.003$, for progression to modified Rankin scale grade III, IV, V, and death, respectively).

Conclusions: The time course and pattern of progression of autonomic failure differed significantly between PAF and MSA. Patients with PAF had slower functional deterioration and a better prognosis.

Pure autonomic failure (PAF) is a sporadic idiopathic neurodegenerative disorder characterised by gradually progressive severe autonomic disturbances without other neurological features. In the past, PAF was defined as severe orthostatic hypotension without other neurological deficits, and was referred to as idiopathic orthostatic hypotension. However, this has proved to be a heterogeneous condition, including diseases such as PAF, acute autonomic neuropathy, the early stages of Shy–Drager syndrome, and Parkinson's disease with autonomic failure.[1–6]

Bannister et al[7] classified primary autonomic failure into three categories: Parkinson's disease with autonomic failure, multiple system atrophy (MSA), and pure autonomic failure. In 1996, a consensus statement was established concerning PAF,[8] but it has remained uncertain whether the autonomic failure of PAF can readily be distinguished from those of MSA and Parkinson's disease with autonomic failure. In addition, although the clinical course of both MSA and Parkinson's disease with autonomic failure has been described to some extent, details of the natural history of PAF have not been fully assessed because of its rarity and very slow progression.[9–11] Previous reports have noted longer survival in patients with PAF than in those with MSA.[3 12–15] Orthostatic hypotension and anhidrosis/hypohidrosis are the main clinical symptoms in PAF, but their severity, prognosis, and progression have been only incompletely assessed. To clarify the clinical features, particularly the natural course of PAF, we observed eight patients who fulfilled the PAF consensus statement and maintained a follow up for at least five years. We show that their features are distinct from those of another form of primary autonomic failure, MSA.

METHODS

Patients

We examined eight patients with PAF (seven men, one woman; mean (SD) age at onset, 57 (14) years; mean age at first evaluation, 68 (12) years; mean duration from onset to most recent evaluation, 19 (10) years) who were referred to the Nagoya University Hospital or its affiliated hospitals in Aichi prefecture between 1988 and 1997. We evaluated these patients clinically from onset for between seven and 32 years. We reviewed the clinical records preceding our own follow up period, and also obtained information by interviewing the patients and family members.

According to the consensus statement,[8] PAF is characterised by orthostatic hypotension, various other autonomic signs without more widespread neurological involvement, and a low resting supine plasma noradrenaline concentration. The statement acknowledged that some patients would later prove to have other disorders such as MSA,[8] but did not state how long a period of follow up was required to confirm a diagnosis of PAF. Early MSA with predominant autonomic failure is particularly difficult to distinguish from PAF. We estimated that most MSA patients can be diagnosed by follow up for five years or more after onset,[8 16] and we therefore serially examined putative PAF patients for more than five years from onset to exclude those with MSA. We also excluded patients with acute autonomic neuropathy, Parkinson's disease with autonomic failure, and other diseases presenting with autonomic signs by neurological examination, imaging (magnetic resonance imaging and positron emission tomography), and neurophysiological tests.

We also investigated 22 probable MSA patients[17] matched according to age at onset (14 men, eight women; mean age at onset, 56 (8) years; mean age at first autonomic test, 61 (7) years; mean interval from onset, 8 (3) years) who had detailed clinical information particularly concerning autonomic features, and follow up intervals from over five years to 16 years after onset. All patients with MSA presented with autonomic failure as an initial symptom or with predominant autonomic failure at their first clinical visit, and fulfilled the criteria for a probable MSA diagnosis.[17]

Abbreviations: AVP, arginine-vasopressin; HUT, head up tilt test; MSA, multiple system atrophy; PAF, pure autonomic failure

Table 1 **Clinical profiles of eight patients with pure autonomic failure at their first visit**

Variable	Patient 1	2	3	4	5	6	7	8
Sex	M	M	M	M	M	M	M	F
Onset age (y)	35	68	72	78	50	52	51	50
Time until first evaluation (y)	17	1	10	5	27	7	5	13
Duration of observation (y)	32	7	12	12	32	14	15	29
Hypohidrosis	+	+	+	+	+	+	+	+
Faintness	+	+	+	+	+	+	+	+
Syncope	−	−	−	−	+	+	+	+
Constipation	+	−	−	−	+	−	+	−
Difficulty in urination	−	−	+	−	+	−	−	−
Incontinence/ urinary urgency	−	−	−	−	−	+	+	+
Respiratory disturbance	−	−	−	−	−	−	−	−
Plasma noradrenaline (pg/ ml) *	30	43	25	83	50	34	14	10
Orthostatic hypotension	+	+	+	+	+	+	+	+
Denervation supersensitivity	+	+	+	+	+	+	+	+
Modified Rankin scale	0	0	0	0	0	0	0	0

*Normal range 150 to 450 pg/ ml.
F, female; M, male; y, years.

Procedures

We evaluated all eight patients with PAF and 22 with MSA with a passive multistage head up tilt test (HUT) and a noradrenaline infusion test. The HUT was performed as follows. Blood pressure and heart rate were measured continuously by tonometry (SA-250; Colin, Komaki, Japan). After blood pressure stabilised at the supine stage, changes in blood pressure and heart rate were recorded continuously through 20°, 40°, and 60° head up tilting for five minutes each. Orthostatic hypotension was defined as a fall in systolic blood pressure of more than 30 mm Hg during the 60° head up tilt.[18]

Blood samples were collected at the rested supine stage and after 60° head up tilting from all patients for evaluation of plasma noradrenaline and arginine-vasopressin (AVP). Differences in AVP between after 60° head up tilting and the supine position were calculated as DAVP. Additionally, a noradrenaline infusion test was carried out as follows. A very low (0.3 ng/min) or a low (3 ng/min) concentration of noradrenaline was infused intravenously while blood pressure was monitored for changes. If diastolic or systolic blood pressure rose by more than 10 mm Hg or 25 mm Hg, respectively, the patient was considered to have denervation supersensitivity involving the sympathetic nervous system.[19] Four patients were re-evaluated two, five, six, and 11 years later, respectively. We also carried out ^{123}I-metaiodebenzylguanidine (MIBG) scintigraphy and evaluated the heart/ mediastinum (H/M) ratio from delayed images, as previously described.[20–22]

We followed up all eight patients and noted the time points when new autonomic symptoms appeared, including hypohidrosis, faintness and syncope, constipation, urinary dysfunction, impotence, and respiratory distress, and considered such clinical features in sequence to assess the natural clinical course. We evaluated hypohidrosis in terms of inspection of the skin and recording of patient symptoms. Dry skin or reduced perspiration was noted on some parts of the body, with compensatory hyperhidrosis elsewhere. Patients often noted their reduced perspiration in summer and felt severe fatigue, which sometimes limited their capacity for outdoor work. Faintness was defined as a floating sensation while in the upright position without loss of consciousness, or as symptomatic orthostatic hypotension during the head up tilt test. Syncope was defined as a blackout or loss of consciousness, including severe blurred vision. Constipation was defined by the passage of stools at intervals of three days or more, or complaints of straining. Urinary dysfunction was defined as urination twice at night or more than five times in the daytime, urinary urgency, incontinence, or difficulty in urination. Impotence was defined as difficulty in achieving normal sexual function. Respiratory disturbances were defined either as the presence of sleep apnoea, including heavy snoring, or as difficulty in respiration. Onset of an autonomic symptom was defined as the time when the patient first noted the symptom.

Statistics

The Mann–Whitney U test for non-parametric statistics was used as appropriate. Kaplan–Meier analyses were employed to estimate the natural course of autonomic features and disease progression, assessed by the modified Rankin scale in both PAF and MSA patients. Log-rank test statistics were used to determine whether the Kaplan–Meier curves differed between PAF and MSA. Calculations were done using the statistical software package Stat View (Abacus Concepts, Berkeley, California, USA). Statistical significance was defined as a probability (p) value of < 0.05.

RESULTS

Clinical profiles of PAF on the first visit to the hospitals

Clinical profiles of the eight patients with PAF at their first examination at our hospital are presented in table 1. They had many complaints suggesting autonomic disturbances, but the specific features varied. The earliest age at onset was 35 years, and the latest was 78 years. The interval from onset to presentation at our hospital varied from one to 27 years. Each patient showed various autonomic disturbances at that time, but faintness and hypohidrosis had been experienced by all patients. Other autonomic symptoms were as follows: urinary dysfunction in five, syncope in four, constipation in three, and impotence in two. All patients had very low plasma noradrenaline concentrations, orthostatic hypotension, and denervation supersensitivity according to the noradrenaline infusion test.

Clinical manifestations of MSA

The initial symptoms in all 22 patients with MSA were those of autonomic failure. Median time from onset to the presence of concomitant autonomic and motor manifestations (evolution from onset to probable MSA) was 2.0 years (range 1 to 10). At the first clinical visit, seven of the 22 patients presented with severe autonomic failure but failed to fulfil consensus diagnostic criteria of MSA.

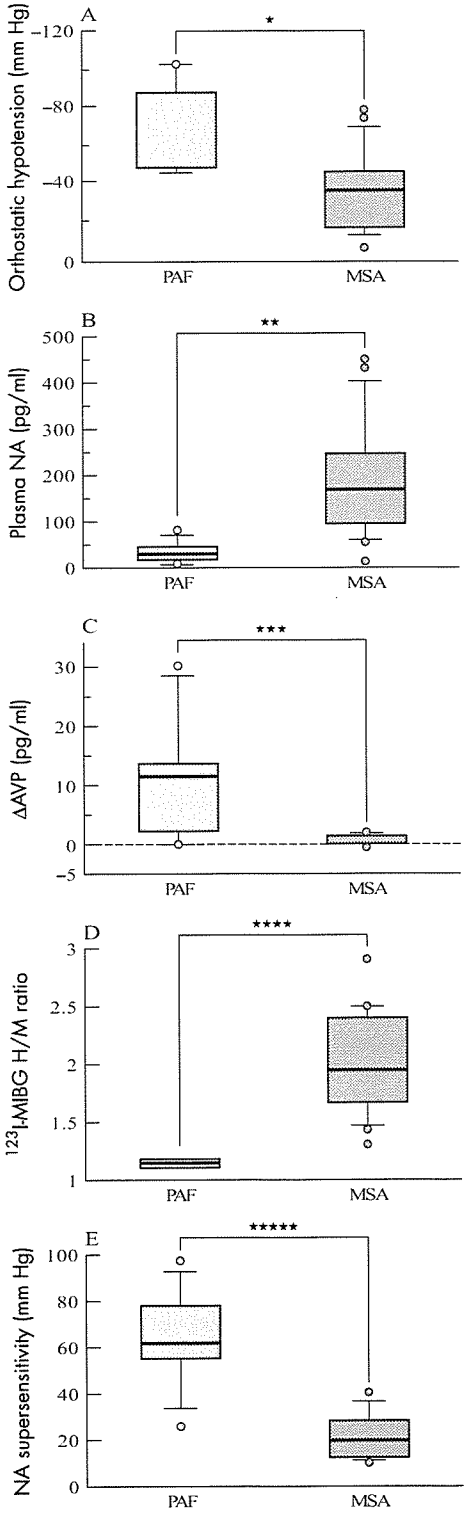

Figure 1 Box and whisker plot of the autonomic nervous testing comparing pure autonomic failure (PAF) with multiple system atrophy (MSA). (A) Systolic blood pressure fall during orthostatic hypotension. (B) Plasma noradrenaline (NA) concentration. (C) Differences in arginine-vasopressin (AVP) concentration between 60° head up tilt and supine posture calculated as DAVP. (D) Heart/ mediastinum (H/ M) ratio from ^{123}I-metaiodobenzylguanidine (MIBG) delayed imaging. (E) Systolic blood pressure increase during noradrenaline infusion test. *p = 0.004, **p = 0.0003, ***p = 0.003, ****p = 0.002, *****p = 0.0004, Mann–Whitney U test.

Autonomic nervous system testing in PAF and MSA

We found significant differences between PAF and MSA patients with respect to the following:

- Orthostatic hypotension evaluated by the head up tilt test (mean (SD): PAF, 68.9 (22.5) mm Hg; MSA, 36.3 (20.4) mm Hg; p = 0.004 (fig 1A);
- Noradrenaline concentration: PAF, 36.1 (23.2) pg/ml; MSA, 189.9 (121.9) pg/ml; p = 0.0003 (fig 1B);
- DAVP: PAF, (10.7) pg/ml; MSA, 0.34 (0.62) pg/ml; p = 0.003 (fig 1C);
- H/M ratio: PAF, 1.15 (0.05); MSA, 2.04 (0.44); p = 0.002 (fig 1D);
- Noradrenaline infusion test: PAF, 70.1 (23.2) mm Hg; MSA, 23.7 (11.0) mm Hg; p = 0.0004 (fig 1E).

Clinical course of autonomic failure

Kaplan–Meier curves depicting the natural clinical course of PAF and MSA are shown in fig 2. Hypohidrosis, faintness and syncope, constipation, urinary dysfunction, and respiratory disturbance were assessed sequentially.

Hypohidrosis

Six patients noted hypohidrosis or anhidrosis as an initial symptom, and seven became aware of hypohidrosis within five years of onset. Hypohidrosis was one of the earliest and most important symptoms of patients with PAF. In contrast, patients with MSA noted hypohidrosis at a significantly later stage of disease (p = 0.027).

Faintness and syncope

These symptoms represented orthostatic hypotension. Usually faintness preceded syncope. Faintness was often noted as an initial autonomic symptom in PAF. Four of eight patients first noted hypohidrosis in the same year as they first experienced faintness. In our series, five patients complained of faintness as an initial symptom, and seven noted faintness within five years of onset. Syncope appeared at (mean (SD)) 6 (7) years after the onset of faintness, and half the patients had experienced syncope within five years. However, two patients first noted syncope more than 19 years after experiencing faintness. In patients with MSA, faintness was observed later in the course of illness, with risk of progression to syncope differing significantly between the two groups (p = 0.002).

Constipation

Constipation was among the early symptoms of PAF. In our series, three patients noted constipation as an initial symptom, and five noted constipation within five years of onset; all patients complained of constipation within 13 years. Constipation was the second earliest symptom in our PAF patients, while patients with MSA also complained of constipation at a relatively early stage of disease. No significant differences were seen between the two groups in time from onset of first symptom to development of constipation (p = 0.46).

Urinary dysfunction

In the early stages few PAF patients noted urinary dysfunction, while at a later stage most patients had this complaint. In our series, urinary dysfunction appeared at (mean (SD)) 9 (9) years after the onset of hypohidrosis, faintness, and constipation. Only three patients noted urinary urgency, urinary frequency, or incontinence in the first five years. Among types of urinary dysfunction, difficulty in urination was rare in PAF patients. We evaluated the results of urodynamic studies in five of the eight PAF patients, at four,

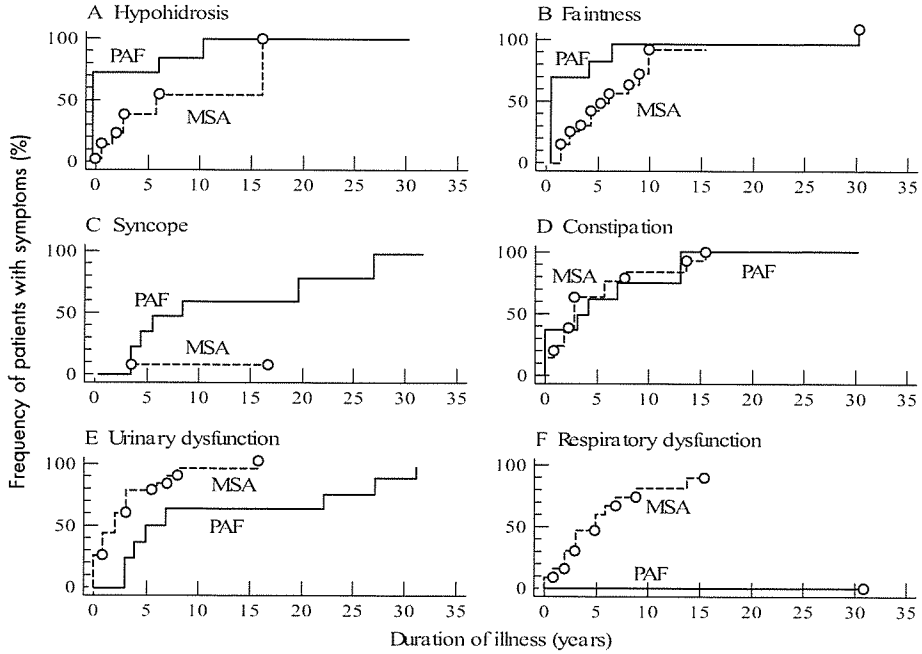

Figure 2 Progression of autonomic symptoms including hypohidrosis (A), faintness (B), syncope (C), constipation (D), urinary dysfunction (E), and respiratory disturbance (F) in patients with pure autonomic failure (PAF) and multiple system atrophy (MSA). Hypohidrosis was an earlier symptom in PAF than in MSA (panel A, $p = 0.027$). Faintness and syncope were earlier symptoms in PAF than in MSA (panel B $p = 0.04$; panel C, $p = 0.002$). Development of constipation was similar between the two diseases (panel D). Urinary dysfunction was a later symptom in PAF than in MSA (panel E, $p = 0.004$). Respiratory disturbance did not occur in our PAF patients, but MSA patients had these problems at an early stage (panel F, $p = 0.0004$).

six, 10, 13, and 17 years after the onset of PAF, respectively. Two of the five patients were essentially asymptomatic and had normal study results. Three patients were symptomatic, one of whom had an overactive bladder and the other an underactive bladder; the third had normal results. In our series, all eight patients had urinary dysfunction by 30 years after onset. Thus urinary dysfunction typically emerged in late stage PAF. In contrast, MSA patients developed urinary dysfunction at a very early stage of their disease ($p = 0.004$), often as an initial autonomic symptom in about a quarter of the patients. Within five years, more than 75% of MSA patients had urinary dysfunction, especially difficulty in urination. Thus urinary symptoms occurred early and were particularly prominent in MSA.

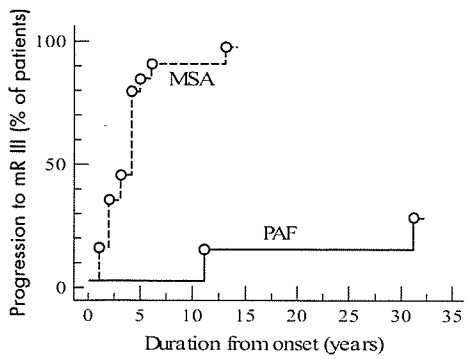

Figure 3 Differences in time remaining independent in activities of daily living (ADL) assessed by the modified Rankin scale between patients with pure autonomic failure (PAF) and multiple system atrophy (MSA). Round symbols represent censored data. Significant differences were seen between PAF and MSA for three ADL milestones and for survival, by Kaplan–Meier analysis and log-rank tests. mR III, modified Rankin scale, grade III (moderate impairment requiring minimal support such as a cane, stair rails, and so on): difference between PAF and MSA significant at p, 0.0001.

Respiratory disturbances

Respiratory disturbances such as sleep apnoea were uncommon in patients with PAF. Indeed, in our series, no patient had respiratory difficulties in 30 years of follow up. In contrast, respiratory disturbance was one of the most important features in patients with MSA ($p = 0.0004$). About half the MSA patients had this complaint within five years, and subsequently the prevalence of respiratory disturbances increased. More than 80% of the MSA patients had respiratory disturbances by 10 years.

Progression of orthostatic hypotension and noradrenaline supersensitivity

Orthostatic hypotension

Orthostatic hypotension (fig 1A) was a major clinical feature in PAF, being marked even in the early stages of the disease. Blood pressure fall varied from 34 to 108 mm Hg at presentation to our hospital, and the extent of orthostatic hypotension progressed markedly in most patients over the next two to 11 years. In seven patients blood pressure fell by more than 50 mm Hg, and most patients experienced syncope.

Noradrenaline supersensitivity

The noradrenaline infusion test estimates denervation supersensitivity at peripheral noradrenaline receptors, suggesting disease involvement of the peripheral sympathetic nervous system. At an early stage, PAF patients all showed excessive rises in blood pressure of 30 mm Hg or more with infusion of a low concentration of noradrenaline (3 or 0.3 ng/min), indicating the presence of denervation supersensitivity (fig 1E). After two to 11 years, however, the extent of blood pressure rise in response to noradrenaline infusion was smaller than at an early stage, suggesting emergence of some compensatory mechanism or secondarily induced insensitivity of noradrenaline receptors.

Activities of daily living and prognosis

PAF patients did not show diminishing capacity for activities of daily living (ADL) up to a late stage (fig 3). In our series three patients died, but they maintained nearly normal ADL throughout their lives. One patient who died at 90 years, 12 years after disease onset, could walk alone without assistive devices until he was 89 years old (modified Rankin scale, 0 to1); rapid deterioration in the last year of life resulted from a subdural haematoma. Another patient who died at 82 years, 32 years after onset, could perform all his daily activities unassisted until he was 81. He was essentially bedridden for the last year of life because of myelodysplastic syndrome. The third patient, who died aged 84 years 12 years after disease onset, remaining healthy and active (modified Rankin scale 0 to 1) until he died suddenly of a severe stroke.

Although both MSA patients and PAF patients have severe autonomic disturbances, functional and survival prognoses[16] were significantly worse in MSA than in PAF. In our series, median time from onset to modified Rankin scale grade III in MSA was four years (p, 0.0001 v PAF); grade IV, seven years ($p = 0.0009$); grade V, nine years (p, 0.0001); and death, 11 years ($p = 0.003$). In contrast to MSA, PAF carried a relatively good prognosis for function and survival.

DISCUSSION

PAF is a chronic progressive neurodegenerative disease characterised by severe autonomic failure without other neurological deficits. Uniquely, PAF patients can maintain a long healthy life, in contrast to patients with other types of primary autonomic failure. Pathological reports of PAF have described Lewy bodies in the intermediolateral grey columns of the thoracolumbar spinal cord, suggesting that PAF is a form of Lewy body disease.[25–30]

Our study is the first assessment of long term progression of autonomic symptoms and ADL status in PAF, particularly in comparison with MSA. Although a consensus has been reached over the diagnostic criteria for PAF,[8] long term follow up observation of the clinical features is important to identify the differences between PAF and autonomic failure in other neurodegenerative diseases, particularly MSA and Parkinson's disease with autonomic failure.[7 8 12] We investigated clinical features of eight patients with PAF over follow up periods ranging from seven to 32 years.

It is generally accepted that patients with PAF have autonomic failure resulting in peripheral but not central involvement. The results of supine noradrenaline levels, DAVP, ^{123}I-MIBG, and the noradrenaline infusion test clearly confirm this. In contrast, patients with MSA have patterns suggesting a predominantly central involvement, although some patients with probable MSA also have low noradrenaline concentrations, increased DAVP, a reduced H/M ratio, and raised blood pressure during the noradrenaline infusion test. These neuropharmacological tests would be useful for differentiating PAF from MSA early in the course of the illness. Further studies are needed to clarify their sensitivity, specificity, and positive predictive value.

In our study, orthostatic hypotension and related faintness and syncope were the most important clinical features of PAF, and developed at a very early stage. Furthermore, orthostatic hypotension worsened gradually as the disease progressed in spite of medical treatment for hypotension. In contrast, MSA patients were less likely to have syncope than PAF patients. Progression of MSA is relatively rapid,[16] so MSA patients are often wheelchair bound or nearly bedridden before showing severe hypotension with syncope.[16] About half the patients with MSA noted faintness by four years after onset, at a time when most of them were wheelchair bound and spent a considerable amount of their waking time lying down. This may limit the exposure of MSA patients to syncope.

Another important autonomic abnormality observed in PAF was sudomotor impairment. Hypohidrosis or anhidrosis was a major complaint in patients with PAF. Emergence of orthostatic hypotension, sometimes with loss of consciousness, and sudomotor dysfunction at a very early stage were striking characteristic features in PAF, in contrast to MSA where these symptoms were absent in the early phase of the disease.

A striking clinical characteristic of PAF was the absence of respiratory dysfunction such as sleep apnoea until a very late phase of disease. This feature again contrasted with MSA, where respiratory dysfunction was a major problem, threatening life in the later phase of disease.

Constipation and urinary dysfunction are among the characteristic symptoms of primary autonomic failure syndrome including PAF, MSA, and Parkinson's disease with autonomic failure.[30 31] Urinary problems have been documented in the past to some extent,[9 11 12] representing a characteristic feature of PAF, especially in the late phase. Sakakibara *et al*[32] reported that all six of their patients with PAF who complained of urinary disturbances showed abnormalities on urodynamic studies. In our series, five of eight patients underwent urodynamic evaluation, and two with urinary symptoms showed a hyperactive or underactive bladder. However, the severity of the urodynamic abnormalities and associated symptoms was mild, in agreement with the previous report.[32] In contrast, patients with MSA have severe urinary dysfunction, especially difficulty in urination[33] and nocturnal urinary frequency, with residual urine, detrusor hyperreflexia, low compliance, and detrusor sphincter dyssynergy on urodynamic studies. Intermittent self catheterisation is often required even early in the course of the illness.

On the basis of these observations, we can assume that orthostatic hypotension and sudomotor dysfunction precede urinary dysfunction and particularly respiratory dysfunction in the development of autonomic disturbances in PAF, while in MSA urinary dysfunction precedes orthostatic hypotension and sudomotor dysfunction, and respiratory dysfunction is a serious problem even at an early stage. Modes of progression of autonomic symptoms seem to be an important way of distinguishing between PAF and MSA.

The evolution of the change in blood pressure during the noradrenaline infusion test in PAF is difficult to explain. While the clinical features became worse over the course of several years in PAF patients, in contrast the degree of blood pressure elevation during the test became smaller with time. The same method was used for the test on each occasion, and no previous reports provide an explanation for this phenomenon. Age related changes such as atherosclerosis or changes in drug treatment might have contributed, but further study is necessary.

Patients with PAF had a better prognosis than those with MSA. Even the three patients with PAF who died during follow up lived independently until one or two years before they died all died of concurrent diseases. Various factors contributed to this advantage in ADL and long term prognosis. First, patients with PAF did not have severe urinary disturbances, which would lessen the risk of recurrent urinary infections, and they also did not have life threatening respiratory failure. Second, while management of orthostatic hypotension remains challenging late in the course of illness, administration of plasma volume expansion fluids, fludrocortisone, and sympathomimetic agents can be effective in ameliorating symptoms and preventing faintness and syncope with resulting head injuries or bone fractures which could compromise ADL and survival. Third, patients

with PAF in this study showed no motor or cognitive impairment. No parkinsonism or dementia, which would have affected daily activities or required additional treatment, was evident during the course of their illness. Further studies are needed to evaluate the significance of the pathological background for temporal features of autonomic, motor, and cognitive involvements.

The precise epidemiology of PAF has not been assessed, either in Japan or in Western countries. To our knowledge, relatively few cases of PAF have been studied or described, and necropsy reports are far less common than for MSA. In our Japanese series, more than 200 patients with MSA were referred to hospital during the course of the study, but only eight patients with PAF were diagnosed during the same period. Although physician referral patterns may have on influence, PAF appears to be uncommon in Japan compared with MSA. Further studies should be undertaken to clarify the incidence and prevalence of PAF worldwide.

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Gene Expression Profile of Spinal Motor Neurons in Sporadic Amyotrophic Lateral Sclerosis

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The causative pathomechanism of sporadic amyotrophic lateral sclerosis (ALS) is not clearly understood. Using microarray technology combined with laser-captured microdissection, gene expression profiles of degenerating spinal motor neurons isolated from autopsied patients with sporadic ALS were examined. Gene expression was quantitatively assessed by real-time reverse transcription polymerase chain reaction and in situ hybridization. Spinal motor neurons showed a distinct gene expression profile from the whole spinal ventral horn. Three percent of genes examined were downregulated, and 1% were upregulated in motor neurons. Downregulated genes included those associated with cytoskeleton/axonal transport, transcription, and cell surface antigens/receptors, such as dynactin, microtubule-associated proteins, and early growth response 3 (EGR3). In contrast, cell death–associated genes were mostly upregulated. Promoters for cell death pathway, death receptor 5, cyclins A1 and C, and caspases-1, -3, and -9, were upregulated, whereas cell death inhibitors, acetyl-CoA transporter, and NF-κB were also upregulated. Moreover, neuroprotective neurotrophic factors such as ciliary neurotrophic factor (CNTF), Hepatocyte growth factor (HGF), and glial cell line–derived neurotrophic factor were upregulated. Inflammation-related genes, such as those belonging to the cytokine family, were not, however, significantly upregulated in either motor neurons or ventral horns. The motor neuron–specific gene expression profile in sporadic ALS can provide direct information on the genes leading to neurodegeneration and neuronal death and are helpful for developing new therapeutic strategies.

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Amyotrophic lateral sclerosis (ALS) is a devastating neurodegenerative disease characterized by loss of motor neurons in the spinal cord, brainstem, and motor cortex.[1] Initial symptoms include weakness of the limbs, abnormalities of speech, and difficulties in swallowing. The weakness ultimately progresses to complete paralysis, and half of the patients die within 3 years after the onset of symptoms, mostly because of respiratory failure. Approximately 10% of all ALS patients show familial traits, and 20 to 30% of familial ALS patients are associated with a mutation in the copper/zinc superoxide dismutase 1 gene (SOD1). However, more than 90% of ALS patients are sporadic, not showing any familial trait. The presence of Bunina bodies in the remaining spinal motor neurons is a hallmark of sporadic ALS cases.[2,3] So far, several hypotheses about the pathogenesis of sporadic ALS have been proposed based on extensive research on sporadic ALS: oxidative stress, glutamate excitotoxicity, impaired axonal transport, mitochondrial dysfunction, neurotrophic deprivation, proteasomal dysfunction, neuroinflammation, autoimmunity, viral infection, and others.[4–11] Nevertheless, the actual pathogenic mechanism of the selective motor neuron degeneration and ultimate cell death in sporadic ALS remains unknown. There have been extensive studies using animal models and culture systems for familial ALS, especially with SOD1 mutations, but no similar approach is available for studying sporadic ALS.

Recently advances in DNA microarray technology make it possible to analyze global gene expression profiles of thousands of genes in normal as well as pathological tissues. Global gene expression studies using DNA microarray technology have generated valuable

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information about cell behavior in tissues consisting of homogeneous cell types, cultured cells, and cancer tissues of monoclonal origin.[12,13] In the case of neuronal tissues, particularly those of patients with neurological diseases, however, the complexity of tissues containing multiple lineages of cells, such as neurons, glial cells, and vascular tissues, places limitations on the use of DNA microarray technology. In the lesions of ALS spinal cords, there are reduced numbers of motor neurons with glial cell proliferation, making it difficult to examine motor neuron–specific gene expression.

Laser-captured microdissection (LCM) has been reported to make it possible to isolate single individual neurons from neural tissues with well-preserved mRNA quality.[14,15] In addition, RNA amplification techniques preserving the relative amounts of individual mRNAs have been developed recently.[16,17] LCM and RNA amplification combined with DNA microarray analyses have been reported to enable studies of cell type–specific gene expression profiles in tissues with multiple cell lineages.[16,18] Such integrated analysis system provide an effective tool for investigating the cellular events affecting cell type–specific gene expression profiles in neurodegenerative diseases such as ALS. Indeed, we and other groups demonstrated that these integrated systems could be applied successfully to describe cell-specific gene expression profiles in neuronal tissues.[15,18]

In this study, we applied integrated LCM, RNA amplification, and DNA microarray analysis to clarify alterations of motor neuron–specific gene expression in sporadic ALS cases and successfully obtained expression gene database in situ from degenerating motor neurons in sporadic ALS spinal cord.

Patients and Methods

Tissues from Amyotrophic Lateral Sclerosis and Control Patients

Fresh specimens of lumbar spinal cord (L4 to L5 segment) from 14 sporadic ALS patients (nine men, five women) and 13 neurologically normal patients (nine men, four women) were obtained at autopsy (Table 1). Diagnosis of ALS was

Table 1. Details of Patients Examined in This Study

Patients	Sex	Age (yr)	Duration of Illness (yr)	Postmortem Delay (hr)	Diseases	Spinal Cord Neuropathology Motor Neuron Loss/Gliosis
ALS1	M	72	3.7	6	ALS (B, UL, LL)	Moderate/mild
ALS2	M	71	2.3	5	ALS (LL, UL)	Moderate/mild
ALS3	M	58	1.8	13	ALS (UL, LL, B)	Severe/severe
ALS4	M	43	2.6	5	ALS (LL, B)	Moderate/mild
ALS5	M	53	2.8	11	ALS (B, UL, LL)	Moderate/severe
ALS6	F	79	4.0	4	ALS (UL, LL, B)	Severe/severe
ALS7	F	59	2.5	3	ALS (UL)	Mild/mild
ALS8	F	67	2.0	7	ALS (UL, B)	Severe/mild
ALS9	M	74	4.3	10	ALS (LL, B)	Severe/mild
ALS10	F	47	1.8	4	ALS (B, UL, LL)	Mild/mild
ALS11	M	74	4.5	12	ALS (UL, LL)	Moderate/mild
ALS12	M	57	3.5	5	ALS (LL, UL)	Severe/mild
ALS13	F	53	3.0	8	ALS (B, UL, LL)	Severe/severe
ALS14	M	63	2.2	5	ALS (UL, B)	Mild/mild
Control1	M	57	—	7	Pneumonia	No
Control2	M	78	—	10	Cerebral infarction	No
Control3	M	72	—	9	Lung cancer	No
Control4	F	52	—	7	Pneumonia	No
Control5	F	65	—	12	Pneumonia	No
Control6	M	75	—	10	Heart failure	No
Control7	M	42	—	5	Heart failure	No
Control8	F	76	—	5	Pancreas cancer	No
Control9	F	84	—	6	Myocardial infarction	No
Control10	M	48	—	13	Heart failure	No
Control11	M	77	—	11	Heart failure	No
Control12	M	66	—	11	Cerebral infarction	No
Control13	M	75	—	4	Pneumonia	No

The age, duration of illness, and postmortem delay are indicated for the ALS and control cases. Predominant clinical features of ALS are shown: UL = upper limbs; LL = lower limbs; B = bulbar. Neuropathological involvement of spinal cords was graded as previously. Ten ALS samples were used for microarray analysis: five of them (1, 7, 10, 11, and 14) were analyzed using 4.8K array for spinal motor neurons; five (2, 4, 5, 8, and 12) using 1.0K for spinal motor neurons; five (1, 3, 10, 13, and 14) using 4.8K for spinal ventral horn gray matter; and five (1, 2, 4, 5, and 13) and five (1, 2, 7, 8, and 10) control samples using 4.8K and 1.0K. Thirteen ALS (1–13) and 11 (1–11) control samples were used for TaqMan reverse transcription polymerase chain reaction analysis. Five ALS (1, 10, 11, 13, and 14) and four control (1, 3, 5, and 12) samples were used for in situ hybridization and immunohistochemistry.
ALS = amyotrophic lateral sclerosis.

confirmed by El Escorial diagnostic criteria defined by the World Federation of Neurology and the histopathological findings, particularly the presence of the Bunina body.[2,3] All cases of ALS were sporadic and did not show any heredity. ALS patients with 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. Tissues were frozen immediately and stored at −80°C until use. The mean ages and standard deviations for ALS and control patients were 62.1 ± 11.0 and 66.7 ± 13.1 years, and the mean postmortem intervals and standard deviations were 7.0 ± 3.3 and 8.5 ± 3.0 hours, respectively. The differences between the means of either age or postmortem interval were not significant between the ALS and control groups. The cause of death in all ALS patients was respiratory failure, and the causes in the control patients were pneumonia, lung cancer, or acute heart failure (see Table 1). 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 and Holzer techniques, and histological assessment was performed. The degree of motor neuron loss and astrogliosis was ranked as mild, moderate or severe according to previously reported.[19,20]

Laser-Captured Microdissection of Spinal Motor Neurons

Sections (10μm) were cut with a standard cryostat, mounted on poly-L-lysin coated slides (Zeiss, Thornwood, NY), and stained with hematoxylin to identify the motor neurons located in the medial and lateral nuclei of the ventral horns of lumber spinal cords. After staining with hematoxylin, the sections were washed in RNase-free water and dried.[21,22] The PALM Robot-Microbeam system (P.A.L.M. Mikrolaser Technology AG, Bernried, Germany) was used for laser capture. The pulsed laser microbeam cut precisely around the targeted motor neurons in the spinal ventral horn (LCM; see Fig 1A–C). The identity of motor neurons was ascertained by reverse transcription polymerase chain reaction (RT-PCR) for choline acetyltransferase (ChAT) as described previously.[15] Each laser-isolated specimen subsequently was ejected from the glass slide with a single or several laser shots and collected directly into the cap of a PCR tube containing denaturing buffer by a process of laser pressure catapulting in the totally noncontact manner previously described.[23] The LCM-isolated cells (approximately 500 pooled cells) were dissolved in denaturing buffer (StrataPrep Total RNA Microprep Kit; Stratagene, San Diego, CA) and stored at −80°C until use.

RNA Extraction of Laser-Captured Microdissection Motor Neuron Samples and Spinal Ventral Horn Homogenates

LCM-isolated cells in denaturing buffer were thawed and centrifuged briefly before the RNA was extracted using a StrataPrep Total RNA Microprep Kit (Stratagene) according to the manufacturer's protocol. RNA was extracted as well from the total homogenates of ventral horn gray matter of spinal cords,[19] which was dissected under a dissecting microscope.

Reverse Transcription and T7 RNA Polymerase Amplification of RNA

Ten microliters of purified RNA obtained as described above was mixed with 1μl of 0.5μg/ml T7-oligodT primer (5′-TCTAGTCGACGGCCAGTGAATTGTAATACGACTCACTATAGGGCGT$_{21}$-3′) to initiate first-strand synthesis. The primer and RNA were incubated in 4μl of 5 × first-strand reaction buffer, 0.1M DTT (2μl), 10mM dNTPs (1μl), 1μl of RNasin, and 1μl of Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA) at 42°C for 1 hour, and then 30μl of 5 × second-strand synthesis buffer, 10mM dNTPs (3μl), 4μl of DNA polymerase, 1μl of Escherichia coli RNase H, and 1μl of E. coli DNA ligase and 91μl of RNase-free H_2O were added, and the mixture was incubated at 16°C for 2 hours and then at 16°C for 10 minutes after the addition of 2μl of T4 DNA polymerase. Next, an Ampliscribe T7 Transcription Kit (Epicentre Technologies, Madison, WI) was used for RNA amplification: 8μl double-stranded cDNA, 2μl of 10 × Ampliscribe T7 buffer, 1.5μl each of 100mM ATP, CTP, GTP, and UTP, 0.1 M DTT (2μl), and 2μl of T7 RNA polymerase were incubated at 42°C for 3 hours.

For second-round amplification, 10μl of amplified RNA (aRNA) from first-round amplification was mixed together with 1μl of 1mg/ml random hexamers (Invitrogen), and then first-stranded cDNA was synthesized, followed by second-stranded cDNA synthesis as described above. The double-stranded cDNA was subjected to second-round T7 in vitro transcription as above and then subsequent third-round aRNA amplification. After third-round amplification, aRNA was treated with DNase (Wako, Kanagawa, Japan) and cleaned up using an RNeasy Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol.

DNA Microarray Analysis

Fluorescent cDNA probes were synthesized from aRNA of laser-captured spinal motor neurons and RNA from ventral spinal tissue homogenates using an Atlas Glass Fluorescent Labeling Kit (Clontech, Palo Alto, CA) according to the manufacturer's protocol. Cy3-labeled cDNA probes were synthesized from ALS samples for spinal motor neurons and homogenates, and Cy5-labeled cDNA probes were synthesized from control samples. BD Atlas Glass Microarray Human 1.0 and 3.8 (Clontech) slides were hybridized with these fluorescent labeled probes overnight at 50°C and then washed four times and dried according to the manufacturer's protocol. Individual Cy3-labeled cDNA probes from ALS RNA samples of spinal motor neurons and homogenates for each patient were coupled with Cy5-labeled cDNA probes from control RNA samples of those tissues, which were prepared by mixing equal amounts of RNA samples amplified from the control patients. The microarrays were scanned in a laser scanner (GenePix 4000; Axon Instruments, Union City, CA), and the resulting signals were quantified and stored using GenePix Pro analysis software (Axon Instruments). The data for each expressed gene obtained from microarray analysis were expressed as the ratios of the values of individual ALS patients or the means of the values of ALS to the values of the control patients. The values of gene expression levels were means-calculated from motor neurons of 5 or 10 inde-

pendent individuals with ALS as well as from spinal ventral horns of 5 individuals with ALS.

Quantitative Real-Time Reverse Transcription Polymerase Chain Reaction

The probe and primers for the real-time PCR were designed using Primer3′ (S. Rozen and H. J. Skaletsky, available at http://www-genome.wi.mit.edu/genome_software/other/primer3.html). TaqMan PCR was conducted using an iCycler system (Bio-Rad Laboratories, Hercules, CA) with a QuantiTect Probe PCR Kit (Qiagen) and the cDNA according to the manufacturer's protocol. The reaction conditions were 95°C for 3 minutes and then 50 cycles of 15 seconds at 95°C followed by 60 seconds at 55°C. All experiments were performed in quadruplicate, and several negative controls were included. For an internal standard control, the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was simultaneously quantified. The primers and probe sequences for the examined genes (acetyl-CoA transporter: D88152; Bak: NM_001188; CRABP1: NM_004378; cyclin C: M74091; dynactin 1: NM_004082; EGR3: NM_004430; ephrin A1: M57730; GAPDH: NM_002046; KIAA0231: D86984; and TrkC: U05012) were described in the legends for Figure 3. The threshold cycles of each gene were determined as the number of PCR cycles at which the increase in reporter fluorescence reached 10 times above the baseline signal. The weight ratio of the target gene to GAPDH gives the standardized expression level.

In Situ Hybridization

Frozen sections (10μm thick) of the spinal cord were prepared and immediately fixed in 4% paraformaldehyde. Then, they were treated with 0.1% diethylpyrocarbonate (DEPC) 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, Genebank accession number, probe position (nucleotide number), and probe size were as follows: acetyl-CoA transporter, D88152, nucleotides 397-741, 345bp; Bak, NM_001188, nucleotides 792–2094, 345bp; CRABP1, NM_004378, nucleotides 210–545, 336bp; dynactin 1, NM_004082, nucleotides 2392–2774, 383bp; DR5, NM_004082, nucleotides 682–1070, 389bp; EGR3, NM_004430, nucleotides 1433–1794, 362bp; KIAA0231, D86984, nucleotides 698–1053, 356bp; TrkC, U05012, nucleotides 1412–1721, 310bp. After prehybridization, the sections were hybridized with each digoxigenin-labeled cRNA probe overnight at 45°C. The washed sections were incubated with alkaline phosphatase-conjugated anti–digoxigenin antibody (Roche Diagnostics). The signal was visualized with NBT/BCIP (Roche Diagnostics).

Immunohistochemistry

Frozen sections (10μm thick) of the spinal cord were prepared and immediately fixed in 4% paraformaldehyde. Then, they were blocked with 2% bovine serum albumin (Sigma) in Tris-buffered saline at room temperature for 20 minutes and incubated with anti–cyclin C (1:200 dilution; Santa Cruz Biotechnology, Santa Cruz, CA) antibody overnight at 4°C. Subsequent procedures were performed using ENVISION++KIT/HRP (diaminobenzidine tetrahydrochloride; DAKO, Carpinteria, CA) according to the manufacturer's protocol.

Statistical Analyses

To assess the correlation of intensity values for each labeling sample, we used scatterplots and measured linear relationships. The correlation coefficient, R^2, that was calculated indicates the variability of intensity values between Cy-5– and Cy-3–labeled samples. To perform cluster analyses of hierarchical clustering, self-organizing maps (SOM) and principal component analysis after logarithmic transformation, we used Acuity 3.0 software (Axon Instruments). The data measured by quantitative real-time RT-PCR analysis were analyzed by Student's t tests.

Results

T7 Amplification Preserves Gene Expression Profiles

Because the amounts of laser-microdissected samples were extremely low and did not contain enough mRNA for further analysis, RNA amplification was required. It was critical to achieve sufficient RNA amplification and yet maintain the expression profiles of mRNAs. We performed experiments to determine how the expression profiles of mRNAs were affected by the T7 amplification procedure. RNA samples were extracted from control spinal cords and a part of RNA samples was amplified using T7 amplification. One flourescently labeled probe was synthesized from an individually amplified RNA (aRNA) or nonamplified RNA (nRNA) and was hybridized to microarrays. Independent amplification of RNA yielded quite similar expression patterns. The correlation of signal intensities between independent amplifications for the third aRNA was $R^2 = 0.9157$, $p < 0.0001$, and on the other hand, the correlation of signal intensities in nRNA was $R^2 = 0.9157$, $p < 0.0001$ (Fig 1D, E). Previous reports using similar amplification procedures as ours also have confirmed the reproducibility of T7 amplification for the preservation of RNA expression profiles.[14,15,17] In this study, the third-round amplification was performed for the LCM-isolated motor neurons, but for the spinal ventral horn homogenates a single amplification produced enough RNA for further analysis, and similar expression patterns were found between the first and third amplifications (data not shown).

Gene Expression Database of Spinal Motor Neurons and Ventral Horn Homogenates of Amyotrophic Lateral Sclerosis

aRNA samples from the motor neurons and the ventral horn homogenates from the lumbar spinal cords were subjected to microarray analysis. The differences of the gene expression levels between ALS and control sam-

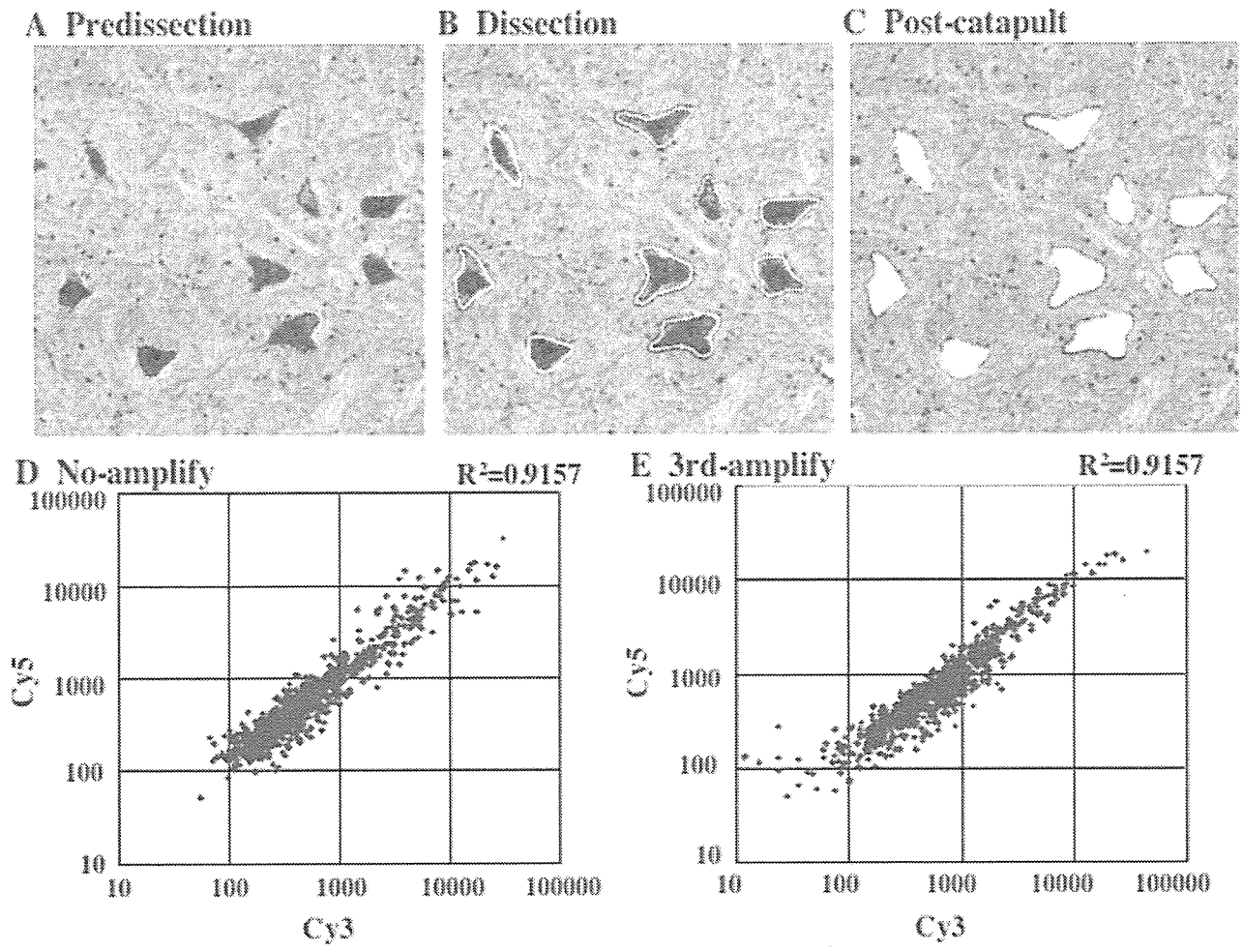

Fig 1. Verification of laser-captured microdissection (LCM) and RNA amplification. Microdissection of motor neurons in spinal ventral horn: sections were stained with hematoxylin (A); margins of motor neurons were dissected by the laser beam (B); and motor neurons were isolated from slides by laser pressure catapulting (C). Scatterplots of nonamplified and amplified RNAs correlations between independent amplifications of control spinal cord samples are shown using nonamplified (D) and third amplified RNAs (E). These RNAs were split into two samples for labeling of Cy5 and Cy3 and hybridized separately to two microarrays. The very high squared correlations reflect the high reproducibility of the hybridization results with the same values between nonamplified and third amplified RNAs.

ples were expressed as ratios of the values of ALS individuals compared with the mean values of the controls. One percent (52/4,845) of genes examined were significantly upregulated in spinal motor neurons of ALS patients and 3% (144/4,845) were downregulated, assuming that the changes of 3.0-fold increase and 0.3-fold decrease were significant, when the mean levels of gene expression were calculated. In contrast with motor neurons, the total spinal ventral horn homogenates demonstrated 0.7% (37/4,845) and 0.2% (8/4,845) significant upregulation and downregulation of gene expression, respectively.

The genes prominently altered in ALS are listed in Tables 2 to 5 for spinal motor neurons and spinal ventral horn homogenates, respectively. Several upregulated genes listed were overlapping between spinal motor neurons (see Table 2) and ventral horns (see Table 4), suggesting that motor neuron overexpression is reflected to some extent by gene expression in ventral horn homogenates. The other genes upregulated in motor neurons were not present in the list for spinal ventral horns, because these gene expression changes were diluted and masked by changes occurring in other cell types. Because the number of spinal motor neurons was decreased in ALS spinal cords, most genes that were listed as downregulated genes in motor neurons (see Table 3) were not found in spinal ventral horns (see Table 5) except for three genes (CRABP1, EGR3, and postmeiotic segregation increased 2-like 11). When we categorized these altered genes in ALS motor neurons into several functional groups, the genes related to cell receptors and intracellular signaling, transcription,

Table 2. Upregulated Genes in ALS Motor Neurons (Top 30)

GeneBank No.	Gene Name	Fold Change (ALS/control)
NM_003419	Zinc finger protein 10 (KOX 1)	8.86
U91618	Neurotensin/neuromedin N precursor	8.33*
NM_004651	Ubiquitin-specific protease 11	8.13
D86984	KIAA0231	7.31*
A26792	Ciliary neuronotrophic factor (CNTF)	6.76
M77830	Desmoplakin I & II (DSP; DPI & DPII)	6.10
NM_005622	SA (rat hypertension-associated) homolog	5.47
NM_004733	Acetyl-coenzyme A transporter	5.33
NM_000021	Presenilin 1 (Alzheimer disease 3)	4.96
K03020	Phenylalanine-4-hydroxylase (PAH)	4.95*
AF016268	Death receptor 5 (DR5); cytotoxic TRAIL receptor, TNFR10b	4.91*
M74091	G1/S-specific cyclin C (CCNC)	4.82*
NM_000275	Oculocutaneous albinism II	4.78
AF000936	SH3-binding protein 2	4.73*
NM_000384	Apolipoprotein B	4.70
M63099	Interleukin-1 receptor antagonist	4.66*
M57730	Ephrin-A1	4.57*
L19067	NF-κ B transcription factor p65 subunit	4.52*
U66838	Cyclin A1 (CCNA1)	4.51*
NM_005021	Ectonucleotide pyrophosphatase/phosphodiesterase 3	4.48
NM_001550	Interferon-related developmental regulator 1	4.45
L25851	Integrin alpha E precursor (ITGAE)	4.43*
X16416	C-abl1 protooncogene	4.41
U08015	Transcription factor NF-ATc	4.40*
U44378	Mothers against dpp homolog 4 (SMAD4)	4.35*
NM_005067	Seven in absentia (Drosophila) homolog 2	4.22
J04536	Leukosialin precursor; sialophorin	4.19*
X06745	DNA polymerase alpha catalytic subunit	4.15*
U09564	Serine kinase	4.09*
U37139	β 3-endonexin	4.06*

Gene expression levels are expressed as means of fold-change, which is calculated by dividing the signals of each ALS sample by those of control samples, in the 5 or 10 (denoted by asterisk) patients with ALS.
ALS = amyotrophic lateral sclerosis.

metabolism, and cytoskeletal architecture were downregulated. The functional categories of secreted and extracellular communication proteins and cell cycle regulators were characteristically upregulated. A complete list of the differentially expressed genes is available online at http://www.med.nagoya-u.ac.jp/neurology/index.html.

Differential Gene Expression Profiles between Spinal Motor Neurons and Ventral Horn Homogenates of Amyotrophic Lateral Sclerosis

To compare the expression profile of motor neurons with that of spinal ventral horn homogenates, we performed cluster analyses. Because the patterns of gene expression from microarray analysis are impossible to discern by eye, data analysis software (Acuity 3.0 software; Axon Instruments) was used based on the dimensionality of the data: hierarchical clustering for high dimensional gene space and principal component analysis and SOM for low one. Hierarchical clustering clearly discriminated the expression profile of isolated motor neurons from that of ventral horn homogenates, showing two grouped branches of the dendrogram with a correlation coefficient of 0.446 (Fig 2A). Moreover, a principal component analysis confirmed the distinction of gene expression profiles between spinal motor neurons and ventral horns (see Fig 2B). The gene expression profile of motor neurons was clustered into a single cluster by the two clustering algorithms, which was well separated from that of spinal ventral horn gray matter, suggesting a relatively uniform degenerating process in spinal motor neurons in ALS.

Motor Neuron–Specific Gene Expression Profiles Identified by the Self-Organizing Map Analysis

To further analyze the expression pattern specific to spinal motor neurons, a SOM was produced as a nonhierarchical clustering.[24,25] The examined genes were subdivided into 25 clustered categories, and the selected genes are shown in a certain group of the SOM (see Fig 2C, Table 6). The genes contained in the clusters reflect the expression pattern in spinal motor neurons as well as that in spinal ventral horns, and these selected genes are somehow different from those in Tables 2 to 5 because of the different bases of classification. Clustering of the SOM showed motor neu-

Table 3. Downregulated Genes in ALS Motor Neurons (Bottom 30)

GeneBank No.	Gene Name	Fold Change (ALS/control)
NM_004378	Cellular retinoic acid–binding protein 1 (CRABP1)	0.12
NM_004430	Early growth response 3 (EGR3)	0.14
NM_005558	Ladinin 1	0.15
NM_003603	Arg/Abl-interacting protein ArgBP2	0.15
NM_000117	Emerin (Emery-Dreifuss muscular dystrophy)	0.15
NM_004357	CD151 antigen	0.15
X06820	Ras homolog gene family member B (RHOB)	0.15*
NM_003834	Regulator of G-protein signalling 11	0.16
NM_002960	S100 calcium-binding protein A3	0.16
NM_006289	Talin	0.16
NM_000964	Retinoic acid receptor, α	0.17
NM_002391	Midkine	0.17
M96944	Paired box protein PAX-5	0.17*
M74178	Hepatocyte growth factor–like protein	0.17*
NM_003822	Nuclear receptor subfamily 5, group A, member 2	0.17
NM_001188	BCL2-antagonist/killer 1; Bak	0.18
NM_000733	CD3E antigen, epsilon polypeptide (TiT3 complex)	0.18
NM_000408	Glycerol-3-phosphate dehydrogenase 2 (mitochondrial)	0.18
NM_000156	Guanidinoacetate N-methyltransferase	0.18*
M11886	Major histocompatibility complex, class I, C	0.18*
NM_003865	Homeo box (expressed in ES cells) 1	0.18
M36340	ADP-ribosylation factor 1 (ARF1)	0.18*
NM_001725	Bactericidal/permeability-increasing protein	0.18
NM_005334	Host cell factor C1 (VP16-accessory protein)	0.19
NM_004192	Acetylserotonin O-methyltransferase–like	0.19
NM_002684	Postmeiotic segregation increased 2–like 11	0.19
M11233	cathepsin D precursor (CTSD)	0.19*
NM_002313	Actin binding LIM protein 1	0.19
NM_002196	Insulinoma-associated 1	0.19
NM_002277	Keratin, hair, acidic, 1	0.19

Gene expression levels are expressed as means of fold-change, which is calculated by dividing the signals of each ALS sample by those of control samples, in the 5 or 10 (denoted by asterisk) patients with ALS.
ALS = amyotrophic lateral sclerosis.

ron–specific upregulated and downregulated gene expression commonly observed in five patients.

Clusters 1 (SOM1) and 6 (SOM6) contains 110 and 169 genes, respectively, that generally are downregulated in spinal motor neurons in all five cases examined, and those are known to be involved in the functional category of cell surface antigens and cell receptors, transcription, and cytoskeleton, whereas clusters 24 (SOM24) and 25 (SOM25) have 191 and 93 genes, respectively, that are predominantly upregulated in spinal motor neurons in all cases and belong to the functional category of cell signaling with extracellular communication, and cell death–associated proteins. The pattern of subcellular localization of their gene products also confirms the characteristics of the functional categories of upregulated and downregulated genes, that is, that plasma membrane and cytoskeletal proteins are more downregulated, and extracellular secreted proteins are more upregulated, in ALS motor neurons. All the genes listed in Table 3 are included in SOM1 and SOM6, whereas SOM24 and SOM25 do not contain all of the genes listed in Table 2. The former group of genes, with downregulation in motor neurons, included BCL2-antagonist/killer 1 (Bak) and TrkC receptor. Regarding genes related to transcription, early growth response 3 (EGR3), cellular retinoic acid–binding protein 1 (CRABP1), retinoic acid receptors, and Musashi 1 were included in SOM1 and SOM6 as downregulated genes. The expression of dynactin and microtubule-associated proteins (MAPs), which belong to the functional category of cytoskeleton and axonal transport, was downregulated in ALS motor neurons. On the other hand, KIAA0231 and acetylcoenzyme A transporter were classified into the upregulated genes in motor neurons of ALS. Regarding genes related to cell death, the expression of cyclins A1 and C, death receptor 5 (DR5), and interleukin-1 receptor antagonist was upregulated together with that of NF-κB, tumor necrosis factor (TNF) receptor–associated factor 6 (TRAF6), and caspase-1, -3, and -9 in SOM24 and SOM25. For genes in the category of trophic factor cell signaling with extracellular communication, CNTF, HGF, and glial cell line–derived neurotrophic factor (GDNF) were upregulated in ALS motor neurons, whereas midkine was downregulated. The expression of vascular endothelial growth factor as

Table 4. Upregulated Genes in Spinal Ventral Horns of ALS (Top 30)

GeneBank	Gene Name	Fold Change (ALS/control)
NM_000508	Fibrinogen, A α polypeptide	8.23
D86984	KIAA0231	6.09
NM_001801	Cysteine dioxygenase, type I	5.81
X02544	α-1-Acid glycoprotein 1 precursor	5.59
NM_001973	ELK4, ETS-domain protein (SRF accessory protein 1)	5.12
NM_000021	Presenilin 1 (Alzheimer disease 3)	5.00
NM_002097	General transcription factor IIIA	4.96
U08015	Transcription factor NF-ATc	4.96
M57730	Ephrin-A1	4.88
U91618	Neurotensin/neuromedin N precursor	4.79
AF000936	SH3-binding protein 2	4.50
NM_002949	Mitochondrial ribosomal protein L12	4.11
NM_002386	Melanocortin 1 receptor	4.03
NM_001991	Enhancer of zeste (Drosophila) homolog 1	3.93
NM_000947	Primase, polypeptide 2A (58kDa)	3.92
NM_000239	Lysozyme (renal amyloidosis)	3.88
NM_001550	Interferon-related developmental regulator 1	3.67
NM_004602	Staufen (Drosophila, RNA-binding protein)	3.66
NM_000063	Complement component 2	3.58
NM_004651	Ubiquitin-specific protease 11	3.54
NM_000397	Cytochrome b-245, β polypeptide	3.51
NM_002056	Glutamine-fructose-6-phosphate transaminase 1	3.41
L25851	Integrin α E precursor (ITGAE)	3.36
NM_004616	Transmembrane 4 superfamily member 3	3.21
NM_003720	Down syndrome critical region gene 2	3.18
J04536	leukosialin precursor; sialophorin	3.15
X06745	DNA polymerase alpha catalytic subunit	3.15
K03020	Phenylalanine-4-hydroxylase (PAH)	3.14
NM_001329	C-terminal binding protein 2	3.14
NM_000276	Oculocerebrorenal syndrome of Lowe	3.13

Gene expression levels are expressed as means of fold-change, which is calculated by dividing the signals of each ALS sample by those of control samples, in the five patients with ALS.
ALS = amyotrophic lateral sclerosis.

well as NT-3 was unchanged. Furthermore, the genes whose expression was altered significantly in spinal ventral horn homogenates as shown in Tables 4 and 5 showed similar alterations to some extent in the remaining motor neurons. However, the upregulated genes, such as integrin αE and sialophorin for cell adhesion, which were demonstrated to be spinal ventral horn–derived (see Table 4) as well as spinal motor neuron–derived (Table 2) genes, were not sorted out into SOM24 and SOM25, indicating that their upregulation occurred predominantly in glial cells.

Data Confirmation with Quantitative Real-Time Reverse Transcription Polymerase Chain Reaction, In Situ Hybridization, and Immunohistochemistry

To assure the validity of the gene expression levels detected by microarray analysis, we performed quantitative real-time RT-PCR analysis on some genes of interest using a TaqMan PCR system. Because LCM-isolated motor neurons did not contain enough RNA to perform real-time RT-PCR analysis, only selected genes were assessed in motor neurons, and for other genes the spinal ventral horn homogenates were used as the template for quantitative RT-PCR. When the extent of increase or decrease of gene expression levels was expressed as the ratio of the genes of interest to GAPDH, acetyl-CoA transporter and KIAA0231 were significantly increased 3.1-fold ($p < 0.001$) and 3.3-fold ($p < 0.01$) in spinal motor neurons of ALS, respectively (Fig 3). EGR3 expression decreased to 0.27-fold ($p < 0.01$) in ALS motor neurons. These mRNA alterations were also detected at comparable levels when using spinal ventral horn homogenates of ALS (acetyl-CoA transporter, 1.8-fold increase [$p < 0.005$]; KIAA0231, 2.3-fold increase [$p < 0.05$]; and EGR3, 0.41-fold decrease [$p < 0.01$]). In addition, the gene expression of Bak and TrkC was downregulated 0.53-fold ($p < 0.01$) and 0.40-fold ($p < 0.05$) in ALS, respectively. Moreover, increases of ephrin A1 and cyclin C expression were observed to the extents of 2.5-fold ($p < 0.05$) and 4.9-fold ($p < 0.01$), whereas dynactin 1 mRNA was downregulated 0.44-fold ($p < 0.01$), and CRABP1 mRNA was also downregulated to 0.59-fold ($p < 0.01$) in ALS.

To further verify the localization and extent of expression of genes of interest, we performed in situ hy-

Table 5. Downregulated Genes in Spinal Ventral Horns of ALS (Bottom 30)

GeneBank	Gene Name	Fold Change (ALS/control)
NM_000843	Glutamate receptor, metabotropic 6	0.22
NM_000730	cholecystokinin A receptor	0.24
NM_003134	Signal recognition particle 14kDa	0.26
NM_003163	Syntaxin 1B	0.27
NM_006476	ATP synthase, H+ transporting, mitochondrial F1F0, subunit g	0.27
NM_001610	Acid phosphatase 2, lysosomal	0.28
NM_003108	SRY (sex determining region Y)-box 11	0.29
NM_001446	Fatty acid binding protein 7, brain	0.30
NM_004583	RAB5C, member RAS oncogene family	0.31
NM_001125	ADP-ribosylarginine hydrolase	0.31
NM_003320	Tubby (mouse) homolog	0.31
NM_001731	B-cell translocation gene 1, antiproliferative	0.31
NM_000999	Ribosomal protein L38	0.32
NM_004128	General transcription factor IIF, polypeptide 2 (30kDa subunit)	0.32
NM_001765	CD1C antigen, c polypeptide	0.32
NM_004430	Early growth response 3 (EGR3)	0.33
K00558	Tubulin, α, ubiquitous	0.33
NM_006732	FBJ murine osteosarcoma viral oncogene homolog B	0.33
NM_002040	GA-binding protein transcription factor, α subunit (60kDa)	0.34
NM_006161	Neurogenin 1	0.35
NM_002684	Postmeiotic segregation increased 2–like 11	0.35
NM_000801	FK506-binding protein 1A (12kDa)	0.35
NM_001051	Somatostatin receptor 3	0.35
NM_005017	Phosphate cytidylyltransferase 1, choline, alpha isoform	0.36
NM_004927	Chromosome 11 open reading frame 4	0.36
NM_000046	Arylsulfatase B	0.37
NM_004378	Cellular retinoic acid–binding protein 1 (CRABP1)	0.37
NM_001998	Fibulin 2	0.38
NM_001839	Calponin 3, acidic	0.38
NM_001183	ATPase, H+ transporting, lysosomal, subunit 1	0.39

Gene expression levels are expressed as means of fold-change, which is calculated by dividing the signals of each ALS sample by those of control samples, in the five patients with ALS.
ALS = amyotrophic lateral sclerosis.

bridization on selected genes. The mRNAs for acetyl-CoA transporter, KIAA0231, and EGR3 were localized in the remaining motor neurons (Fig 4). Spinal motor neurons overexpressed acetyl-CoA transporter and KIAA0231 in ALS, whereas EGR3 was underexpressed. Moreover, TrkC, CRABP1, Bak, and dynactin 1 gene expression was found in motor neurons, and those signals were reduced in ALS. DR5 signals were increased in motor neurons in ALS. Cyclin C signals with punctate immunoreactivity were increased in the cytoplasm as well as in nuclei in ALS motor neurons. The nuclear staining of motor neurons for cyclin C was more prominent in ALS compared with controls.

Discussion

Although reports about differential gene expression using the postmortem spinal cords, including those of patients with ALS, have been published,[26,27] the precise gene expression profiles of the degenerating motor neurons themselves have remained to be elucidated. Laser-captured dissection of motor neurons and subsequent microarray analysis are the most appropriate approaches to understanding the motor neuron–specific gene expression profile related to the motor neuron degeneration process in sporadic ALS, because these approaches eliminate bias of motor neuron loss, reactive astroglial proliferation, and other cellular reactions. Indeed, serine kinase has been reported to be underexpressed in ALS spinal cord gray matter,[27] but this study showed it was overexpressed in isolated motor neurons, suggesting that the reported underexpression in whole gray matter was influenced by the decreased motor neuron population. In contrast, cathepsin D expression was downregulated in the ALS motor neurons in this study, whereas it was increased in spinal cord gray matter in a previous report,[27] indicating its upregulation in glial cells. In addition, clustering analyses showed that the gene expression profile in the spinal motor neurons was substantially different from that in the whole homogenates of spinal ventral horn gray matter.

The overall microarray analysis using spinal ventral horn homogenates showed gene expression changes in less than 1% of genes examined with more genes showing increased than decreased expression. On the other hand, the motor neuron–specific microarray analysis

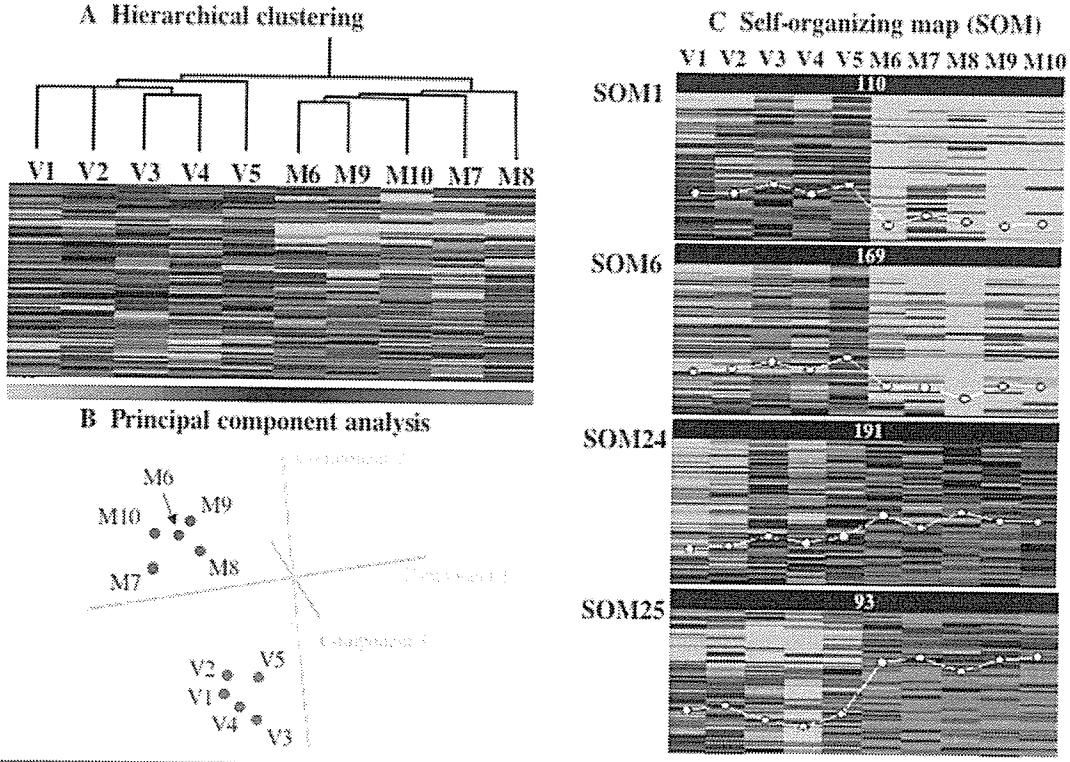

Fig 2. Clustering of gene expression in spinal motor neurons and spinal ventral horns. (A) Hierarchical clustering of gene expression in spinal motor neurons and ventral horns. The dendrogram was produced by hierarchical clustering of relative expression levels of 4,845 genes (rows) in five spinal homogenate and five motor neuron samples (columns) in making a total of 48,450 data points. Visual representation is shown with green representing downregulated (<0.44), black representing intermediate, and red representing upregulated (>2.28). The hierarchical clustering successfully detects two large clusters of amyotrophic lateral sclerosis (ALS), discriminating between spinal homogenates of ventral horns (samples V1 [ALS1], V2 [ALS10], V3 [ALS3], V4 [ALS14], and V5 [ALS13]) and motor neurons (samples M6 [ALS1], M7 [ALS10], M8 [ALS14], M9 [ALS11], and M10 [ALS7]), with a correlation coefficient of 0.446 at the branching point. (B) Principal component analysis of spinal motor neurons and ventral horns. Principal component analysis by six components for the 4,845 genes shows two main clusters consisting of spinal motor neurons (M6–10) and homogenates (V1–5). The number of patients corresponds to those in the dendrogram. (C) Self-organizing map (SOM) analysis of spinal motor neurons and ventral horns. The 4,845 genes are grouped into 25 clusters, the optimal size of which is calculated from gap statistics analysis. In SOM1 and SOM6, most genes are downregulated and in SOM24 and SOM25 the majority of genes are upregulated commonly in isolated motor neurons of five cases (M6–10). The numbers of genes are given at the top, and selected genes are listed for clusters 1, 6, 24, and 25 in Table 6.

showed that the proportion of significantly downregulated genes was 3% of the examined genes, whereas that of upregulated genes was one third of the downregulated genes. Moreover, the genes found to be downregulated specifically in motor neurons were not found to be downregulated in ventral horns, except for three genes with high expression levels. These results strongly support the notion that microarray analysis of laser-captured isolated spinal motor neurons has an advantage especially for the detection of motor neuron–specific downregulated transcripts.

In the differentially expressed genes, cell death–associated genes and genes related to cell signaling were characteristically upregulated in ALS motor neurons, whereas the genes categorized into cytoskeleton and transcription were downregulated. In the prominently altered genes of interest related to the cell death pathway, acetyl-coenzyme A transporter, which has been cloned and shown to encode a protein with multitransmembranous spanning domains,[28] was overexpressed in ALS motor neurons. Acetyl-CoA transporter functions as a cofactor for acetylation of gangliosides as well as vesicular transport of acetylcholine, which is synthesized from acetyl-CoA and choline. Acetylation has been documented to suppress proapoptotic activity of GD3 ganglioside, which increased in ALS neural tissues, as previously shown.[29,30] These results suggest that enhanced expression of acetyl-CoA transporter may be related to the antiapoptotic mechanism for cholinergic motor neuron degeneration in ALS.