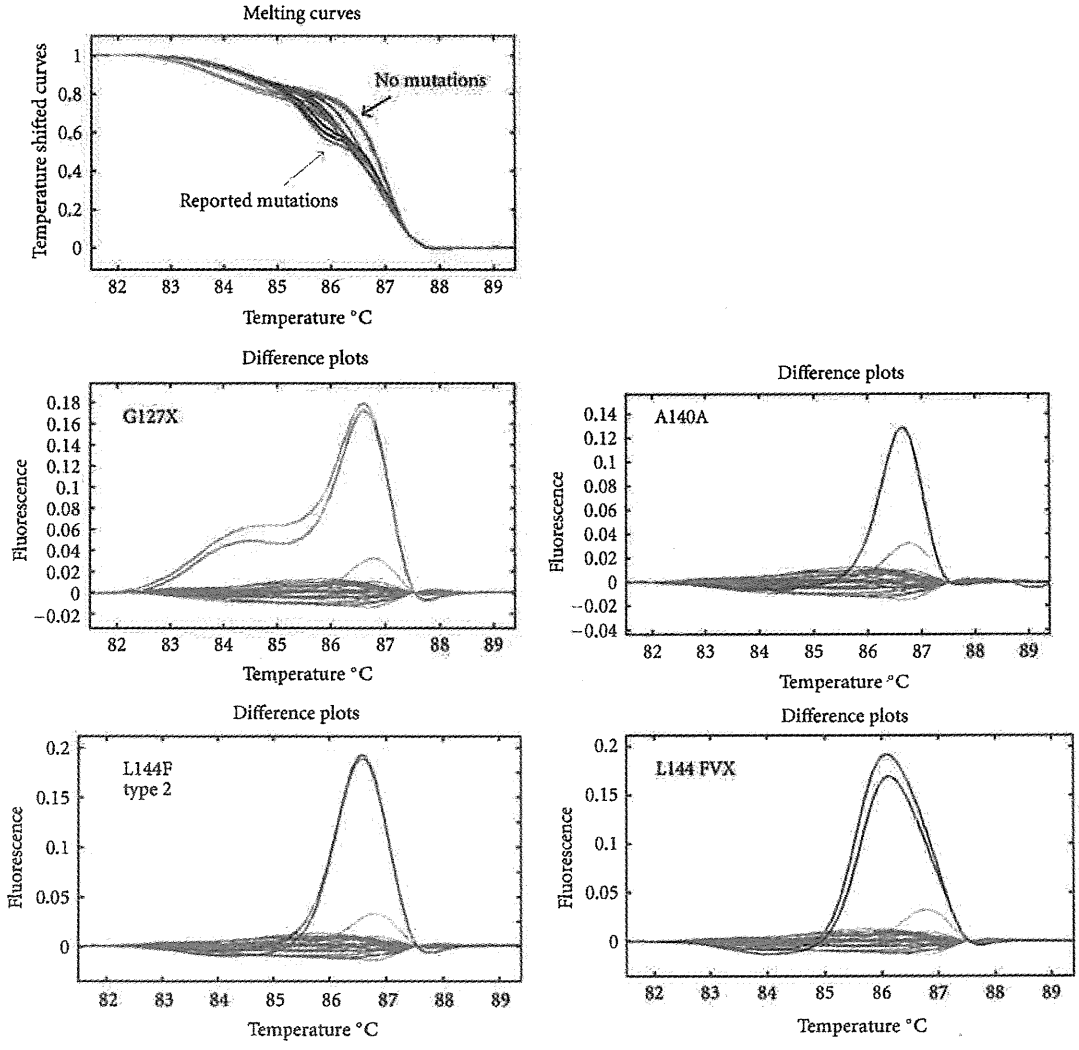
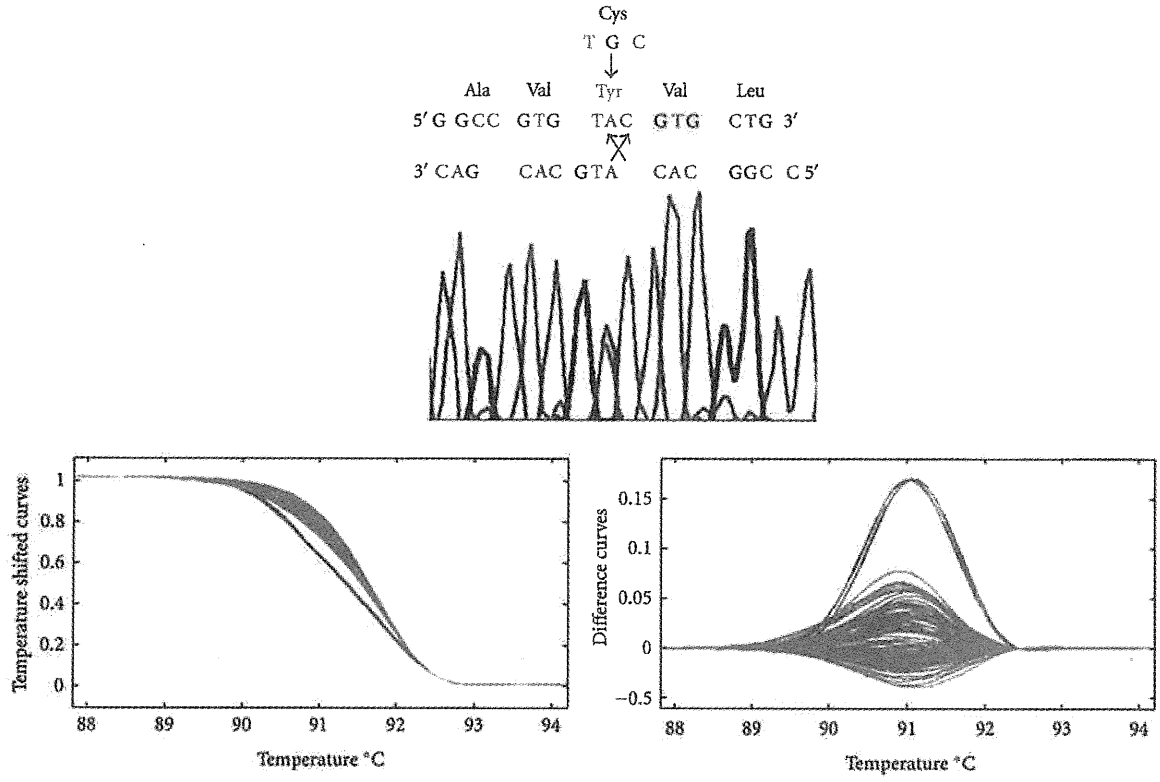


FIGURE 1: Melting curves and subtractive fluorescent difference plots of a wild type (gray lines) and reported *SOD1* mutations (colour lines). Difference plots were easily identified for the mutations.

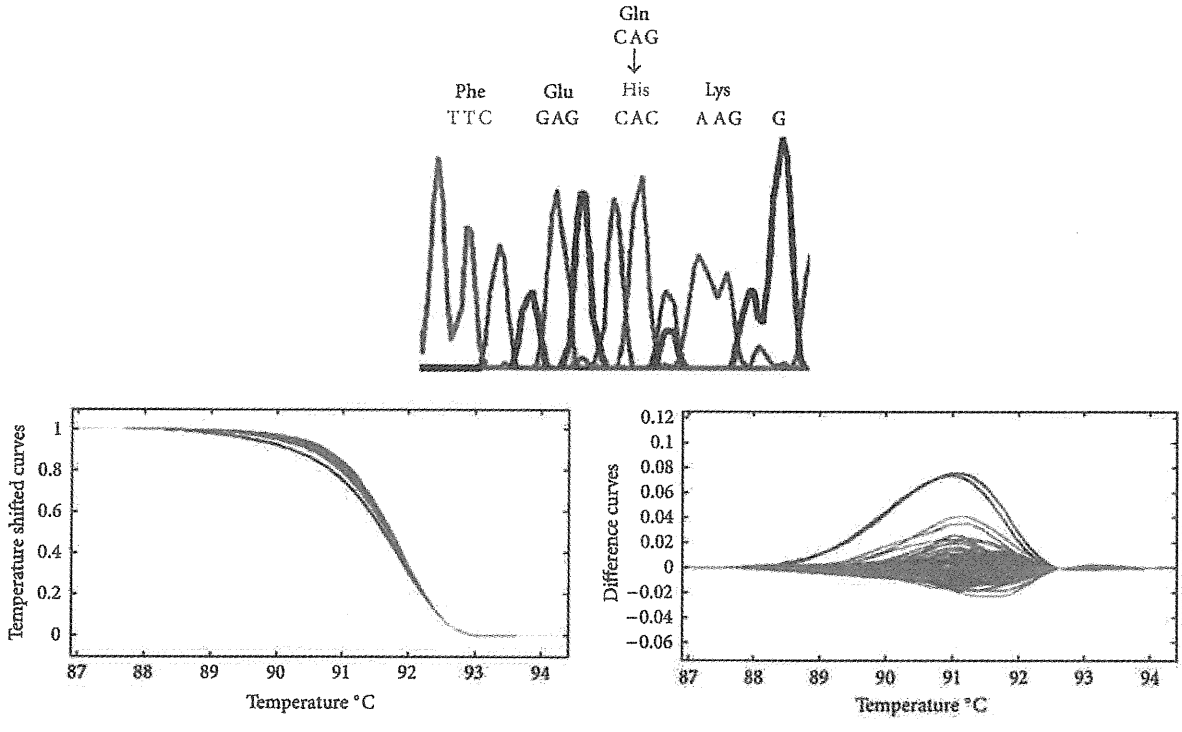
gamma globulin, cyclophosphamide, and plasmapheresis. The disease course was rapid and the bulbar symptom developed in the last stage. She expired 3 years after disease onset.

In case 2, a 48-year-old man, there was a single-base pair substitution in exon 1 at codon 22 (CAG to CAC). This change created a glutamine 22 to histidine missense mutation (Q22H). He developed left leg weakness and atrophy at the age of 46 years. Two years after the onset, neurological examination showed muscle weakness, atrophy and fasciculation were observed in the left leg. Tendon reflexes were brisk in the right leg and both arms. The weakness and atrophy spread to the right leg, confining him to a wheelchair at 51 years old and to bed at 52 years old. He underwent tracheotomy because of progressive respiratory failure, and artificial ventilation support was started eight years after disease onset. Five years after artificial ventilation support was started, he moved to another hospital and thus we could not follow him further.

In case 3, a 69-year-old man, there was a single-base pair substitution in exon 5 at codon 134 (AGT to ACT). This change created a serine 134 to threonine missense mutation (S134T). He noticed gait disturbance due to muscle weakness of the lower limbs at the age of 62 years. The weakness progressively worsened, and he could not walk by himself at 67 years old. Neurological examination showed muscle weakness, and fasciculation were evident in the upper and lower limbs. Tendon reflexes were diminished and plantar responses were flexor. No sensory abnormalities were noted. Nerve conduction studies demonstrated normal motor and sensory nerve conduction velocities. Electromyographic analysis revealed fasciculation and denervation in the upper and lower limbs. Although upper motor neuron impairment was not confirmed, ALS was considered as the most probable

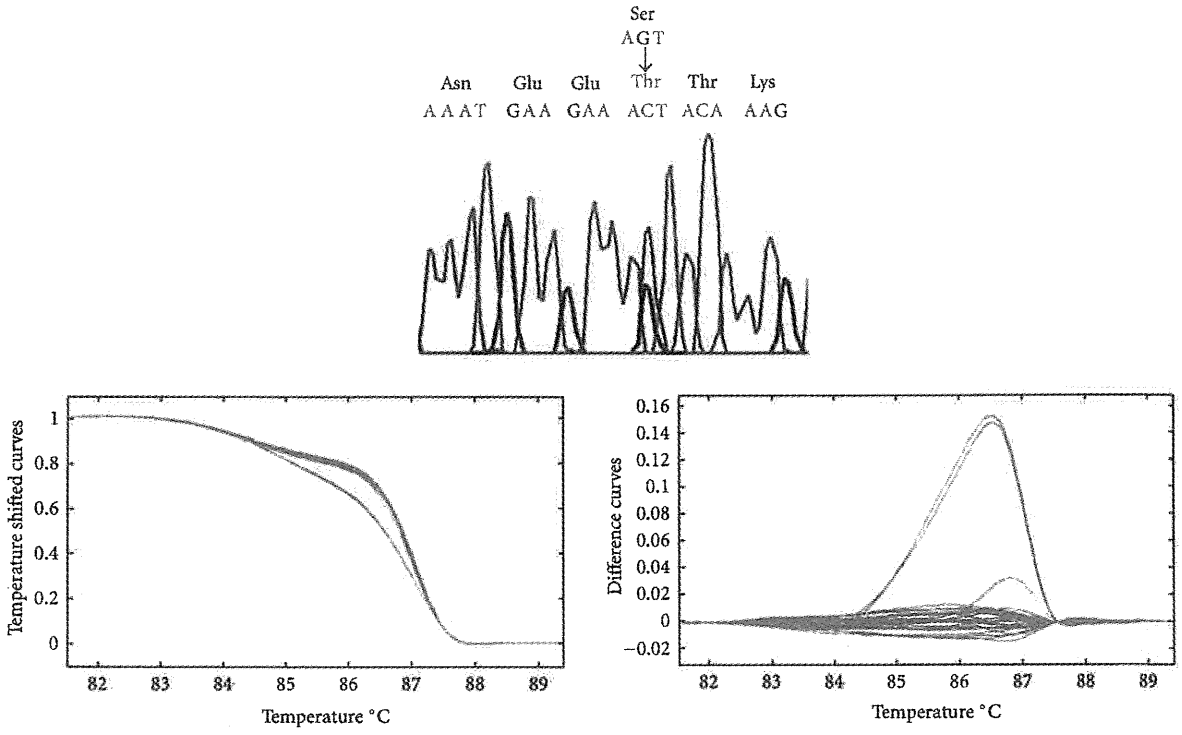


(a) Case 1 (20 G > A; C6Y)



(b) Case 2 (69 G > C; Q22H)

Figure 2: Continued.



(c) Case 3 (404 G > C; S134T)

FIGURE 2: Sequence (upper), melting curves (left lower) and subtractive fluorescent difference plots (right lower) of the three novel mutations.

diagnosis. The weakness progressed very slowly, and he died of respiratory insufficiency seven years after disease onset.

3.3. SOD1 Mutations in Group 2. We found *SOD1* mutations in eight out of 265 cases. Of these, four had family histories, mutations being Leu 38 Val (L38V) and His 46 Arg (H46R) in exon 2, Gly 93 Ser (G93S) in exon 3 and Gly 141 Ala (G141A) in exon 5. The G141A found in a woman whose brother probably died of ALS was a novel mutation. In this case, left hand weakness occurred at 57 years old. The clinical course was rapid that she died at 3 years and 11 months after the onset. The remaining four *SOD1* mutations were found in sporadic cases, mutations being Lys 3 Glu (K3E) in exon 1 and Gly 93 Ser (G93S) in exon 3. K3E was a novel mutation found in a woman who noticed right leg weakness at 52 years old, and artificial ventilation support was started 6 years after the onset. The G93S mutation was found in three unrelated patients. The prevalence of *SOD1* mutations in the SALS cases was 1.56% (4 of 255 SALS cases) in the group 2.

4. Discussion

4.1. HRM Analysis on SOD1. This is the first report of HRM analysis for *SOD1* mutation screening. HRM analysis could clearly distinguish 18 of 19 reported *SOD1* mutations from normal controls. We have demonstrated that HRM analysis is a rapid and sensitive (94.7% sensitivity) method for mutation scanning of *SOD1*. SSCP is a method that most laboratories use for the screening of gene mutations, but the sensitivity is not high (80% to 90%) [7]. DHPLC using WAVE system is also a screening method, but it cannot detect the D90A mutation [6], which is one of the worldwide detected *SOD1* mutations, and the most appropriate condition for analysis is difficult to determine. Using HRM analysis, we can analyze within 5 to 10 minutes on 96 samples and the running cost is not expensive.

The one mutation that HRM analysis could not detect was guanine to cytosine at nucleotide 341 substituting glycine (GGC) to alanine (GCC) at codon 114. On the other hand, guanine (TTG) to cytosine (TTC) mutations (L144F), and alanine (GCT) to alanine (GCA) mutations (A140A) in other samples were detected with this method, indicating the possibility that the G to C mutation detection failure may be a sequence-specific phenomenon.

4.2. SOD1 Mutations in SALS. We applied this method to our own 184 (group 1) and 255 (group 2) Japanese cases of SALS, finding three different novel *SOD1* mutations in three cases in the former (mutation prevalence, 1.63%), and one novel and three known mutations in four cases in the latter (mutation prevalence, 1.57%). We listed the prevalence and identified mutations of *SOD1* in SALS cases in other

Table 2: *SOD1* mutations in SALS patients of the different countries.

Country	Total SALS	No. of *SOD1*	*SOD1*/Total	Mutations identified	Screening method	Author, year
North England	46	1	2.1	D101N	SSCP	Jones et al. 1994 [14]
Scotland	57	4	7.0	E21K, I113T	SSCP	Jones et al. 1995 [2, 15]
Scandinavia	355	14	3.9	V14G, D90A (hetero & homo)	SSCP	Andersen et al. 1997 [16]
England	155	4	2.6	D90A, I113T, V118KTGPX	SSCP	Jackson et al. 1997 [17]
England	175	5	2.8	G72S	SSCP	Shaw et al. 1998 [18]
Belgium	69	3	4.3	D90A, N139N, IVS + 19A > G	SSCP	Aguirre et al. 1999 [3]
Italy	48	3	6.3	D90A (homo), I113T, A95T	DS	Gellera et al. 2001 [22]
Spain	87	1	1.2	N65S	SSCP	García-Redondo et al. 2002 [19]
Italy	225	0	0		SSCP	Batlistini et al. 2005 [20]
Spain (Catalonia)	94	4	4.2	D90A, N139H, A140A	DS	Gamez et al. 2006 [4]
Italy	66	3	4.5	K135X, N65S, A95T	DHPLC	Corrado et al. 2006 [5]
Italy	303	2	066	N19S, E133ΔE	DHPLC	Chiò et al. 2008 [6]
Japan	184	3	1.6	C6Y, Q22H, S134T	HRM	This article group1
Japan	255	4	1.5	K3E, G93S	HRM	This article group2
Total	2119	51	2.4			

DS: direct sequence (no screening method in the article).

countries (Table 2). The prevalence was high in the Scottish population (7%) and widely ranged in Italy (0%–6%), but in other countries, it was 2 to 4%, similar to our data. This time we found four novel mutations in SALS cases, and these mutations were not found in the Japanese control group.

In a sporadic ALS patient carrying an *SOD1* mutation, it is also difficult to ascertain whether it is a genuine sporadic case, a case due to a mutation, or a familial case with incomplete penetrance. To date, an SALS case with H80A is the only one with a proven de novo mutation [23]. In our analysis, the G93S mutation was found in three unrelated patients from the Tokai district of Japan (personal communication). There are at least 6 Japanese families with G93S, 4 of the 6 families being reported to be residents of the Tokai district [24–26]. The accumulation of G93S in Japanese SALS cases suggests the possibility of decreased penetrance or an incomplete family history rather than a de novo mutation.

4.3. Clinical Characteristics of SALS Involving SOD1 Mutations. Clinical characteristics such as onset age, onset symptoms, and clinical course of so far reported SALS patients having *SOD1* mutations are summarized in Table 3. Since A4V, D90A, and I113T have been observed worldwide and are considered to be the most common mutations in both familial and sporadic ALS cases [4, 7]. Because of the difficulty to define true sporadic, we did not include these three mutations in the table. Based on the results of analysis of these 20 *SOD1* mutations in 27 sporadic ALS patients (13 men, 10 women, and 4 unknown), the average age at onset was 43.8 (range 18–77) years, which is about 10 years younger that the mean age at onset reported for the sporadic ALS population [22]. The onset symptom was limb weakness in 21 cases and bulbar dysfunction only in one case. The clinical courses were under three years (rapid) in seven cases, over six years (slow) in nine cases, and three to six years (moderate) in five cases. The clinical characteristics of SALS involving *SOD1* mutations indicate a relatively young onset age and a high percentage of limb involvement at onset. These characteristics are similar to the features of ALSOD (ALS patients having *SOD1* mutations), not those of sporadic ALS [29].

The C6Y mutation in our case was difficult to diagnose because the main symptom was lower motor neuron dysfunction and the onset age was young (midthirties). But this clinical course was similar to that in the case of de novo mutation H80A [23]. There were nine (bold) patients whose onset ages were under forty, and eight of them had rapid or moderate clinical course (Table 3). On the other hand, there are four (underlined) patients whose onset ages were over 55, and three of them had slow clinical course (Table 3). Gamez and his colleagues reported [4] there were three types of sporadic ALS patients who were particular candidates for genetic testing for *SOD1*: (a) those with the typical Scandinavian phenotype, (b) those with clinical onset before 55 years of age, and (c) patients with slow progression/long survival. Compare with this theory (b) and (c), only one patient (N19S) is an exception for *SOD1* screening.

5. Conclusion

We have demonstrated that HRM analysis is a rapid and sensitive method for the mutation scanning of *SOD1*. With this method, four novel *SOD1* mutations were found in SALS cases, the prevalence of *SOD1* mutations in Japanese SALS cases being 1.6%. The clinical characteristics of SALS involving *SOD1* mutations are a young onset age and a high percentage of limb involvement at onset. We will screen other causative genes for ALS (*TDP-43, ANG, FUS/TLS, OPTN* and others) by HRM analysis and determine the cause of disease appearance.

Table 3: Clinical characteristics of the SALS patients having *SOD1* mutations.

Amino acid change	Sequence change	No. of pt.	Onset age	Onset symptom	Disease course/Disease duration	Author/Reference
K3E	AAG > GAG	1	52	Right leg weakness	Moderate, 6y	This article
C6Y	TGC > TAC	1	**34**	Right leg weakness	Moderate, 3y	This article
V14G	GTG > GGG	1	**39**	Both legs fatigue	ND, 16m~	Andersen et al. [16]
G16S	GGC > AGC	1	**18**	Hand paresis	Rapid, 1y	Kawamata et al. [27]
N19S	AAT > AGT	2	**32** 41	Both legs weakness Left arm weakness	Moderate, 36m ND	Mayeux et al. [28]
		1	77	Hand paresis	Rapid, 15m	Chiò et al. [6]
E21K	GAG > AAG	1	ND	ND	ND	Jones et al. [2]
Q22H	CAG > CAC	1	46	Left leg weakness	Slow, 8y	This article
N65S	AAT > AGT	1	44	Left leg weakness	Slow, 14y	García-Redondo et al. [19]
		1	40	Drop foot	Slow, 11y	Corrado et al. [5]
G72S	GGT > AGT	1	**29**	Left leg weakness	Rapid, 15m	Shaw et al. [18]
H80A	CAT > CGT	1	**24**	Left leg weakness	Rapid, 18m	Alexander et al. [23]
G93S	GGT > AGT	3	44 55 64	Both legs weakness Left leg weakness Right leg weakness	ND, 6y~ Slow, 8y~ Slow, 12y~	This article
A95T	GCC > ACC	1	**26**	Both legs weakness	Slow	Gellera et al. [22]
		1	45	Left drop foot	Slow, 20y	Corrado et al. [5]
D101N	GAT > AAT	1	53	ND	ND	Jones et al. [14]
V118 KTGPX	GTG > AAAACTG	1	**34**	ND	Rapid, 16m	Jackson et al. [17]
E133ΔE	GAA del GAA	1	54	Left leg weakness	Moderate, 4y	Chiò et al. [6]
S134T	AGT > ACT	1	62	Both legs weakness	Slow, 7y	This article
K136X	AAG > TAG	1	45	Left leg weakness	Rapid,12m	Corrado et al. [5]
N139H	AAG > CAC	1	53	ND	ND	Gamez et al. [4]
N139N	AAC > AAT	1	**33**	ND	Moderate, 3y	Aguirre et al. [3]
A140A	GCT > GCA	2	52 ND	Bulbar palsy Limb weakness	Rapid, 22m Slow	Gamez et al. [4]
Total/Average	20	27	43.8	21 Extremity 1 Bulbar 5 No data	7 Rapid 5 Moderate 9 Slow	

ND: no data, y: year or years, m: month or months, and y~ or m~: alive at the reported time.
Age: **under forty** (bold) and over fifty-five (underlined).
Disease course (until invasive ventilation support): ~2 years, rapid; 3–6 years, moderate; 7~ years, slow.

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Nationwide survey on the epidemiology of syringomyelia in Japan

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ABSTRACT

Background: Syringomyelia is a rare disease characterized by abnormal fluid-filled cavities within the spinal cord, and is associated with Chiari malformations, arachnoiditis, or spinal cord tumors. The widespread availability of magnetic resonance imaging (MRI) in Japan has allowed for easy identification of syrinxes. The aim of this study was to survey the clinicoepidemiological characteristics of syringomyelia in Japan.
Methods: A 2-stage postal survey was conducted in late 2009. The first survey aimed to estimate the number of patients with syringomyelia, and the second survey aimed to elucidate clinicoepidemiological characteristics. Diagnosis of syringomyelia was based on the findings of MRI or computed tomographic myelography.
Results: In the first survey, we received 2133 responses from 2937 randomly selected departments and collected data of 1215 syringomyelia patients (543 men and 672 women). The total response rate for the first survey was 73%. The estimated prevalence of ambulatory syringomyelia patients in Japan was 1.94 per 100000. In the second survey, the proportion of asymptomatic syringomyelia patients was 22.7%. Chiari type I malformations and idiopathic syringomyelia were the first and second most common etiologies.
Conclusions: Our nationwide survey indicated that widespread MRI availability has contributed to the diagnosis of both asymptomatic and idiopathic cases.

1. Introduction

Syringomyelia is a heterogeneous disorder characterized by abnormal fluid-filled cavities or cysts within the spinal cord. The etiologies of syringomyelia can include Chiari malformations, arachnoiditis, trauma, and spinal cord tumors [1–3], but the pathophysiology of syrinx development remains enigmatic. Some cases with Chiari Type I malformations manifested asymptomatic syringomyelia [4]. The reported prevalence was 8.2 to 8.4 per 100 000 in Western countries [5,6]. An epidemiologic survey that collected data from 1243 patients between 1982 and 1991 in Japan showed the predominance of Chiari Type I malformations in syringomyelia, and identified a few cases of spontaneous remission [7]. Surgical treatment for syringomyelia is essential to stop the progression of the disease and further cavity enlargement. However, the previous epidemiologic survey did not determine the prevalence of the disease in the Japanese population [7].

The diagnosis of syringomyelia has been greatly aided by the development and widespread availability of magnetic resonance imaging (MRI) scanners, which have allowed for the relatively easy identification of syrinxes. Japan has the highest number of magnetic resonance imaging (MRI) scanners per capita, with national healthcare insurance coverage allowing universal access to outpatient hospital care. Hence, both symptomatic and asymptomatic syringomyelia patients can be more adequately examined than was possible prior to MRI facilities becoming widely accessible.

The characteristics of asymptomatic syringomyelia have not been sufficiently investigated. The aim of this study, therefore, was to estimate the prevalence of syringomyelia in Japan and identify its clinicoepidemiological characteristics by taking advantage of the current widespread availability of MRI facilities.

2. Methods

We conducted a 2-stage postal survey according to methods described previously [8,9] in late 2009. The first survey aimed to estimate the number of individuals with syringomyelia, and the second survey aimed to elucidate the clinicoepidemiological characteristics

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of syringomyelia. We collected data from patients diagnosed with syringomyelia by neuroimaging from the departments of neurosurgery, neurology, orthopedics, and pediatrics. We requested the numbers of male and female ambulatory syringomyelia patients from each department in the past year (August 2008 to July 2009).

In the first survey, we adopted a definition of syringomyelia based on neuroimaging: a central or lateralized syrinx detected on MRI (including syrinxes with septums), or a syrinx detected with computed tomographic myelography in patients who could not undergo MRI because of metal in the body. The number of patients with syringomyelia in each institution was counted based on this definition. The departments surveyed were randomly selected by stratified sampling from a list of all hospitals with 20 or more beds; the list was obtained from the Ministry of Health and Welfare. Sampling rates were approximately 5%, 10%, 20%, 40%, 80%, and 100% for the stratum of general hospitals with 20 to 99 beds, 100 to 199 beds, 200 to 299 beds, 300 to 399 beds, 400 to 499 beds, and 500+ beds, respectively. Additionally, all university hospitals in Japan were surveyed.

In the second stage of the survey, we requested details of individual patients from each department that had 1 or more syringomyelia patients. The detailed information for each patient was reported based on a retrospective chart review. Epidemiological items included sex, date of birth, time of onset and diagnosis, family history, symptoms and signs, imaging findings, treatment, and clinical course. Symptoms included motor function, sensory disturbance, autonomic failure, cranial nerve disturbance, and skeletal deformity. Motor functions included weakness, muscle atrophy, spasticity, hypotonus, and planter reflex. Autonomic failure included Horner syndrome, anisocoria, dyshidrosis, abnormal nail development, limb hypertrophy, bladder and rectal disturbance, orthostatic hypotension, impotence, and neurogenic arthropathy.

This study was approved by the Institutional Review Board of Hokkaido University.

2.1. Estimation and statistical analysis

We estimated the prevalence of syringomyelia based on the results from the first stage of the survey. The estimation was based on the assumption that the responses of the departments were independent of the frequency of patients [8,10]. Formulas used to estimate the total number of patients, and the 95% confidence intervals are described below.

The point estimation of prevalence was calculated using the following equation, where SRT_k, RRT_k, NS_k, n_k, N_k, and N_{ki} denote the sampling rate, response rate, the number of sampling departments, the total number of departments, the number of responding departments, and the number of departments with i patients in stratum k, respectively.

$$\hat{\alpha}_k = \frac{1}{SRT_k RRT_k}\sum_i iN_{ki} = \frac{1}{\frac{NS_k}{n_k}\frac{N_k}{NS_k}}\sum_i iN_{ki} = \frac{n_k}{N_k}\sum_i iN_{ki}.$$

3. Results

In the first survey, we received 2133 responses from 2937 randomly selected departments, and collected data regarding 1215 syringomyelia patients (543 men and 672 women). The total response rate of the first survey was 73%.

Results from the first survey (Table 1) showed that the number of syringomyelia patients who were referred to a hospital between August 2008 and July 2009 was 2475 (95% CI: 2051–2899). The

Table 1
Summary of data collected in the first stage of the survey.

Type s of departments	Type s of hospitals and beds	Total no. of departments	Sampling rate (%)	No. of surveyed departments	No. of departments that responded	Response rate (%)	No. of reported patients	No. of estimated patients
Neurosurgery	General hospitals with ≤99 beds	710	5%	35	22	63%	0	0
	General hospitals with 100–199 beds	528	10%	52	27	52%	7	137
	General hospitals with 200–299 beds	298	20%	59	37	63%	26	209
	General hospitals with 300–399 beds	296	40%	119	73	61%	23	93
	General hospitals with 400–499 beds	167	80%	133	94	71%	40	71
	General hospitals with ≥500 beds	216	100%	216	147	68%	133	195
	University hospitals	113	100%	113	94	83%	267	321
	Subtotal	2328		727	494	68%	496	1027
Neurology	General hospitals with ≤99 beds	506	5%	25	13	52%	0	0
	General hospitals with 100–199 beds	335	10%	34	18	53%	3	56
	General hospitals with 200–299 beds	170	20%	34	27	79%	6	38
	General hospitals with 300–399 beds	170	40%	68	38	56%	7	31
	General hospitals with 400–499 beds	91	100%	91	59	65%	21	32
	General hospitals with ≥500 beds	93	100%	93	60	65%	25	39
	University hospitals	118	100%	118	103	87%	53	61
	Subtotal	1483		463	318	69%	115	257
Orthopedics	General hospitals with ≤99 beds	2278	5%	114	66	58%	4	138
	General hospitals with 100–199 beds	1047	10%	105	70	67%	10	150
	General hospitals with 200–299 beds	436	20%	87	63	72%	10	69
	General hospitals with 300–399 beds	362	40%	145	110	76%	48	158
	General hospitals with 400–499 beds	190	80%	152	107	70%	20	36
	General hospitals with ≥500 beds	228	100%	228	178	78%	120	154
	University hospitals	118	100%	118	98	83%	300	361
	Subtotal	4659		949	692	73%	512	1065
Pediatrics	General hospitals with ≤99 beds	1069	5%	54	32	59%	0	0
	General hospitals with 100–199 beds	613	10%	62	41	66%	0	0
	General hospitals with 200–299 beds	356	20%	71	49	69%	0	0
	General hospitals with 300–399 beds	339	40%	136	105	77%	7	23
	General hospitals with 400–499 beds	184	80%	147	120	82%	11	17
	General hospitals with ≥500 beds	214	100%	214	183	86%	58	68
	University hospitals	114	100%	114	99	87%	16	18
	Subtotal	2889		798	629	79%	92	126
	Total	11359	26%	2937	2133	73%	1215	2475

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estimated prevalence of ambulatory syringomyelia patients in Japan was 1.94 per 100 000. In the second survey, we collected reports from 720 of the 1215 patients from the first survey. The response rate for the second survey was 59%. There were 12 duplicated reports, and thus, we integrated the data reported in them.

Results of the second survey (Table 2) described the characteristics of both symptomatic and asymptomatic syringomyelia. The proportion of patients with asymptomatic syringomyelia was 22.7% (161 cases). The mean ages at survey and diagnosis of asymptomatic syringomyelia (28.9 ± 23.3 and 24.4 ± 24.1 years, respectively) were lower than those of patients with symptomatic syringomyelia (40.8 ± 22.8 and 35.3 ± 22.5 years, respectively). Asymptomatic syringomyelia tended to be primarily associated with localized cavities. The proportion of syringomyelia cases with a Chiari type I malformation etiology was higher among symptomatic than asymptomatic syringomyelia patients. Conversely, the proportion of cases with idiopathic etiologies was higher in asymptomatic than in symptomatic syringomyelia.

A subset of patients with symptomatic syringomyelia (Table 3) included both those who had, and those who had not undergone surgical treatment. The mean age at onset and diagnosis of patients who had undergone surgical treatment (29.4 ± 21.0 and 31.6 ± 21.5 years, respectively) was lesser than that of patients who had not received surgical treatment (40.1 ± 22.6 and 44.8 ± 22.3 years, respectively). There were only 2 cases with a family history of the disease. Approximately 11% of patients in each group experienced an improvement in their symptoms. The most common symptom was sensory disturbance, which was reported in 75.3% of patients with surgical treatment and 68.8% of those without surgical treatment. Motor disturbance was the second most common symptom in each group (59.8% and 51.0%, respectively). Patient histories showed that approximately one-tenth of the patients in each group had previous injuries of the head or spine.

The characteristics of patients in each age group (Table 4) showed that the prevalence of idiopathic syringomyelia was higher in adults, particularly in the elderly, than in children.

Fig. 1 shows the distributions of patient's ages at the time of survey (Fig. 1A), age at diagnosis (Fig. 1B), age at surgical treatment (Fig. 1C), and year of diagnosis (Fig. 1D). The distribution of ages at survey consisted of 2 peaks, at 10 to 20 years of age, and at 60 to 70 years of age. The distribution of age at diagnosis showed a higher proportion of 0- to 20-year-olds. Finally, the distribution of diagnosis year showed an acute increment in the number of cases diagnosed in more recent years.

Table 2
Demographics of patients in the second stage of the survey.

	Symptomatic (N = 543)	Asymptomatic (N = 161)	Total (N = 708[a])	Missing
Age at survey (Mean ± SD)	40.8 ± 22.8	28.9 ± 23.3	38.0 ± 23.5	35
Age at diagnosis (mean ± SD)	35.3 ± 22.5	24.4 ± 24.1	32.7 ± 23.4	66
Sex (%)				
Male	41.6	44.1	42.1	1
Female	57.3	53.4	56.5	3
Missing	1.1	2.5	1.4	0
Morphology (%)				
Asymmetry	31.3	8.1	25.8	0
Symmetry	58.9	83.2	64.4	2
Missing	9.8	8.7	9.7	2
Distribution (%)				
Syringobulbia				
Bulbus only	1.5	0.6	1.3	0
Bulbus and spinal cord	5.7	1.2	4.8	1
Syringomyelia				
Cervical cord only	18.6	32.9	21.8	0
Thoracic cord only	7.9	8.7	8.2	1
Lumbosacral cord only	0.9	9.9	3.1	1
Cervical–thoracic	49.4	27.3	44.1	0
Thoracic–lumbosacral	2.6	4.3	3.0	0
Cervical–lumbosacral	4.6	4.3	4.5	0
Missing	8.8	10.6	9.3	1
Etiology (%)				
Chiari type I	53.6	30.4	48.0	0
Chiari type II	4.4	20.5	8.1	0
Bone anomaly	1.1	0.6	1.0	0
Arachnoiditis	5.7	2.5	4.9	0
Trauma	9.6	0.6	7.5	0
Spinal cord tumor	5.2	5.6	5.2	0
Idiopathic	12.9	24.8	15.7	1
Other	6.1	13.0	7.9	2
Suspected two or more	1.1	1.2	1.1	0
Missing	0.4	0.6	0.6	1

[a] Four patients who did not report on the existence of symptoms were excluded.

Table 3
Demographics, clinical history, and manifestations of symptomatic patients.

		Surgical treatment			
		Yes	No	Total	Missing
Number of cases		376	157	543	10
Age at onset (mean ± SD)		29.4 ± 21.0	40.1 ± 22.6	32.3 ± 22.0	
Age at diagnosis (mean ± SD)		31.6 ± 21.5	44.8 ± 22.3	35.3 ± 22.5	
Age at surgery (mean ± SD)		32.6 ± 21.0			
Family history (%)	Yes	0.3	0.6	0.4	0
	No	64.4	59.9	62.2	2
	Unknown/missing	31.1	35.0	32.4	
Course of symptoms after initial diagnosis (%)					
Worsen		51.1	22.3	42.2	2
Unchanged		26.3	56.7	35.5	5
Improved		11.2	10.8	10.9	0
Stop after progression		4.8	5.7	5.0	0
Missing		6.6	4.5	6.4	
Symptoms (%)					
Motor	Yes	59.8	51.0	57.5	7
	No	37.8	45.9	39.4	0
	Unknown/missing	2.4	3.2	3.1	
Sensory	Yes	75.3	68.8	72.7	4
	No	19.9	21.0	19.9	0
	Unknown/missing	4.8	10.2	7.4	
Autonomic	Yes	20.7	19.1	19.9	0
	No	65.2	65.6	64.6	3
	Unknown/Missing	14.1	15.3	15.5	
Cranial nerves	Yes	10.1	7.0	9.2	1
	No	83.2	80.9	81.4	2
	Unknown/missing	6.6	12.1	9.4	
Skeletal deformity	Yes	31.4	22.9	29.3	5
	No	64.9	75.2	67.4	4
	Missing	3.7	1.9	3.3	
Past history (%)					
CNS infections	Yes	3.5	3.8	3.7	1
	No	80.6	74.5	78.3	5
	Unknown/missing	16.0	21.7	18.0	4
Injuries of head or spine	Yes	11.4	10.2	10.9	0
	No	76.3	75.8	75.7	5
	Unknown/missing	12.2	14.0	13.4	
Surgery of head or spine	Yes	13.8	12.1	13.4	2
	No	77.4	77.7	77.0	5
	Unknown/missing	8.8	10.2	9.6	
Problems at delivery	Yes	2.1	1.3	2.0	1
	No	66.2	59.9	63.9	4
	Unknown/missing	31.6	38.9	34.1	

4. Discussion

This study revealed the prevalence (1.94 per 100 000) and characteristics of ambulatory syringomyelia patients in Japan. Among these patients, the prevalence of asymptomatic syringomyelia was 22.6%,

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Table 4
Summary of characteristics of patients according to age group.

Age	Female (%)	Asymptomatic (%)	Etiology 1st / 2nd / 3rd	%	Localized cavity (%)
<10	51.11	40.9	Chiari type I	40.6	36.9
			Chiari type II	34.8	
			Other	14.5	
10–19	66.07	23.9	Chiari type I	78.8	36.2
			Idiopathic	6.2	
			Other	5.3	
20–29	52.63	14.0	Chiari type I	47.4	47.1
			Idiopathic	22.8	
			Trauma	14.0	
30–39	46.34	20.5	Chiari type I	49.4	39.0
			Idiopathic	17.3	
			Trauma	14.8	
40–49	55.17	15.3	Chiari type I	55.9	27.8
			Idiopathic	18.6	
			Spinal cord tumor	10.2	
50–59	69.74	14.5	Chiari type I	42.1	40.6
			Idiopathic	23.7	
			Spinal cord tumor	10.5	
60–69	54.55	11.5	Chiari type I	28.2	42.9
			Idiopathic	24.4	
			Trauma	16.7	
>70	66.67	24.3	Idiopathic	37.8	40.0
			Chiari type I	27.0	
			Arachnoiditis	13.5	

and that of idiopathic syringomyelia was 15.8% according to the second survey.

The prevalence of syringomyelia in this survey is lower than that in previous studies that used different methods for estimation [5,6]. Estimation of prevalence in this survey was based on patients who were referred to a hospital for evaluation or treatment. Therefore, the data from patients whose syringomyelia was stable and who had discontinued their ambulatory care were not collected in this study. It is noteworthy that the early detection of syringomyelia by MRI can allow for early interventions, including surgery. Early diagnosis and intervention are more likely to lead to a positive outcome, and may therefore reduce the number of patients requiring ambulatory care. The lower number of patients diagnosed in the years preceding 2005 (Fig. 1-D) is consistent with our speculation. However, these results show the characteristics of ambulatory care among syringomyelia patients.

The etiology of syringomyelia can include Chiari malformation, trauma, arachnoiditis, and idiopathic origin, among other causes. In our study, Chiari malformations, including both types I and II, were the most common cause in both children and adults, and this finding is consistent with those of previous studies [7,11]. In particular, Chiari malformation is more frequent in children than in adults. These results may be associated with the widespread availability of MRI, which contributes to early diagnoses in cases of syringomyelia caused by Chiari malformation. Interestingly, idiopathic syringomyelia was the second most common cause according to our survey. Bogdanov et al. suggested that idiopathic syringomyelia is associated with a small posterior fossa with a narrow cerebrospinal fluid (CSF) space as well as with Chiari I malformation [12]. It is possible that some of the cases of idiopathic syringomyelia in our survey may be attributable to a small posterior

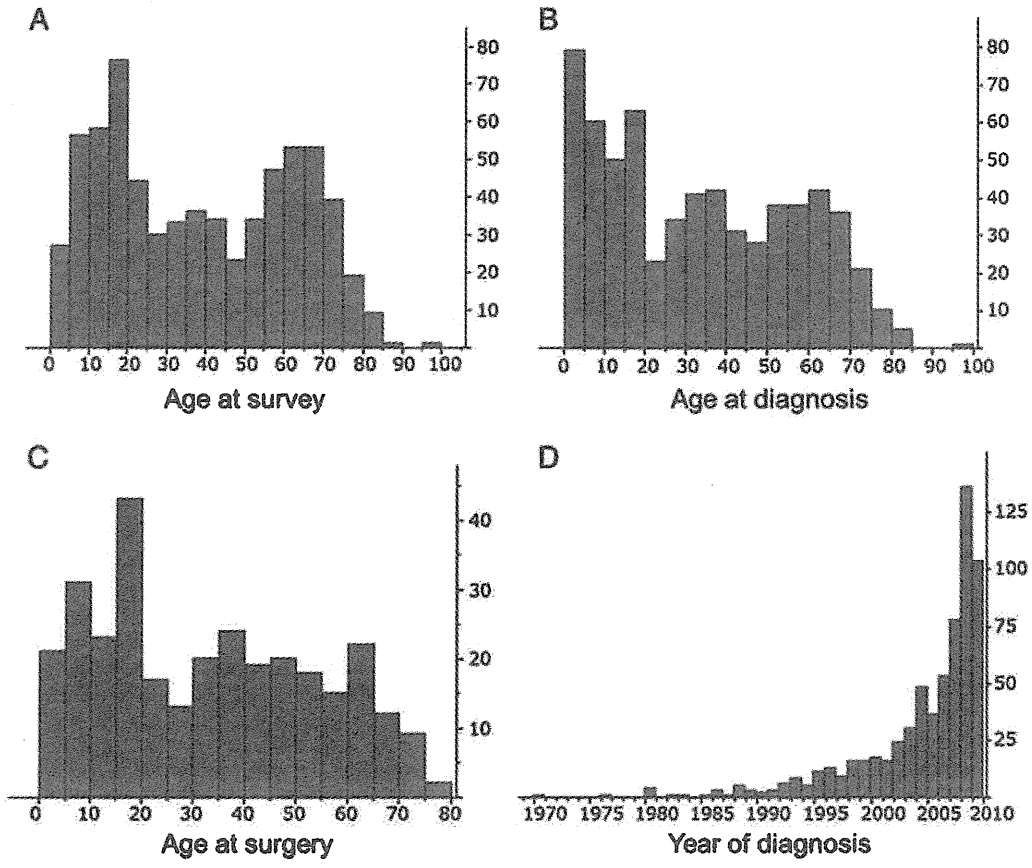

Fig. 1. (A) Histogram showing age distribution of patients at time of survey. (B) Histogram showing age distribution of patients at diagnosis. (C) Histogram showing age distribution at time of surgery. (D) Histogram showing the diagnosis by year.

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fossa. Holly et al. described slit-like syrinx cavities characterized by remnants of the central canal and an asymptomatic clinical course [13]. Therefore, idiopathic syringomyelia has several potential causes, including congenital remnants of the central canal and acquired dilations by a small posterior fossa. Hida et al. reported an association between syringomyelia with Chiari I malformation and birth injuries [14]. In this study, patients with problem at delivery accounted for 2.0% of symptomatic syringomyelia cases, but it had a higher unknown/missing proportion in the past history. Nakamura et al. discuss 2 types of idiopathic syringomyelia: localized and extended. Localized syringomyelia is associated with congenital enlargement of the central canal of the spinal cord and can be managed conservatively [15]. Actually, most of the patients with idiopathic cases in our study did not undergo surgical treatment. Idiopathic syringomyelia might be less progressive than syringomyelia with other causes.

Asymptomatic syringomyelia comprised 22.7% of all syringomyelia cases in our second survey. Prior to this survey, the proportion of asymptomatic syringomyelia cases was unknown. Cases of a few patients with asymptomatic syringomyelia caused by a brain tumor of the posterior fossa have been previously reported [16–18]. The infrequency of asymptomatic syringomyelia seems inconsistent with our survey results. There are 2 possible explanations for the relatively high proportion of asymptomatic syringomyelia in our survey. Firstly, the symptoms of patients who did not complain because of their age were underestimated. Secondly, the availability of MRI in Japan has resulted in an increase in the number of incidental diagnoses of asymptomatic syringomyelia including slit-like syrinx cavities.

Resolution of syringomyelia without surgical treatment was observed in 17 patients (3.2% of symptomatic patients) in our second survey. Spontaneous resolution of syringomyelia has recently been found to be more common than previously thought [19]. The mechanisms involved in the development and spontaneous resolution of syringomyelia are unclear despite multiple hypotheses [20]. The number of patients with spontaneous resolution may be underestimated because cases of asymptomatic syringomyelia patients who had not sought consultation were not evaluated in our survey.

Symptoms of syringomyelia include pain, sensory disturbance, and amyotrophy. Bogdanov et al. reported that 90% of patients had unilateral or bilateral sensory disturbances, while 79% of patients experienced weakness or wasting of the upper limbs [21].

Familial syringomyelia cases with autosomal dominant or recessive inheritance have been reported [22,23]. Chatel et al. suggested that the incidence of familial syringomyelia is approximately 2% [24]. However, a large-scale survey has not yet been conducted to determine the proportion of familial cases. In our study, familial syringomyelia comprised only 2 cases (0.6%) of patients with a reported family history. Although a potentially large number of patients who have been lost to follow-up affect the accuracy of the proportion of syringomyelia, familial syringomyelia cases are extremely rare.

This study has several limitations. Firstly, the prevalence of syringomyelia reported in this study was calculated using the estimated number of ambulatory patients. Cases of patients who did not receive ambulatory care in the past year were not evaluated. Therefore, the potential number of syringomyelia patients may be larger than that reported in this study. Secondly, this cross-sectional survey could not evaluate the entire clinical course of syringomyelia. The disease progression from asymptomatic to symptomatic is particularly unclear. The clinical course of idiopathic cases is also unclear. Further investigation is required to determine the most appropriate evaluations and treatments for these patients. Thirdly, the response rates in this study were 73% and 59% in the first and second stage surveys, respectively. Characteristics of patients whose cases were not reported in the second survey are unknown. The effect of this selection bias on our results is also unknown.

Finally, the definition of syringomyelia associated with spinal cord tumor has been changing, and peritumoral cysts have been differentiated from other distinct forms of syringomyelia. In this study, syringomyelia associated with spinal cord tumor was regarded as merely 1 type of syringomyelia.

Taken together, the findings of our survey can contribute to the development of healthcare services for syringomyelia patients. Knowledge of the characteristics of asymptomatic and symptomatic syringomyelia patients without surgical treatment can be useful for the optimization of those services. Further evaluations of the potential number of non-ambulatory syringomyelia patients should be performed to estimate the precise prevalence of syringomyelia.

In conclusion, we have investigated the epidemiology of syringomyelia in Japan. Asymptomatic and idiopathic syringomyelia cases are more common than was previously believed. The widespread availability of MRI scanners has potentially contributed to the early diagnosis of these cases.

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A functional variant in *ZNF512B* is associated with susceptibility to amyotrophic lateral sclerosis in Japanese

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Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease characterized by the selective loss of motor neurons. Several susceptibility genes for ALS have been reported; however, ALS etiology and pathogenesis remain largely unknown. To identify further ALS-susceptibility genes, we conducted a large-scale case–control association study using gene-based tag single-nucleotide polymorphisms (SNPs). A functional SNP (rs2275294) was found to be significantly associated with ALS through a stepwise screening approach (combined $P = 9.3 \times 10^{-10}$, odds ratio = 1.32). The SNP was located in an enhancer region of *ZNF512B*, a transcription factor of unknown biological function, and the susceptibility allele showed decreased activity and decreased binding to nuclear proteins. ZNF512B over-expression increased transforming growth factor-β (TGF-β) signaling, while knockdown had the opposite effect. ZNF512B expression was increased in the anterior horn motor neurons of the spinal cord of ALS patients when compared with controls. Our results strongly suggest that ZNF512B is an important positive regulator of TGF-β signaling and that decreased *ZNF512B* expression increases susceptibility to ALS.

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INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a heterogeneous motor neuron disease that results from selective death of motor neurons in the brain and spinal cord (1). The predominant clinical feature of ALS is progressive wasting and weakness of limb, bulbar and respiratory muscles. The mean survival of patients after onset of symptoms is 3–5 years. Its worldwide incidence and prevalence are 0.3–2.4 and 0.7–7.0 per 100 000 each year (2). The heritability of ALS is high, with twin studies estimating it at 0.61 and the unshared environment component at 0.39 (3).

Approximately 10% of ALS cases are familial (fALS), and the remaining 90% are sporadic (sALS). Genetic factors have been reported in ALS. Detailed information regarding ALS-related genes is available via amyotrophic lateral sclerosis online genetics database and the ALS mutation database (4,5). Most fALS is monogenic in origin. At least 15 fALS loci, under various modes of inheritance, have been identified by linkage studies, and pathogenic mutations have been described in 11 genes, *SOD1*, *NEFH*, *ALS2*, *DCTN1*, *VAPB*, *SETX*, *ANG*, *TARDBP*, *FUS*, *OPTN* and *DAO*, in fALS (6–19). Despite the abundance of genes and loci identified in fALS, mutations in these genes explain only a small minority of sALS (20).

Regarding susceptibility genes for sALS, >30 association studies based on the candidate-gene approach have been reported (21,22). Among them, *NEFH*, *APEX* and *ANG* have the most evidence; associations of these genes have been found in Caucasians (23–25) and replicated in several studies (7,22,26). However, many of the reported genes are still controversial. For example, the association of non-synonymous substitution (P413L) in the chromogranin B gene (*CHGB*) is reported in French, French-Canadian and Scandinavian ALS populations (27), but has not been found in a Dutch and another French population (28,29).

The genome-wide association study (GWAS) has identified five ALS-susceptibility genes (*FGGY*, *ITPR2*, *DPP6*, *KIFAP3* and *UNC13A*) and two loci (9p21.2 and 10q26.3) in Caucasian (30–35). These results are promising, but remain slightly controversial (36–39). The association of the 9p21.2 locus has been independently replicated in three studies (34,40,41), but is not found in all populations, including those from Japan and China (42). More studies are necessary to evaluate and confirm these previously reported ALS-susceptibility genes.

To identify novel susceptibility genes for ALS, we conducted a large-scale genetic association study in Japanese ALS patients using gene-based single-nucleotide polymorphisms (SNPs) (43). We identified a functional SNP that was significantly associated with ALS. The SNP was located in an enhancer region of *ZNF512B*, a previously uncharacterized transcription factor, and the susceptibility allele of the SNP had decreased enhancer activity for the *ZNF512B* promoter and decreased binding capacity to nuclear proteins. We found that in neuron cells, ZNF512B acts as a positive regulator of transforming growth factor-β (TGF-β) signaling, which is known to be neuroprotective and critical for maintenance and/or survival of neurons (44–46). We demonstrated the localization of ZNF512B in the spinal cord of ALS patients and it showed enhanced expression in motor neuron cells of the anterior horn when compared with controls.

RESULTS

Genome screening

We carried out a stepwise case–control association study (Supplementary Material, Fig. S1) as previously described (47–51). In stage 1 of the discovery series, 92 ALS and 233 control subjects were analyzed at 52 608 gene-based SNP loci selected from the JSNP database (43). Genotype information was successfully obtained for 48 939 SNPs on autosomal chromosomes passed after the quality control. Either the Chi-square test or Fisher's exact test was performed for three genetic models: dominant, recessive and allelic. Comparison of observed and expected distributions showed no evidence for inflation of the trend test statistics (inflation factor, lambda = 1.04; Supplementary Material, Fig. S2). Also, principal component analysis (52) in stage 1 and HapMap samples showed no evidence of population stratification between the case and control groups (Supplementary Material, Fig. S3). In stage 2 of the discovery series, 893 SNPs that showed *P*-values of ≤0.01 in stage 1 were genotyped for an additional 1087 subjects (362 ALS cases and 725 controls). Subsequently, 10 SNPs with *P*-values <0.001 were identified by the Chi-square test for the three models (Supplementary Material, Table S1).

Identification of genetic association between rs2275294 and ALS

We validated the association of these SNPs using independent subjects from Biobank Japan (sample set 1). In all, 249 ALS cases and 1030 controls were genotyped and validated the association in rs2275294 (allele model, $P = 1.8 \times 10^{-3}$). The SNP was then genotyped in an independent Japanese population consisting of 602 ALS cases and 2256 controls (sample set 2). Significant association was replicated in this population (allele model $P = 5.6 \times 10^{-5}$). The combined *P*-values for the stepwise association study calculated by the Mantel–Haenszel method and the joint analysis were 9.3×10^{-10} and 6.7×10^{-10}, respectively (Table 1). The combined *P*-values remained significant after Bonferroni correction ($9.3 \times 10^{-10} \times 52\,608 \times 3 = 1.47 \times 10^{-4}$). The *P*-values from the Mantel–Haenszel method and the joint analysis were very similar, supporting the fact that there is no hidden confounder in our population. The minor allele frequency (MAF) of rs2275294 in 744 samples of the Japan Biological Informatics Consortium (JBIC)-genotyping data deposited in the dbSNP database was similar to that of our controls (0.414).

Evaluation of rs2275294

We assessed the stratification using principal component analysis (52). The top six principal components were associated with case–control status. The association of rs2275294 with the top six principal components included as covariates (trend model $P = 0.00287$) was similar to that in stage 1 (trend model $P = 0.00246$), suggesting no stratification. Population stratification was also assessed by evaluating differences in population structure among all case and control sample sets using Wright's F statistics (53). There was no difference in the population structure among these groups (Supplementary Material, Table S2). Potential confounding factors were also

Table 1. Association of rs2275294 in *ZNF512B* with ALS

	No. of subjects		Risk allele frequency			Odds ratio (95% CI)
	Case	Control	Case	Control	*P*-value	
Discovery series	454	958	0.491	0.422	6.3×10^{-4}	1.32 (1.13–1.55)
Sample set 1	249	1030	0.512	0.434	1.8×10^{-3}	1.37 (1.12–1.66)
Sample set 2	602	2256	0.481	0.416	5.6×10^{-5}	1.30 (1.14–1.48)
Combined	1305	4244				
Meta-analysis[a]					9.3×10^{-10}	1.32 (1.21–1.44)
Joint analysis					6.7×10^{-10}	1.32 (1.21–1.44)

[a]By the Mantel–Haenszel method.

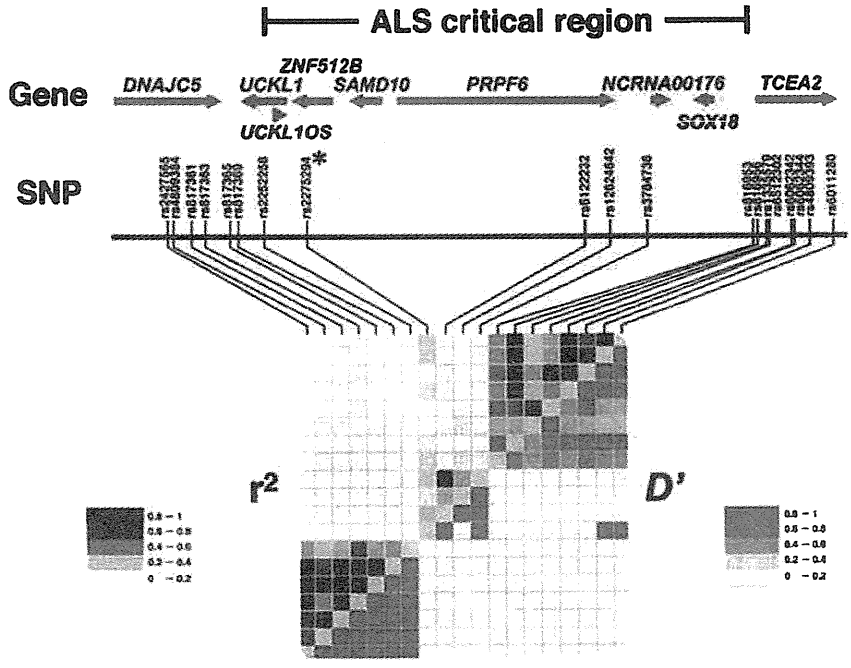

Figure 1. Genomic structure and linkage disequilibrium (LD) map in the ALS critical region. Top, an SNP map of a 111 kb genomic region containing *ZNF512B*. The orientation of each gene is indicated by a green arrow. An asterisk shows the landmark SNP. Bottom, an LD map as measured by D' (lower right triangle) and r^2 (upper left triangle).

examined and no significant differences in age and gender distribution were found among rs2275294 genotyped. The associations with rs2275294 were significant in two sample sets ($P = 4.1 \times 10^{-3}$ and 1.4×10^{-4}), even after adjusting for age and gender in a logistic regression analysis.

Genome analysis of the ALS critical region containing rs2275294

We constructed a linkage disequilibrium (LD) map around rs2275294 on the basis of the genotyping data for Japanese subjects used in HapMap (HapMap JPT). Because rs2275294 was unmapped in the HapMap data, we genotyped the SNP for the HapMap JPT samples and integrated the data with the HapMap JPT data. We found that rs2275294 was in strong LD with the two SNPs rs6122232 and rs3764736 ($D' > 0.85$). Subsequently, the critical region could be confined to a 111 kb interval flanked by rs2252258 and rs816953 on chromosome 20q13.33 (Fig. 1). This region included four genes (*ZNF512B*, *SAMD10*, *PRPF6* and *SOX18*) and a part of *UCKL1*, as well as two non-protein-coding RNAs (*UCKL1OS* and *NCRNA00176*). In order to identify a more significantly associated SNP, we searched for SNPs in each gene by re-sequencing genomic DNA of 48 ALS subjects. A total of 24 SNPs were identified and their level of association was examined using 455 cases and 452 controls, but rs2275294 remained the most significantly associated (Supplementary Material, Table S3).

Functional analysis of rs2275294

To gain insight into the biological significance of rs2275294, luciferase reporter plasmids corresponding to a genomic

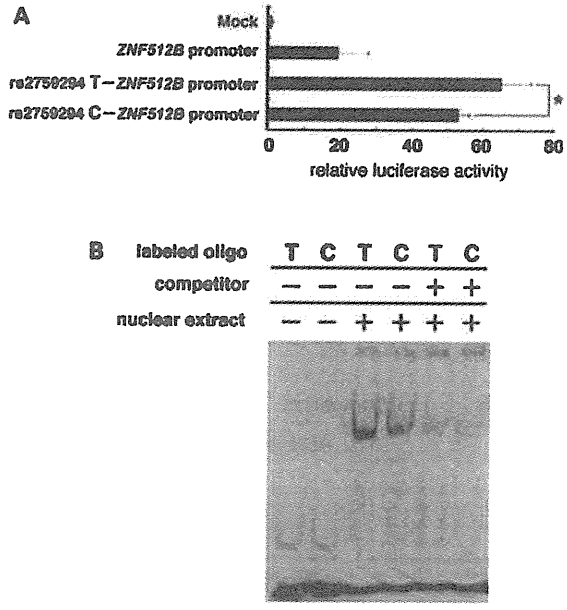

Figure 2. Functional analysis for rs2275294 in *ZNF512B*. (A) Difference in the enhancer activity of genomic DNA segments containing rs2275294. Luciferase assay in SK_N_Be(2)C cells. Enhancer activity was lower in the ALS-susceptibility allele (C allele). *ZNF512B* promoter:native promoter (nts −820 to −74) of *ZNF512B*. Data represent the mean ± SEM ($n = 6$). *$P < 0.01$ (Student's t-test). (B) Difference in binding of nuclear proteins to a cis-element containing rs2275294. An EMSA using nuclear extracts from SK_N_AS cells. The specific band was weaker in the ALS-susceptibility allele (C allele).

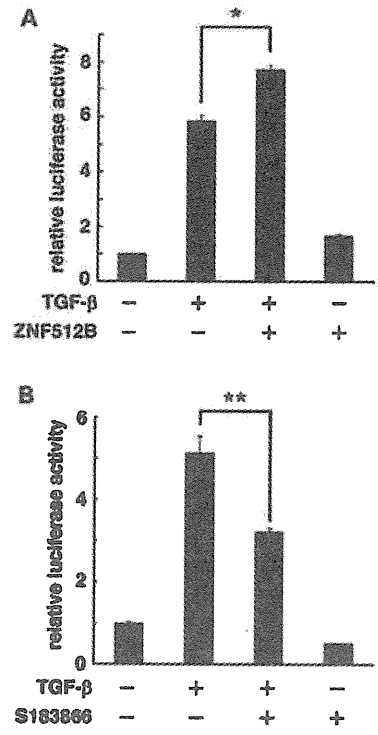

Figure 3. ZNF512B is a positive regulator of the TGF-β signal. (A) Luciferase assay using SBE4-luciferase. ZNF512B trans-activated the TGF-β-induced SMAD transcriptional activity in the SK_N_AS cell line (*$P < 0.0005$). (B) S183866, a *ZNF512B*-targeting siRNA oligonucleotide repressed the TGF-β-dependent SBE4-luciferase activity (**$P < 0.005$).

DNA fragment containing rs2275294 were constructed and a luciferase assay using the human neuroblastoma cell line SK_N_Be(2)C was performed. Constructs containing the ALS-susceptibility allele (C allele) of rs2275294 showed lower enhancer activity than those containing the non-susceptibility allele, indicating that the SNP affects the *ZNF512B* transcription level (Fig. 2A). We then examined the allelic difference in the binding of genomic DNA containing rs2275294 to nuclear proteins by the electrophoretic mobility shift assay (EMSA). The DNA–protein complex from the C allele showed weaker binding (Fig. 2B). Thus, it is feasible that the presence of the susceptibility allele leads to lower *ZNF512B* levels as a consequence of decreased enhancer activity.

ZNF512B is a positive regulator in the TGF-β signaling pathway

Proteomics analysis has suggested that ZNF512B functions as a regulator of the TGF-β signaling pathway (54). We examined the effect of ZNF512B on TGF-β signaling using the TGF-β-dependent SMAD2/3-specific luciferase assay (55) in a HepG2 cell (data not shown). SMAD2/3-mediated reporter activity after TGF-β stimulation was enhanced by ZNF512B over-expression. The TGF-β-dependent reporter activity was activated by ZNF512B over-expression in a neuroblastoma cell line SK_N_AS (Fig. 3A) and a glioblastoma cell line U87MG (Supplementary Material, Fig. S4). Next, we knocked down expression of the endogenous *ZNF512B* in SK_N_AS by using the short-interfering RNA (siRNA) technique. Real time polymerase chain reaction (PCR) showed that *ZNF512B* siRNA significantly reduced *ZNF512B* transcription, and TGF-β-dependent reporter activity was repressed by the siRNA (Fig. 3B).

ZNF512B expression in the spinal cord of ALS

The localization of ZNF512B in the spinal cord of ALS patients was investigated by immuno-histochemical studies. The immuno-reactivity for an anti-ZNF512B polyclonal antibody was intense in motor neuron cells in the anterior horn of the spinal cords of ALS patients, while it was barely detectable in those of controls (Fig. 4A–D). Glial cells in the anterior horn did not show ZNF512B immuno-reactivity.

DISCUSSION

By a large-scale case–control association study using gene-based SNPs and enrolling a total of more than 5500 subjects, we identified *ZNF512B* at chromosome 20q13.33 as a new susceptibility gene for ALS. rs2275294 in *ZNF512B* had

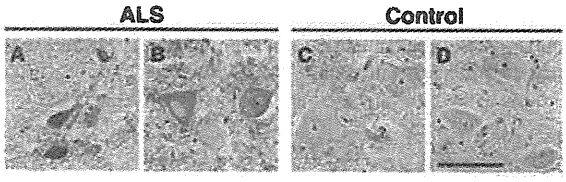

Figure 4. Immuno-histochemical localization of ZNF512B in the spinal cord of ALS patients (A and B) and controls (C and D). The ZNF512B immuno-reactivity was intense in motor neuron cells in the anterior horn of ALS patients, while it was hardly detectable in those of controls. Glial cells in the anterior horn did not show ZNF512B immuno-reactivity. Scale bar, 100 μm.

significant association that satisfied a genome-wide significance level ($P = 9.3 \times 10^{-10}$). rs2275294 affected *ZNF512B* transcription *in vitro*, and the ALS-susceptibility allele (C allele) showed lower enhancer activity for the *ZNF512B* promoter. Therefore, *ZNF512B* is presumably lower in those who have the susceptibility allele than in those who have the non-susceptibility allele. *ZNF512B* over-expression enhanced TGF-β signaling, while its knockdown decreased the signal. Our findings suggest that ZNF512B is an important positive regulator of TGF-β and that lowered ZNF512B expression is implicated in the pathogenesis of ALS susceptibility via decreased TGF-β signal.

In this study, we screened the genic regions using >52 000 gene-based SNPs from the JSNP database. The number of SNPs and their coverage are not sufficient to screen the entire genome. Our study must have many false negatives. Current commercial GWAS platforms are considered superior to ours in terms of the study power and the coverage of SNPs in the human genome. In contrast, the false-positive association of rs2275294 is unlikely. The inflation factor was low and principal component analysis showed no evidence of population stratification. We validated the association in independent Japanese panels. The statistical significance of the association for the combined *P*-values by two different methods fulfilled criteria of the genome-wide significance level. The results of the two analyses were very similar, which further shows that a hidden confounder in our population is unlikely. In addition, there was no difference in the population structures among the case–control sets by Wright's F statistics (53) throughout the study. The MAF of rs2275294 in 744 Japanese samples deposited in dbSNP is similar to that of our controls (0.414).

In spite of its very significant association in our study, rs2275294 in *ZNF512B* has not been found in the previous GWASs. Several explanations can be considered. The main reason is that rs2275294 was not included in the platforms of the previous GWASs. Only 15 SNPs in Illumina 610K SNP Array were mapped to the 111 kb genomic region (1 SNP/7.4 kb) corresponding to the ALS critical region we determined. Also, only 10 SNPs in Affymetrix SNP Array 6.0 were mapped to the genomic region (1 SNP/11.1 kb). In addition, rs2275294 is not even mapped in the HapMap JPT database, nor included in the CEU and YRI HapMap data sets. In the Illumina and Affymetrix SNP arrays, the numbers of SNPs in the *ZNF512B* locus are only two and one, respectively. Their coverages of *ZNF512B*-SNPs in the ALS critical region were very low, 2/15 (13%) and 1/10 (10%), respectively. The low coverage of the region might have led to the false-negative association in the previous GWASs. No SNP was in strong LD ($r^2 > 0.8$) with rs2275294 in CHB-JPT, CEU and YRI in the 1000 Genomes data (Supplementary Material, Table S4). Hence, we speculate that rs2275294 has been identified by virtue of our platform. Still another explanation is the ethnic difference of ALS susceptibility.

A number of GWASs in ALS have been performed recently. They report the identification of five candidate genes and two candidate loci (30–35). Among them, only five gene loci (*DPP6, ITPR2, FLJ10986, KIFAP3* and *UNC13A*) were included in our platform. We checked 16 SNPs in *DPP6*, 23 in *ITPR2*, 2 in *FLJ10986*, 9 in *KIFAP3* and 4 in *UNC13A*; however, their associations were not replicated in our study (Supplementary Material, Table S5). The small number of samples and the low coverage of SNPs in our platform may have resulted in false-negative association. Ethnic differences may be another reason for no replication. The 9p21.2 SNP that has been reported in the previous study (42) was not included in the present study. The tested SNPs for previous associations were negative, but no evidence can be provided for the chromosome 9p21.2 locus. Because the powers of Japanese and Chinese were only 0.37 and 0.11, respectively (42), the negative association may be due to a lack of power in the study. More extensive association studies using larger panels of Japanese samples will be required to conclude the associations between previous candidate genes and ALS.

ZNF512B was originally identified as a *KIAA1196* in the course of the Kazusa Human cDNA project (56). The *ZNF512B* cDNA is 5919 bp long and encodes an 893 amino-acid protein that is ubiquitously expressed in various tissues, including the brain and spinal cord (56). Our immunohistochemical studies confirmed its localization in the spinal cord. The ZNF512B protein showed no significant homology with any proteins in the public database. It contains six C2H2-type zinc finger domains and is predicted to act as a transcription factor. The ALS-susceptibility SNP rs2275294 was localized to intron 12 of *ZNF512B*. We have demonstrated that the genomic region containing rs2275294 can act as an enhancer of the *ZNF512B* promoter and that the susceptibility allele of rs2275294 had reduced transcriptional activity, which was likely due to its decreased binding capacity to trans-factors. Further studies for the upstream factors of *ZNF512B* are necessary to clarify the molecular pathogenesis of ALS related to *ZNF512B*.

We showed that ZNF512B is a positive regulator of the canonical TGF-β signaling pathway through SMAD2/3. TGF-β signal is essential for the survival of neurons (44–46). Upregulation of PAI-1 by SMAD3-dependent induction in astrocytes mediates the neuroprotective activity of TGF-β against NMDA receptor-mediated excitotoxicity (57). TGF-β signal has been implicated in the pathogenesis of ALS. Plasma TGF-β1 level is significantly increased in ALS patients compared with healthy controls, and there is a significant positive correlation between TGF-β1 concentration in ALS patients and duration of their disease (58). A microarray analysis showed a 4.8-fold increased expression of *SMAD4* in sALS compared with neurologically normal controls (59).

Also, phosphorylated SMAD2/3 immuno-reactivity is increased in the remaining spinal motor neurons and glial cells in sporadic and familial ALSs, as well as in *Sod1* transgenic mice (60). These findings suggest that the TGF-β signal is increased in ALS.

Several studies have shown an association between duration of ALS and TGF-β levels. Houi *et al.* (58) found a positive correlation between the plasma concentration of TGF-β1 in ALS patients and the duration of disease. Another group reported that TGF-β1 concentrations in serum and cerebrospinal fluid did not differ between ALS patients and controls, but were higher in ALS patients with a terminal clinical status than in controls (61). These data suggest that TGF-β is increased in the motor neuron cells of ALS patients during the disease process. As ZNF512B is a critical enhancer of TGF-β signaling, its genetic association may be related to the progression of the disease rather than its onset.

We have demonstrated the localization of ZNF512B in the spinal cord of ALS patients, and that ZNF512B expression in the motor neurons of ALS patients was significantly increased compared with that of controls (Fig. 4). It is biologically plausible that ZNF512B is a positive regulator (co-activator) of neuroprotective TGF-β signaling (Fig. 3) and may act as a protector against ALS. Taken together with the results of luciferase assay and EMSA that showed allelic differences in *ZNF512B* expression level (Fig. 2), a patient harboring the susceptibility allele would have decreased *ZNF512B* expression level compared with a patient harboring non-susceptibility alleles. The decreased *ZNF512B* enhancer activity by the susceptibility allele leads to insufficient increase in ZNF512B, which leads to insufficient increase in the TGF-β signal that results in decreased potential for survival and/or recovery of motor neurons. The discovery of this ALS-susceptibility gene and its pathway should shed light on ALS pathogenesis and facilitate development of targeted therapies.

MATERIALS AND METHODS

Subjects

A total of 1305 ALS patients diagnosed as having probable, probable and laboratory-supported, or definite ALS according to the El Escorial revised criteria (62) were included in the study. All subjects were unrelated Japanese individuals. We obtained a total of 703 DNA samples from the Biobank Japan project (63). All patients were screened for mutations in *SOD1*, *TARDBP* and *ANG* and none was detected. The mean age of cases was 60.8 years (range: 28–82 years), and 66.1% were male. 74.4% had a spinal onset, 19.6% a bulbar one and 6% a multiple and the others. We obtained a total of 602 DNA samples from the Japanese Consortium for Amyotrophic Lateral Sclerosis Research (JaCALS), Jichi Medical University and The University of Tokyo. The mean age was 61.5 years (range: 27–89 years), and 62.0% were male. 70.4% of the patients had a spinal onset and the remaining had a bulbar one. We recruited 4244 controls through several medical institutes in Japan. Their mean age was 66.8 years (range: 18–98 years), and 48.0% were male. All controls had negative medical and family histories for neurodegenerative disorders. Written informed consent was obtained from all the subjects. The ethical committees at the participating institutions approved this project.

SNP genotyping

Using standard protocols, genomic DNA was extracted from the peripheral blood leukocytes. SNPs were genotyped using the multiplex PCR-based invader assay (Third Wave Technologies) as described previously (64). A total of 52 608 gene-based SNPs were selected from the JSNP database on the basis of the haplotype block structure reported previously (43,65). We calculated the total number of independent SNPs in this study to be 43 052 (the SNPs in LD: $r^2 > 0.80$ were considered as one SNP). We checked the cryptic relatedness for each pair of samples by identity-by-state by estimating the average number of shared alleles between two individuals (V_I) using 48 884 autosomal SNPs. Six individuals in controls were related ($V_I > 1.65$). They were excluded from the analysis. A stepwise screening method was adopted to increase the statistical power (66). In stage 1, 92 ALS and 233 control subjects were analyzed. We applied the SNP quality control filters of call rate of ≥ 0.95 in both cases and controls and *P*-value of Hardy–Weinberg equilibrium (HWE) test of $\geq 1.0 \times 10^{-2}$ in controls. A total of 48 939 SNPs on autosomal chromosomes passed the quality control filters and were analyzed for the association. The data of this study are available at the JSNP database (http://snp.ims.u-tokyo.ac.jp/). Among the SNPs analyzed in stage 1, 893 SNPs showing the smallest *P*-values (0.01 or smaller) were selected for stage 2. Three models (i.e. allelic, dominant and recessive) were tested for the association. Since these three models are not independent, 893 SNPs were isolated. In stage 2, we genotyped an additional 1087 subjects consisting of 362 ALS cases and 725 controls. Stage 1 and stage 2 were defined as the discovery series of this research and the following sample sets were defined as sample set 1 and sample set 2.

SNP discovery

Appropriate genome sequences were extracted from the UCSC Genome Bioinformatics website. The critical region contained five genes (*ZNF512B, SAMD10, PRPF6, SOX18* and part of *UCKL1*) and two non-protein-coding RNAs (*UCKL1OS* and *NCRNA00176*). We defined the exon–intron boundaries of each gene and designed PCR primer sets for the critical region except for repetitive sequence regions. Each PCR was performed with 5 ng of mixed genomic DNA derived from three ALS subjects; 16 mixed samples were amplified in the GeneAmp PCR system 9700 (PE Applied Biosystems) under the following conditions: initial denaturation at 95°C for 2 min, followed by 35 cycles of denaturation at 96°C for 30 s, annealing at 60–65°C for 30 s, extension at 72°C for 2 min and post-extension at 72°C for 7 min. PCR products served as templates for direct sequencing by the fluorescent dye-terminator cycle sequencing method.

Statistical analysis

For general statistical analyses, we used R statistical environment version 2.6.1 and programs created by our group. The Chi-square test or Fisher's exact test was applied to a two-by-two contingency table in three genetic models: an allele frequency model, a dominant-effect model and a recessive-effect model. Principal component analysis was performed using the smartpca program (52). We calculated the association in case–control status of stage 1 by using a twstats of EIGENSOFT (52). The top six principal components were associated with case–control status. Genotype data from the HapMap project were used (67) to estimate the population structure. The significance of stratification was determined using the Wright method (53). The Mantel–Haenszel method was used for meta-analysis. An automated laboratory system and bar-coding were employed to reduce clerical errors. The accuracy of our system has been guaranteed in data of the HapMap project (67). We checked HWE and personally retyped some SNPs from genome screening in duplicated samples. We also obtained age- and gender-adjusted odds ratios by logistic regression analysis by program R. Haploview 4.1 was used to infer the LD structure of the ALS critical region. An LD pattern was created based on the JPT HapMap data. Luciferase assay data were analyzed by Student's *t*-test.

Luciferase assay

We cloned DNA fragments containing rs2275294, nucleotides (nts) 190–208 of intron 12 of *ZNF512B*. The fragments for both alleles as three tandem copies were inserted into pGL3-Basic vector (Promega) upstream of its luciferase gene in 5′–>3′ orientation together with the *ZNF512B* core promoter of nts −820 to −74 of its 5′ flanking region. We transfected SK_N_Be(2)C cells with 400 ng of each reporter construct using FuGene 6 transfection reagent (Roche) together with 8 ng of pRL-TK vector (Promega) as a control. After 24 h, the cells were lysed in a passive lysis buffer and luciferase activities were measured using Dual-Luciferase Reporter Assay System (Toyo Ink). The entire coding sequence of ZNF512B was cloned into pcDNA3.1, which had a Myc-tag sequence. We also co-transfected with SBE4 (four copies of Smad Binding Element) luciferase reporter vector (55)/Myc-tagged ZNF512B or SBE_4-luciferase reporter vector/Myc-tagged pcDNA3.1, and pRL-TK vector using Trans-IT LT reagent (TAKARA Bio). After 24 h, we treated the cells with 10 ng/ml of TGF-β for 24 h. The cells were lysed in a passive lysis buffer and luciferase activities were measured using Dual-Luciferase Reporter Assay System (Toyo Ink).

Electrophoretic mobility shift assay

A nuclear extract from SK_N_AS cells was prepared as previously described (68) and incubated with oligonucleotides (nts 184–203 of intron 12 of *ZNF512B*) that were labeled with digoxigenin-11-ddUTP using the Dig Gel Shift Kit (Roche). The reaction was carried out at a room temperature with an additional 1 mg/ml of poly[d(I-C)]. For the competition assay, the nuclear extract was pre-incubated with unlabeled oligonucleotides (200-fold molar excess) before adding digoxigenin-labeled oligonucleotide. The protein–DNA complex was separated on a non-denaturing 6% polyacrylamide gel in 0.25× Tris-borate-EDTA buffer. We transferred the gel to membrane and detected the signal with a chemiluminescent detection system (Roche) according to the manufacturer's instructions.

RNAi experiment

Double-strand stealth RNAi oligonucleotides (ZNF512B-S183866 for ZNF512B and negative universal control medium GC duplex for negative control) were purchased from Invitrogen. The RNAi oligonucleotides were transfected into a cell line using Lipofectamine RNAiMAX reagent according to the manufacturer's instructions (Invitrogen). After 24 h, we also transfected with SBE_4-luciferase reporter vector and pRL-TK vector. We treated the cells with TGF-β (10 ng/ml) for 24 h, collected the cells and measured luciferase activity using the Dual-Luciferase Reporter Assay System (Toyo Ink).

Immuno-histochemistry

Autopsy specimens of lumbar spinal cord were obtained from clinically and histopathologically diagnosed ALS patients (13 males and 9 females, age 41–79 years) and from neurologically normal patients (4 males and 3 females, age 42–76 years). The autopsy times in relation to death for the cases and controls (average ± SD) were 4.0 ± 2.8 h and 4.5 ± 5.2 h, respectively. 6-μm-thick sections were prepared from paraffin-embedded tissues. The sections were microwaved for 20 min in 50 mM citrate buffer (pH 6.0) and then treated with a TNB blocking buffer (PerkinElmer) before incubation with an anti-ZNF512B antibody (Santa Cruz Biotechnology, 1:200). The immuno-reactivity was detected using EnVision+ System-HRP (Dako). The sections were photographed with an optical microscope (BX51, Olympus).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement. None declared.

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