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CROSS-SECTIONAL AND LONGITUDINAL ANALYSIS OF AN OXIDATIVE STRESS BIOMARKER FOR SPINAL AND BULBAR MUSCULAR ATROPHY

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ABSTRACT: *Introduction:* Spinal and bulbar muscular atrophy (SBMA) is an adult-onset motor neuron disease caused by a CAG repeat expansion in the androgen receptor gene. The aim of this study was to verify whether urinary 8-hydroxydeoxyguanosine (8-OHdG), an oxidative stress marker, is a biomarker for SBMA. *Methods:* We measured the levels of urinary 8-OHdG in 33 genetically confirmed SBMA patients and 32 age-matched controls over a 24-month period at 6-month intervals. *Results:* Urinary 8-OHdG levels in SBMA patients were significantly elevated compared with those of controls and correlated well with motor function scores. During the follow-up period, urinary 8-OHdG levels increased and correlated with motor function at each time-point. In addition, urinary 8-OHdG levels at baseline were correlated with changes in the 6-minute walk test during 24 months. *Conclusions:* Urinary 8-OHdG is a biomarker for SBMA, reflecting the severity and possibly predicting the deterioration of motor function.

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Spinal and bulbar muscular atrophy (SBMA) is an hereditary, adult-onset, lower motor neuron disease. It is caused by aberrant elongation of a trinucleotide CAG repeat, which encodes a polyglutamine tract in the first exon of the androgen receptor (AR) gene.[1–3] The main symptoms are slowly progressive muscle weakness and atrophy of the bulbar, facial, and limb muscles. In general, the interval between the onset of weakness and death is 10–20 years.[4] An expanded CAG repeat has been identified as the cause of several neurodegenerative disorders, including SBMA, Huntington disease (HD), and several forms of spinocerebellar ataxia.[5] Although the causative genes show little homology other than the presence of a CAG repeat, these polyglutamine-mediated disorders share common pathways of molecular pathogenesis, such as transcriptional dysregulation, axonal transport defects, and mitochondrial dysfunction.[6,7]

Although animal studies have indicated the beneficial effects of androgen deprivation for SBMA, the results of clinical trials were inconclusive.[8–10] This is likely attributable to the difficulties in evaluating the disease-modifying effects of the tested drugs due to the slow progression of the neurological symptoms in SBMA. Therefore, appropriate surrogate endpoints are needed to facilitate the proof-of-concept of potential therapies for this disease. In this regard, it is important to identify biomarkers for SBMA that reflect the pathogenic processes and can be used as indicators of therapeutic efficacy. Although the nuclear accumulation of mutant AR protein in the scrotal skin has been shown to be a candidate histopathological biomarker, its practical use is limited due to the invasive nature of the procedure.[11,12] Conversely, non-invasive serum or urinary markers to evaluate disease severity have not been established for SBMA.

Oxidative stress resulting from mitochondrial dysfunction has been implicated in aging and neurodegeneration.[13] Pathogenic huntingtin, the causative protein of HD, induces oxidative stress through its direct association with mitochondria and downregulation of mitochondrial transcriptional regulators.[14,15] In a cellular model of SBMA, the expression of pathogenic AR is associated with depolarization of the mitochondrial membrane and an increase in the levels of reactive oxygen species (ROS), which is attenuated by the antioxidants coenzyme Q10 and idebenone.[16] ROS, such as hydroxyl radicals and H_2O_2, react with guanine residues in DNA and produce 8-hydroxydeoxyguanosine (8-OHdG), which is excreted in the urine, thereby serving as a biomarker of oxidative DNA damage.[17]

The aim of this study was to evaluate the validity of urinary 8-OHdG as a biomarker for SBMA. In particular, we investigated whether the urinary levels of 8-OHdG reflect the disease severity of SBMA patients. We also investigated the natural history of this parameter in order to determine whether it can be used to monitor disease progression.

METHODS

Participants. We studied 33 patients with SBMA and 32 age-matched, normal controls (Table 1). The inclusion criteria were: a clinical diagnosis of

Abbreviations: 6MWT, 6-minute walk test; 8-OHdG, 8-hydroxydeoxyguanosine; ALS, amyotrophic lateral sclerosis; ALSFRS-R, ALS Functional Rating Scale-revised; AR, androgen receptor; BMI, body mass index; HbA_{1c}, glycated hemoglobin; HD, Huntington disease; LNS, limb Norris score; NBS, Norris bulbar score; PCR, polymerase chain reaction; ROS, reactive oxygen species; SBMA, spinal and bulbar muscular atrophy

Key words: androgen receptor, biomarker, motor neuron, oxidative stress, spinal and bulbar muscular atrophy

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Table 1. Clinical and genetic features of the SBMA patients and controls.

Clinical and genetic features	SBMA	Control	*P*-value
Number of subjects	33	32	
Age at examination (years)	54.7 ± 10.1 (27–72)	52.8 ± 12.8 (36–74)	0.879
BMI*	22.8 ± 3.4 (15.1–27.6)	25.0 ± 6.7 (17.9–29.0)	0.465
CAG repeat length in AR gene	48.4 ± 3.9 (40–57)		
Duration from onset (years)	10.0 ± 6.8 (2–22)		
Hypertension	11 (34.4%)	12 (36.4%)	0.099
Hyperlipidemia	5 (15.6%)	7 (21.2%)	0.299
Diabetes	2 (6.3%)	3 (9.1%)	0.509
HbA_{1c} (%)	5.3 ± 0.5 (4.3–7.2)	5.3 ± 0.4 (4.8–6.1)	0.953

Data are shown as number, mean ± SD (range), or number (%). BMI, body mass index; AR, androgen receptor; HbA_{1c}, glycated hemoglobin.

SBMA with more than 1 motor symptom (i.e., muscle weakness, muscle atrophy, bulbar palsy, and hand tremor) and confirmation by genetic analysis; age between 25 and 75 years; and the ability to walk with or without a cane. We evaluated disease severity using the clinical scales for amyotrophic lateral sclerosis (ALS), such as the limb Norris score (LNS), the Norris bulbar score (NBS), and the ALS Functional Rating Scale-revised; (ALSFRS-R) score. We also measured motor function using clinical tests such as the 6-minute walk test (6MWT) and grip power.[18,19] We defined the onset of disease as the time when muscle weakness began, but not when tremor of the fingers appeared. The first examination was performed between June and November 2008. All patients were outpatients. In this longitudinal study, the SBMA patients were followed for 24 months at 6-month intervals. During follow-up, 3 patients did not complete the assessment due to difficulty in visiting the hospital regularly. This study conformed to the ethics guidelines for human genome/gene analysis research and epidemiological studies endorsed by the Japanese government. The institutional review board of the Nagoya University Graduate School of Medicine approved the study, and all SBMA patients and normal subjects gave informed consent for participation in the investigation.

Urinary 8-OHdG. Urine samples were obtained from each individual in the morning (9:00 a.m. to 12:00 noon) and were immediately stored at −20°C. Age-matched controls were selected from male examinees undergoing health check-ups between March and April 2009, at the Chuo Clinic, Nagoya. We included control subjects with ages between 25 and 75 years, and those who had any neurological symptoms or findings were excluded. We instructed the patients and controls to avoid strenuous exercise in the 24 h before providing the sample, because it was shown that exercise increases the urinary levels of 8-OHdG.[20,21] All samples from the same time-point were measured simultaneously. We de-identified the samples, and the urinary levels of 8-OHdG and creatinine were anonymously measured using an enzyme-linked immunosorbent assay (ELISA) with a specific monoclonal antibody (N45.1; Japan Institute for the Control of Aging, Furoi, Japan) at Mitsubishi Chemical Medicine Co. (Tokyo, Japan).[22] The urinary levels of 8-OHdG were normalized in relation to creatinine levels to adjust for the urine volume.

Genetic Analysis. Genomic DNA was extracted from the peripheral blood of SBMA patients using conventional techniques. Polymerase chain reaction (PCR) amplification of the CAG repeat in the AR gene was performed using a fluorescein-labeled forward primer (5′-TCCAGAATCTGTTCCAGAGCG TGC-3′) and an unlabeled reverse primer (5′-TGG CCTCGCTCAGGATGTCTTTAAG-3′). The detailed PCR conditions and measurement of the CAG-repeat size have been described elsewhere.[23]

Data Analysis. All data are presented as mean ± SD. Patient–control differences in categorical variables were assessed using the chi-square test. Longitudinal changes in the parameters were compared using a paired *t*-test, where the null hypothesis was that there was no change between baseline and the end of the 24-month follow-up. Correlations among the parameters were analyzed using Pearson correlation coefficients. We used multiple regression analysis for multiple classification analysis. $P < 0.05$ and correlation coefficients (r) > 0.4 were considered significant. Calculations were performed using SPSS version 14.0J (SPSS Japan, Tokyo, Japan) statistical software.

RESULTS

Clinical Backgrounds and Urinary 8-OHdG Levels in the SBMA Patients. The clinical characteristics of the study population are listed in Table 1. There were a total of 33 SBMA patients in the study. All participants were male and of Japanese nationality. There was no substantial difference between the median CAG repeat length in the SBMA population enrolled in this study and those reported previously.[4,9,24] Two of the 33 patients needed a cane

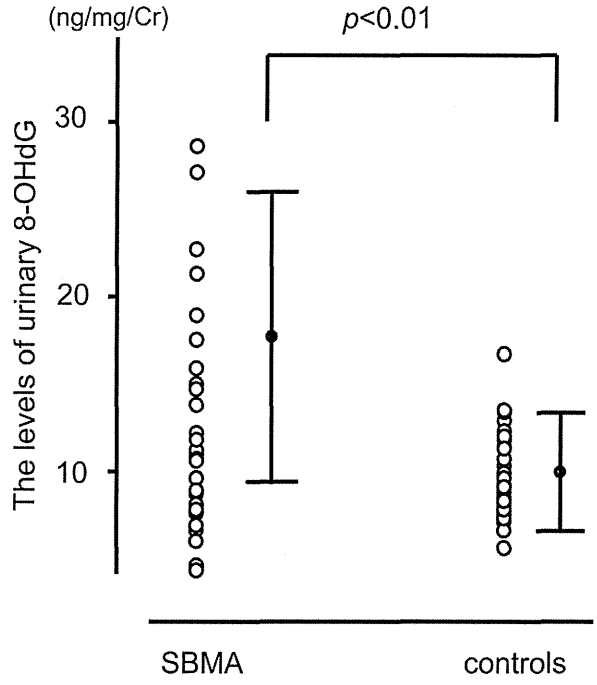

FIGURE 1. Urinary levels of 8-OHdG in SBMA patients and controls. Levels of urinary 8-OHdG in 33 SBMA patients were significantly higher than in the 32 age-matched controls ($P < 0.01$).

for ambulation. None were bedridden or wheelchair-bound. The prevalence of hypertension, hyperlipidemia, and diabetes mellitus as well as the value of glycated hemoglobin (HbA_{1c}) were equivalent between the SBMA patients and controls. Levels of urinary 8-OHdG in the SBMA patients (11.8 ± 6.2 ng/mg) were significantly higher than in the controls (9.7 ± 2.5 ng/mg) ($P < 0.01$) (Fig. 1). Two patients with especially high levels of urinary 8-OHdG (28.6 and 27.1 ng/mg) were at an advanced age (58 and 60 years, respectively) and had diabetes mellitus.

Correlation between Urinary 8-OHdG and Clinical Severity. The urinary 8-OHdG levels correlated well with all clinical scales and measures of motor function, namely ALSFRS-R, 6MWT, LNS, NBS, and grip power (Fig. 2 and Table 2). There was no correlation between urinary 8-OHdG levels and body mass index (BMI). Although age at examination was correlated with urinary 8-OHdG levels in the SBMA patients ($r = -0.401$), this relationship was not observed in the controls ($r = -0.124$). No relationship was found between the urinary 8-OHdG levels and the age at onset, the number of

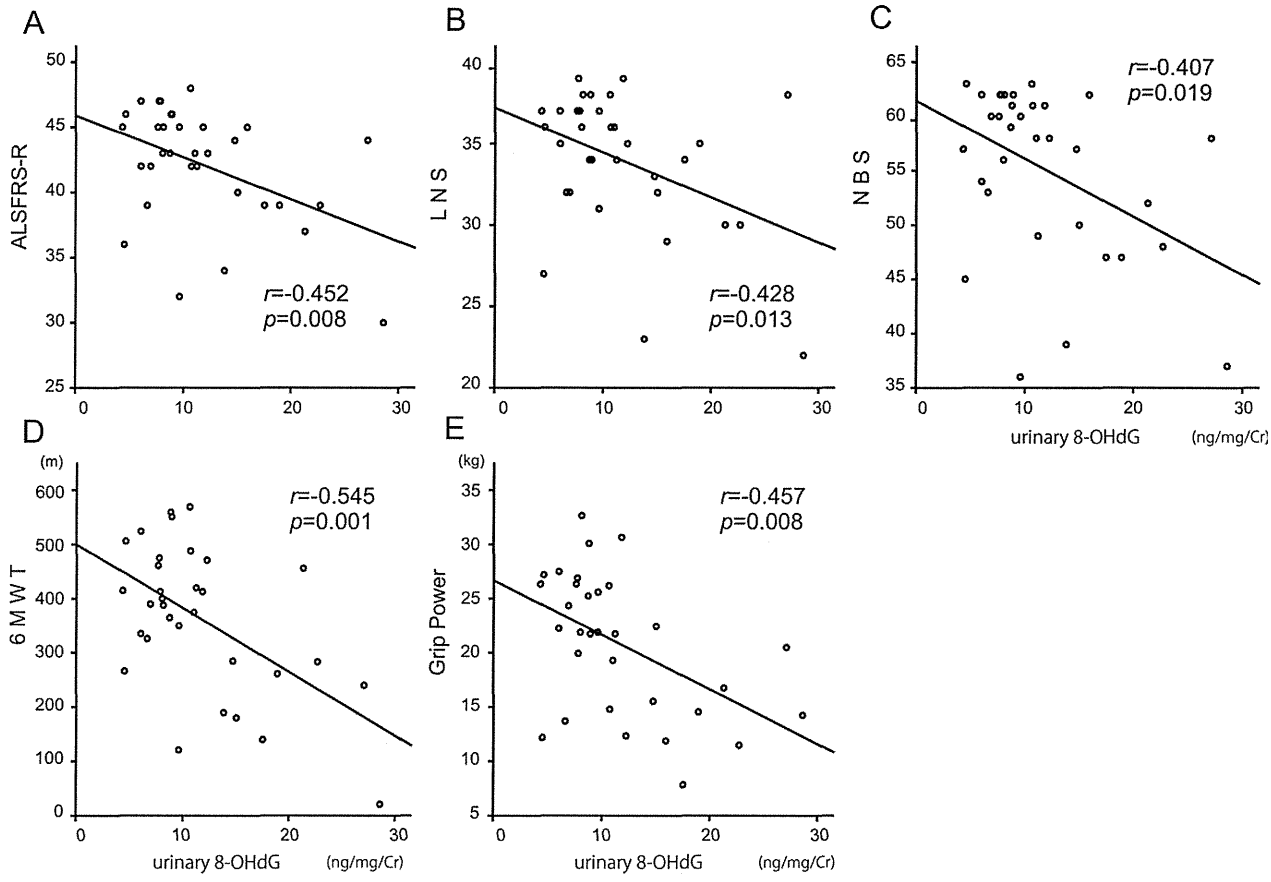

FIGURE 2. Correlation between levels of urinary 8-OHdG and motor function parameters. **(A–C)** Urinary 8-OHdG levels correlated with the scores of motor function scales, including the ALS Functional Rating Scale-revised; (ALSFRS-R) **(A)**, limb Norris score (LNS) **(B)**, and Norris bulbar score (NBS) **(C)** in the SBMA patients. **(D–E)** Similarly, there was a correlation between the levels of urinary 8-OHdG and the results of motor function tests, such as the 6-minute walk test (6MWT) **(D)** and grip power **(E)**.

Table 2. Correlation of urinary 8-OHdG and other clinical parameters in SBMA patients at baseline ($n = 33$).

	Measured value	Correlation with urinary 8-OHdG	*P*-value
ALSFRS-R	42.1 ± 4.4	$r = -0.452$	0.008
LNS	55.2 ± 7.8	$r = -0.428$	0.013
NBS	34.0 ± 4.2	$r = -0.407$	0.019
6MWT (m)	363.7 ± 135.9	$r = -0.545$	0.001
Grip power (kg)*	20.2 ± 7.3	$r = -0.457$	0.008
Age at examination (years)	54.7 ± 10.1	$r = -0.401$	0.021
Number of CAG repeats	48.4 ± 3.9	$r = -0.221$	0.223
Duration from onset (years)	10.0 ± 6.8	$r = -0.03$	0.891
Age at onset (years)	42.2 ± 12.4	$r = -0.271$	0.186
BMI	22.8 ± 3.4	$r = 0.186$	0.326
HbA_{1c} (%)	5.3 ± 0.5	$r = 0.309$	0.080

Data are presented as mean ± SD. ALSFRS-R, ALS Functional Rating Scale-revised; (normal score = 48); LNS, limb Norris score (normal score = 63); NBS, Norris bulbar score (normal score = 39); 6MWT, 6-minute walk test.
**Average of both hands.*

CAG repeats, or disease duration in the SBMA patients. In multiple regression analysis, clinical tests of general motor function, such as grip power and 6MWT, correlated with the urinary levels of 8-OHdG (grip power, $r = -0.512$; 6MWT, $r = -0.442$). There was no detectable correlation between urinary 8-OHdG levels and the other parameters, including HbA_{1c}.

Longitudinal Analysis of Urinary 8-OHdG levels in the SBMA Patients. Due to the withdrawal of 3 patients, we analyzed the remaining 30 patients in the longitudinal study. Urinary 8-OHdG levels showed a significant increase during the 24-month follow-up period ($P < 0.05$) (Table 3). The other clinical scales and measures of motor function also showed a significant increase during this period ($P < 0.05$) (Table 3). During the follow-up period, urinary 8-OHdG levels increased and were correlated with each measure of motor function at all time-points examined (Table 4). Next, we investigated whether the change in the levels of urinary 8-OHdG reflects the degree of disease progression. The percentage of change from baseline was used to analyze each parameter. The levels of urinary 8-OHdG at baseline correlated with the changes of 6MWT during 24 months, suggesting that the intensity of oxidative stress at baseline is associated with the prognosis for ambulatory capacity (Table 5). There was, however, no correlation between the change in urinary 8-OHdG levels and that of the clinical scores or measures (Table 5).

DISCUSSION

This study has shown that urinary 8-OHdG levels are significantly increased in patients with SBMA compared with controls. Moreover, the urinary 8-OHdG levels in SBMA patients correlated with the motor function scores in our cross-sectional study. Our results thus show that urinary 8-OHdG, an oxidative stress marker, is a biomarker that reflects disease severity in patients with SBMA. 8-OHdG is produced by the reaction between ROS and guanine residues in DNA. The ROS-mediated formation of mutated mtDNA has been implicated in the pathogenesis of age-related disorders such as neurodegenerative disorders, type II diabetes, cancer, and cardiac diseases.[25] Generally, the levels of urinary 8-OHdG are not strongly elevated by aging.[26] Our results, however, show that the urinary 8-OHdG levels correlated with age in SBMA patients. Given the strong correlation between the urinary 8-OHdG levels and the motor function scores, this may be due to the fact that the condition of aged SBMA patients is more severe than that of younger patients. In support of this view, multiple regression analysis showed that the urinary levels of 8-OHdG were correlated with motor function parameters, but not with the age of SBMA patients. Diabetes mellitus, a putative trigger of oxidative stress,[27] was found in the 2 SBMA patients who had elevated levels of urinary 8-OHdG. Nevertheless, there was no strong correlation between the levels of urinary 8-OHdG and HbA_{1c} levels.

Increased oxidative stress has been detected in various diseases, including neurological disorders.

Table 3. Changes in urinary 8-OHdG and other parameters in SBMA patients during 24-month follow-up ($n = 30$).

	0 month ($n = 30$)	6 months ($n = 30$)	12 months ($n = 30$)	18 months ($n = 30$)	24 months ($n = 30$)	Change in 24 months
Urinary 8-OHdG (ng/mg/Cr)	11.6 ± 6.5	16.9 ± 7.8	16.5 ± 7.8	18.8 ± 8.1	17.5 ± 7.9	6.0 ± 5.1*
ALSFRS-R	42.2 ± 4.2	42.4 ± 4.0	41.8 ± 4.0	41.0 ± 3.9	40.7 ± 3.8	−1.5 ± 1.9*
LNS	56.0 ± 6.9	56.0 ± 6.6	54.1 ± 7.7	52.9 ± 7.9	51.8 ± 7.8	−3.5 ± 3.9*
NBS	33.6 ± 4.4	33.9 ± 4.1	33.4 ± 4.3	33.0 ± 4.9	32.4 ± 4.6	−2.0 ± 2.4*
6MWT (m)	367.8 ± 136.4	357.8 ± 137.9	348.8 ± 142.3	337.7 ± 138.3	331.7 ± 132.3	−36.1 ± 2.5*
Grip power (kg)	21.0 ± 6.6	20.7 ± 6.6	20.2 ± 6.4	18.9 ± 7.2	18.9 ± 7.1	−1.9 ± 2.9†

Data are presented as mean ± SD. ALSFRS-R, ALS Functional Rating Scale-revised; LBS, limb Norris score; NBS, Norris bulbar score; 6MWT, 6-minute walk test.
**P < 0.001 for comparison between 0 month and 24 months (paired t-test).*
†P = 0.001 for comparison between 0 month and 24 months (paired t-test).

Table 4. Correlations between urinary 8-OHdG and other parameters in SBMA patients during 24-month follow-up.

	Correlation with urinary 8-OHdG			
	6 months ($n = 30$)	12 months ($n = 30$)	18 months ($n = 30$)	24 months ($n = 30$)
ALSFRS-R	$r = -0.454$	$r = -0.472$	$r = -0.586$	$r = -0.665$
	$P = 0.012$	$P = 0.008$	$P = 0.001$	$P < 0.001$
LNS	$r = -0.482$	$r = -0.408$	$r = -0.550$	$r = -0.610$
	$P = 0.007$	$P = 0.025$	$P = 0.002$	$P < 0.001$
NBS	$r = -0.390$	$r = -0.531$	$r = -0.550$	$r = -0.593$
	$P = 0.033$	$P = 0.003$	$P = 0.002$	$P = 0.001$
6MWT (m)	$r = -0.457$	$r = -0.372$	$r = -0.552$	$r = -0.542$
	$P = 0.011$	$P = 0.043$	$P = 0.002$	$P = 0.002$
Grip power (kg)	$r = -0.605$	$r = -0.409$	$r = -0.457$	$r = -0.508$
	$P < 0.001$	$P = 0.025$	$P = 0.011$	$P = 0.004$

ALSFRS-R, ALS Functional Rating Scale-revised; LNS, limb Norris score; NBS, Norris bulbar score; 6MWT, 6-minute walk test.

Recent studies have suggested that oxidative stress appears be a common process in a number of neurodegenerative diseases, such as ALS, Alzheimer disease, Parkinson disease, and HD.[28–32] The association between neurodegeneration and oxidative stress has been best described in Parkinson disease, autosomal recessive forms of which are caused by mutations in the genes *Parkin*, *DJ1*, and PTEN-induced putative kinasel (*PINK1*), which play a role in the degradation of abnormal mitochondria via autophagy.[33] Patients with polyglutamine diseases, including SBMA, have a relatively low mitochondrial DNA copy number in leukocytes, which is inversely correlated with the length of the CAG repeat in causative genes.[34,35] Abnormal mitochondria have been detected in a knock-in mouse model of SBMA.[16] The amino-terminal fragment of polyglutamine-expanded AR induces mitochondrial damage through the activation of c-Jun N-terminal kinase.[36] Pathogenic AR also reduces the mRNA levels of peroxisome proliferator–activated receptor γ coactivator-1beta (PGC-1β) as well as those of various antioxidant genes in cellular and animal models of SBMA.[16] The increased levels of 8-OHdG in our study confirm the implication of oxidative stress in SBMA.

Urinary 8-OHdG is considered to be a reliable biomarker of oxidative stress, because the excised 8-OHdG is excreted in the urine without compensation by anti-oxidative agents in the plasma. Thus, the measure of urinary 8-OHdG has gained attention as a biomarker of neurodegenerative disease. The urinary levels of 8-OHdG were shown to be correlated with disease severity in Parkinson disease and ALS.[37,38] In this study, the urinary levels of 8-OHdG correlated significantly with the scores of motor function and other functional parameters at all timepoints, suggesting that this parameter appears to be a biomarker that reflects the severity of SBMA.

There have been a limited number of studies on the chronological changes in oxidative stress markers in neurodegenerative diseases.[29] In our longitudinal analysis, we confirmed the significant increase in urinary levels of 8-OHdG, suggesting the accumulation of oxidative stress in SBMA patients during the observation period. Furthermore, levels of urinary 8-OHdG at baseline correlated with changes in the 6MWT during the 24 months of observation, indicating that increased oxidative stress is associated with a poor prognosis for ambulatory function in SBMA patients. In support of this view, chemical induction of oxidative stress was shown to exacerbate neurodegeneration in rodents.[39] However, baseline levels of urinary 8-OHdG did not correlate with changes of other functional measures over the 24 months of the present study. Neither were the changes of urinary 8-OHdG levels paralleled by those of the motor function parameters. This discrepancy may result from intrasubject fluctuations of the functional scores or the relatively short observation period of the study. Alternatively, the fact that the functional scales, but not the 6MWT, are vulnerable to patient and rater subjectivity may mask the correlation between levels of urinary 8-OHdG and changes in motor function scores.[40]

Validated biomarkers are key to the development of disease-modifying therapies for neurodegenerative disorders.[41] Although a number of candidate therapeutics have emerged from basic studies, most have failed to show positive results in clinical trials for neurodegenerative diseases. A lack of sensitive and reliable measures of pharmacological effects is one of the factors preventing successful translation of animal studies to the clinic. The use of biomarkers in exploratory-phase clinical trials may facilitate the selection of agents for further testing in confirmatory-phase trials and

Table 5. Correlation between changes in urinary 8-OHdG and other parameters ($n = 30$).

	8-OHdG (0 month)	Δ8-OHdG (0–6 months)	Δ8-OHdG (0–24 months)
ΔALSFRS-R*	$r = -0.70$	$r = 0.167$	$r = 0.173$
	($P = 0.713$)	($P = 0.379$)	($P = 0.389$)
ΔLNB*	$r = -0.288$	$r = 0.051$	$r = 0.081$
	($P = 0.123$)	($P = 0.787$)	($P = 0.671$)
ΔNBS*	$r = -0.113$	$r = 0.041$	$r = 0.032$
	($P = 0.551$)	($P = 0.830$)	($P = 0.865$)
Δ6MWT*	$r = -0.376$	$r = 0.102$	$r = 0.037$
	($P = 0.040$)	($P = 0.591$)	($P = 0.847$)
ΔGrip power*	$r = -0.050$	$r = -0.107$	$r = -0.108$
	($P = 0.793$)	($P = 0.575$)	($P = 0.570$)

ALSFRS-R, ALS Functional Rating Scale-revised; LNS, limb Norris score; NBS, Norris bulbar score; 6MWT, 6-minute walk test.
**Changes between 0 and 24 months.*

the stratification of those patients who will be most likely to benefit from the therapy.[42] The urinary levels of 8-OHdG have been used as an endpoint in clinical trials for neurological disorders.[29,43,44] Given that several drugs have been developed for SBMA, the plausibility of urinary 8-OHdG as a biomarker should also be examined in clinical trials.

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Longitudinal changes of outcome measures in spinal and bulbar muscular atrophy

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Spinal and bulbar muscular atrophy is an adult-onset, hereditary motor neuron disease caused by the expansion of a trinucleotide CAG repeat within the gene encoding the androgen receptor. To date, several agents have been shown to prevent or slow disease progression in animal models of this disease. For the translational research of these agents, it is necessary to perform the detailed analysis of natural history with quantitative outcome measures and to establish sensitive and validated disease-specific endpoints in the clinical trials. To this end, we performed a prospective observation of disease progression over 3 years in 34 genetically confirmed Japanese patients with spinal and bulbar muscular atrophy by using quantitative outcome measures, including functional and blood parameters. The baseline evaluation revealed that CAG repeat length in the androgen receptor gene correlated not only with the age of onset but also with the timing of substantial changes in activity of daily living. Multiple regression analyses indicated that the serum level of creatinine is the most useful blood parameter that reflects the severity of motor dysfunction in spinal and bulbar muscular atrophy. In 3-year prospective analyses, a slow but steady progression was affirmed in most of the outcome measures we examined. In the analyses using random coefficient models that summarize the individual data into a representative line, disease progression was not affected by CAG repeat length or onset age. These models showed large interindividual variation, which was also independent of the differences of CAG repeat size. Analyses using these models also demonstrated that the subtle neurological deficits at an early or preclinical stage were more likely to be detected by objective motor functional tests such as the 6-min walk test and grip power or serum creatinine levels than by functional rating scales, such as the revised amyotrophic lateral sclerosis functional rating scale or modified Norris scale. Categorization of the clinical phenotypes using factor analysis showed that upper limb function is closely related to bulbar function, but not to lower limb function at baseline, whereas the site of onset had no substantial effects on disease progression. These results suggest that patients with spinal and bulbar muscular atrophy show a slow but steady progression of motor dysfunction over time that is independent of CAG repeat length or clinical phenotype, and that objective outcome measures may be used to evaluate disease severity at an early stage of this disease.

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Keywords: spinal and bulbar muscular atrophy; natural history; biomarker; random coefficient linear regression model; CAG repeat

Abbreviations: ALSAQ-5 = five-item amyotrophic lateral sclerosis assessment questionnaire; ALSFRS-R = revised amyotrophic lateral sclerosis functional rating scale

Introduction

Spinal and bulbar muscular atrophy, also known as Kennedy's disease, is an adult-onset, hereditary motor neuron disease characterized by muscle atrophy, weakness, contraction fasciculation and bulbar involvement (Kennedy *et al.*, 1968; Sobue *et al.*, 1989; Sperfeld *et al.*, 2002). The progression of neurological deficits is usually slow in spinal and bulbar muscular atrophy, with the average interval between the onset of symptoms and death being ~20 years (Atsuta *et al.*, 2006). Life-threatening respiratory tract infections due to bulbar palsy often occur in an advanced stage of the disease, resulting in premature death. In blood profiles, the elevation of serum creatine kinase levels is a characteristic blood finding of spinal and bulbar muscular atrophy and is occasionally detectable many years prior to the onset of clinical symptoms (Sorarù *et al.*, 2008; Chahin and Sorenson, 2009; Rhodes *et al.*, 2009). In addition, patients often have non-neurological conditions, such as hyperlipidaemia and diabetes mellitus (Barkhaus *et al.*, 1982; Dejager *et al.*, 2002; Sinnreich *et al.*, 2004).

Spinal and bulbar muscular atrophy is caused by the expansion of a CAG repeat, encoding a polyglutamine tract, within the first exon of the androgen receptor gene (La Spada *et al.*, 1991). CAG repeat numbers range among 38–62 in patients with spinal and bulbar muscular atrophy, whereas normal individuals have 9–36 CAG repeats (La Spada *et al.*, 1991; Fischbeck *et al.*, 1997; Atsuta *et al.*, 2006). To date, nine polyglutamine diseases have been identified: Huntington's disease, dentatorubral–pallidoluysian atrophy, spinocerebellar ataxia 1, 2, 3, 6, 7 and 17, and spinal and bulbar muscular atrophy. Although the clinical features vary for each disorder, corresponding to the pathological distribution of neurodegeneration, the symptoms generally appear in mid-life and progressively deteriorate until death from fatal complications (La Spada and Taylor, 2003). Although several studies showed that CAG repeat size correlated with the age of onset in polyglutamine diseases, including spinal and bulbar muscular atrophy, considerable controversy surrounds whether the length of the CAG repeat influences the speed of disease progression (Atsuta *et al.*, 2006; Orr and Zoghbi, 2007; Walker, 2007).

The androgen-dependent accumulation of pathogenic androgen receptor proteins in the nucleus of lower motor neurons is thought to be crucial in inducing neuronal cell dysfunction and eventual degeneration, and underlie the gender dependency in the manifestation of the disease (Schmidt *et al.*, 2002; Katsuno *et al.*, 2012). In mouse models of spinal and bulbar muscular atrophy, surgical castration delays disease onset and reverses the neuromuscular phenotype (Katsuno *et al.*, 2002; Chevalier-Larsen *et al.*, 2004). Similar effects emerged when these mice are treated with leuprorelin, a luteinizing hormone-releasing hormone agonist that reduces testosterone release (Katsuno *et al.*, 2003). In a phase II clinical trial, leuprorelin suppressed the accumulation of pathogenic androgen receptor and slowed the deterioration of motor function in patients with spinal and bulbar muscular atrophy (Banno *et al.*, 2006, 2009). However, in a large-scale phase III randomized controlled trial, the efficacy of leuprorelin on clinical endpoints was not clearly demonstrated, although it was suggested that early intervention might be beneficial (Katsuno *et al.*, 2010). Similarly, the 5α-reductase inhibitor dutasteride, which blocks the conversion of testosterone to dihydrotestosterone, did not show a significant effect in a phase II clinical trial (Fernández-Rhodes *et al.*, 2011). These results appear to be partly attributable to the characteristics of spinal and bulbar muscular atrophy, such as its notably slow progression, and the lack of established outcome measures for the evaluation of therapeutic efficacy. In slowly progressive neurodegenerative diseases, the efficacy of a disease-modifying therapy is difficult to detect in short-term trials (Rascol, 2009). To facilitate the development of disease-modifying therapy for spinal and bulbar muscular atrophy, it is necessary to have a detailed description of the natural history of the disease to design appropriate clinical trials and to evaluate drug efficacy in patients. However, there are limited published data on the natural history of spinal and bulbar muscular atrophy, particularly for objective and quantitative measures, mainly because of its rarity (Rhodes *et al.*, 2009; Hashizume *et al.*, 2012).

In the present study, we performed prospective, quantitative analyses of the natural course of the disease in 34 genetically-confirmed Japanese patients with spinal and bulbar muscular atrophy over 3 years. Our results demonstrated longitudinal progression of quantitative outcome measures such as motor functional scales and tests as well as serum levels of creatinine. Although CAG repeat size in the androgen receptor gene correlates with activity of daily living milestones, i.e. hand tremor, muscular weakness, requirement of a handrail, dysarthria and dysphagia; its effect on disease progression rate was not demonstrated using random coefficient linear regression models. The analyses using these models also revealed that objective motor function tests and the serum levels of creatinine, but not subjective functional scales, are sensitive measures to detect neurological deficits at an early or preclinical stage of spinal and bulbar muscular atrophy. In addition, our findings indicated that upper limb function closely related to bulbar function, but not to lower limb function in spinal and bulbar muscular atrophy, whereas the site of onset had no substantial effect on disease progression.

Materials and methods

Patients

A total of 34 male patients with a diagnosis of spinal and bulbar muscular atrophy were recruited and followed with no specific treatment. The inclusion criteria were as follows: (i) genetically confirmed male Japanese patients with spinal and bulbar muscular atrophy with more than one of the following symptoms: muscle weakness, muscle

atrophy or bulbar palsy; and (ii) patients who were 25–75 years old at the time of informed consent. The patients were excluded if they met any of the following criteria: (i) unable to attend periodic follow-up visits; (ii) unable to stand upright for 6 min without assistance; (iii) tachycardia (>120 beats/min) or uncontrolled hypertension (>180/100 mmHg); (iv) experienced angina pectoris or myocardial infarction; (v) severe complications, such as malignancy and heart failure; (vi) severe bulbar palsy or other neurological complications; (vii) medical history of allergy to barium; (viii) taken hormonal agents within 48 weeks before informed consent; (ix) castrated; and (x) participated in any other clinical trials before informed consent. All of the patients were followed in Nagoya University Hospital. The data were collected between January 2007 and February 2011.

This study adhered to the ethics guidelines for human genome/gene analysis research and those for epidemiological studies endorsed by the Japanese government. The Ethics Committee of Nagoya University Graduate School of Medicine approved the study, and all participants gave their written informed consent.

Activity of daily living milestones

The initial symptoms and onset of nine activities of daily living milestones were assessed to evaluate the clinical course of the disease as previously described (Atsuta *et al.*, 2006). The activity of daily living milestones were defined as follows: hand tremor (patient awareness of hand tremor), muscular weakness (initial patient awareness of muscular weakness in any part of the body), requirement of a handrail (patient was unable to ascend stairs without the use of a handrail), dysarthria (patient was unable to articulate properly and had intelligible speech only with repetition), dysphagia (patient choked occasionally at meals), use of a cane (patient used a cane constantly when away from home), use of a wheelchair (patient used a wheelchair when away from home) and development of pneumonia (patient developed pneumonia that required in-hospital care). We assessed the age at which the activity of daily living milestones first occurred by direct interview at the first evaluation, since the inter-rater reliability of this method has been validated (Atsuta *et al.*, 2006). The activity of daily living milestones that occurred before the initial examination were checked and analysed. In this study, the age at which muscular weakness first occurred was defined as age at disease onset.

Outcome measures

The outcome measures of this study consist of functional and blood parameters, which were measured every 6 months during the 3-year follow-up. We used the following functional parameters in the present study: the revised amyotrophic lateral sclerosis functional rating scale (ALSFRS-R), modified Norris scale (Limb Norris score and Norris Bulbar score), modified quantitative myasthenia gravis score, grip power, 6-min walk test, five-item amyotrophic lateral sclerosis assessment questionnaire (ALSAQ-5), timed walking test (15 ft) and pharyngeal barium residue.

The ALSFRS is a validated questionnaire-based scale that measures physical function in patients with amyotrophic lateral sclerosis performing activity of daily living [The ALS CNTF treatment study (ACTS) phase I–II Study Group, 1996]. The revised version of this scale, ALSFRS-R, was generated to improve the disproportion of weighting to the limbs and bulbar system compared with respiratory dysfunction. The ALSFRS-R was translated into Japanese and validated (Ohashi *et al.*, 2001). The ALSFRS-R is divided into five domains: bulbar-related (three items: speech, salivation and swallowing), upper limb-related (two items: handwriting, and cutting food and handling utensils), trunk-related (two items: dressing and hygiene, and turning in bed and adjusting bed clothing), lower limb-related (two items: walking and climbing stairs) and respiration-related (three items: dyspnoea, orthopnoea and respiratory insufficiency).

The modified Norris scale is another rating scale for amyotrophic lateral sclerosis, which consists of two parts: the Limb Norris Score and the Norris Bulbar Score. The former has 21 items to evaluate limb function and the latter has 13 items to assess bulbar function. Each item is rated in four ordinal categories, and thus the possible best score is 63 and 39, respectively. The original version was translated into Japanese and validated (Oda *et al.*, 1996).

The quantitative myasthenia gravis score is an objective measure to detect fatigue of enduring muscle power that was originally designed for myasthenia gravis (Besinger *et al.*, 1983). We used a part of the quantitative myasthenia gravis score that measures the muscle power of the extremities and neck flexion as a modified quantitative myasthenia gravis score. Therefore, the best possible score is 0 and the worst possible score is 15. Although this scale has not been previously validated in patients with spinal and bulbar muscular atrophy, the contents of the modified quantitative myasthenia gravis score are suitable for the evaluation of spinal and bulbar muscular atrophy symptoms, and we thus considered them to be applicable to this disease (Katsuno *et al.*, 2010).

Grip power was measured using an electronic hand dynamometer. The patients were instructed to keep their elbows at 90°, their forearms in neutral rotation and their wrists not flexed or pronated. The measurements were performed twice on each side and the larger value was adopted as the grip power on each side. Grip power has been recommended as an acceptable endpoint for amyotrophic lateral sclerosis clinical trials (James *et al.*, 1997).

The 6-min walk test is a popular clinical test that has been used to assess the functional capacity of gait. The distance travelled during 6 min, i.e. the 6-min walk distance is a parameter that evaluates the global and integrated responses of all the systems involved in walking, including the neuromuscular, pulmonary and cardiovascular systems. The validity of this test has been verified in various neuromuscular disorders, including spinal and bulbar muscular atrophy (Takeuchi *et al.*, 2008; Montes *et al.*, 2010).

The timed walking test measures the time required to walk 15 feet. It has been recommended as a test for amyotrophic lateral sclerosis clinical trials (James *et al.*, 1997).

The ALSAQ-5 is a subjective health measure that was designed to evaluate the quality of life in patients with amyotrophic lateral sclerosis. This questionnaire was developed from the original version (ALSAQ-40) using item reduction (Jenkinson *et al.*, 1999; Jenkinson and Fitzpatrick, 2001). The validity of the Japanese version of the ALSAQ-40 has been confirmed (Yamaguchi *et al.*, 2004).

Pharyngeal barium residue was examined to evaluate swallowing function. In the videofluorography examinations, the patients were instructed to swallow 3 ml of 40% w/v barium sulphate twice while standing. Pharyngeal barium residue was measured for the first 3 ml swallowed because the first residue directly affects the second one. Pharyngeal barium residue after initial swallowing was measured by two masked independent investigators according to standard procedures using a semiquantitative scale: 0, 2, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100% (Logemann *et al.*, 1989, 2000; Katsuno *et al.*, 2010). Previous studies have shown high intra- and inter-rater reliability for the measurement of videofluorographic swallowing, although little is known about the reproducibility of this parameter (Kuhlemeier *et al.*, 1998).

Blood sampling was performed at an outpatient clinic without any fasting.

Longitudinal analyses

The data of the patients who were evaluated only once during the follow-up period were eliminated from the longitudinal analyses. Follow-up data were defined as the values of the last evaluation. The differences between baseline and follow-up were analysed using a paired *t*-test. The disease progression rate per year was defined as the difference between the baseline and follow-up data divided by the follow-up period (years).

Genetic analysis

Genomic DNA was extracted from peripheral blood of the patients with spinal and bulbar muscular atrophy using conventional techniques. PCR amplification of the CAG repeat in the androgen receptor gene was performed using a fluorescent-labelled forward primer (5′-TCCAG AATCTGTTCCAGAGGTGC-3′) and a non-labelled reverse primer (5′-TGGCCTCGCTCAGGATGTCTTTAAG-3′). The detailed PCR conditions were described previously (Doyu *et al.*, 1992). Aliquots of the PCR products were combined with loading dye and separated by electrophoresis using an autoread sequencer (SQ-5500; Hitachi Electronics Engineering). The size of the PCR standards was determined by direct sequencing, as described previously (Doyu *et al.*, 1992).

Statistics

Statistical analyses were performed using SPSS Statistics 17.0 (SPSS Japan Inc.) or SAS Software version 9.2 (SAS Institute). Descriptive variables such as the mean, median, standard deviation, standard error of the mean and range were used to summarize the quantitative measures. Spearman's correlation coefficient was used to assess the correlations between the age at the appearance of each activity of daily living milestone and CAG repeat number, and the baseline characteristics and disease progression rate. For multivariate analyses, stepwise multiple linear regression was first performed to select the best subset of covariates. Covariates that strongly correlated with each other (Spearmann's correlation coefficient > 0.7) were eliminated to avoid the multicollinearity that may affect the precise selection of factors. In the 'Results' section, only associations that were selected by stepwise analysis and found to be significant ($P < 0.05$) are shown.

In order to address the representative disease progression most effectively, random coefficient regression models were used as the primary statistical method to evaluate the longitudinal relationship of outcome measures (Laird and Ware, 1982). Although its mathematical formulation is somewhat different, the theory underlying these models is essentially the same as that for a traditional univariate repeated measures ANOVA (Searle, 1988; McLean *et al.*, 1991). Random coefficient regression models utilize familiar designs such as ANOVA, but their hypotheses and designs are related to regression lines rather than to single observations and can deal with random variation of entry scores and rates of progression among subjects. These models also attend to 'random effects', that is, unmeasured, uncontrolled sources of variability and have been used for analysing natural history of neurodegenerative diseases (Nandhagopal *et al.*, 2009). Ignoring random effects can increase the chance of declaring statistical significance in error because the pooling of intra-subject with inter-subject variability falsely reduces the estimate of the error of variance.

Factor analysis was performed using the baseline data of ALSFRS-R to classify the clinical phenotypes of spinal and bulbar muscular atrophy. We selected the factors with loadings that were >1, and performed a 'Varimax' rotation on these factors to maximize the number of variables with high loadings for each factor (Williams *et al.*, 2005). The loadings of each variable on both of these factors were plotted against each other, and two groups of variables in different areas of the plot were selected for further analyses.

Results

Patient demographics and blood profiles

A total of 34 patients with spinal and bulbar muscular atrophy were included (Table 1). The characteristics of the present study population, such as age at the first evaluation, age at onset and CAG repeat length, were similar to those of previous studies (Atsuta *et al.*, 2006; Katsuno *et al.*, 2010; Fernández-Rhodes *et al.*, 2011). Blood count and biochemical and hormonal profiles are shown in Table 2. The most characteristic observations of the blood tests were the elevated levels of creatine kinase and the decreased levels of creatinine. A total of 29 cases (85.3%) showed abnormalities in both parameters, whereas none had a normal value for both creatine kinase and creatinine. Aspartate and alanine aminotransferase were elevated above the reference range in ~70% of the patients. The total testosterone level was also elevated in 23.5% of the patients, while no case showed an abnormally low level of this hormone.

Activity of daily living milestones

The timing of activity of daily living milestones in this cohort was equivalent to that in a previous study (Supplementary Table 1) (Atsuta *et al.*, 2006). For instance, the age at onset of muscle weakness was between 22 and 66 years, which was preceded by hand tremor in most cases, and the intervals between the onset of weakness and the requirement of handrails for stair climbing were 0 to 18 years. As previously reported (Atsuta *et al.*, 2006), all of the milestones including the onset of muscular weakness were significantly correlated with CAG repeat length (Spearman's correlation

Table 1 Clinical and genetic features of 34 patients with spinal and bulbar muscular atrophy at baseline

Demographic	Mean ± SD (range)
Age at the first evaluation (years)	53.6 ± 12.6 (27–74)
Disease duration (years)	9.2 ± 5.3 (2–21)
Age at onset (years)	44.4 ± 12.6 (19–66)
CAG repeat length in the androgen receptor gene (number)	47.9 ± 4.0 (40–57)
Functional parameters	
ALSFRS-R	42.6 ± 4.1 (32–48)
Limb Norris score	55.9 ± 6.9 (39–63)
Norris Bulbar score	34.6 ± 4.3 (22–39)
Modified quantitative myasthenia gravis score	6.1 ± 3.4 (0–13)
Grip power	42.2 ± 13.5 (16.6–70.6)
6-Min walking distance	350 ± 130 (60–569)
ALSAQ-5	10.3 ± 3.7 (5–18)
Timed walking (15 ft)	4.48 ± 2.51 (2.29–13.08)
Barium residue	13.7 ± 17.5 (0–65)

coefficient = −0.568 to −0.713), except for three milestones, i.e. use of a cane, developed pneumonia and use of a wheelchair, which were experienced by less than seven patients (Supplementary Table 1).

Baseline data of outcome measures

To clarify the clinical parameters that are associated with disease severity, we investigated the correlations between the value of the clinical parameters and the following demographic and anthropometric variables: age at first evaluation, CAG repeat length, disease duration, age at onset and body mass index. We also examined whether the value of the functional parameters correlated with blood parameters which are often abnormal in patients with spinal and bulbar muscular atrophy (Table 2 and Supplementary material). As a result, age at the first evaluation, serum creatinine, HbA1c, prothrombin time and body mass index were selected by the stepwise analyses as candidate variables that reflected disease severity in the patients with spinal and bulbar muscular atrophy examined in the present study (Supplementary Table 2). The age at baseline was correlated with the scores in the functional rating scales and walking capacity. Moreover, the serum levels of creatinine were strongly correlated with all of the clinical parameters, except for the ALSAQ-5 and pharyngeal barium residue, suggesting that this parameter is likely to be the most reliable and valid blood parameter that reflects disease severity. By contrast, no correlation was detected between the functional parameters and creatine kinase, although it is thought to be the most characteristic biomarker of spinal and bulbar muscular atrophy (Chahin and Sorenson, 2009). The scatter diagrams also showed strong simple correlations between the serum creatinine and the clinical parameters at baseline (Fig. 1).

Longitudinal assessment of outcome measures

Based on the results of baseline correlations, we prospectively analysed the longitudinal change of the functional parameters and the serum levels of creatinine. The results of longitudinal observation showed a slow but steady disease progression in all of the outcome measures we examined, except for the quality of life score (ALSAQ-5) and swallowing function (barium residue after initial swallowing) (Table 3). We also performed sample size estimation using the outcome measures that showed significant longitudinal changes (Supplementary Table 3). The results demonstrated that the functional rating scales require a smaller sample size than objective measures, although a larger number of patients have to be enrolled in clinical trials of disease-modifying therapies that slow disease progression in comparison with those of symptomatic therapies expected to improve motor function.

Next, we investigated the correlations between the baseline characteristics, such as age at onset, age at the first evaluation, disease duration and CAG repeat length and the disease progression rate of the outcome measures that showed significant changes during the 3-year follow-up (Table 3). The baseline characteristics we evaluated did not correlate with the longitudinal changes of any outcome measure, suggesting that disease progression may not be affected by the characteristics of these patients (Supplementary Table 4).

Furthermore, we analysed the longitudinal data in terms of the disease duration in each patient. The score of each outcome measure was plotted over disease duration for each subject (Fig. 2). The trajectory for each subject was expressed with a connected line over the plot. We applied modelling processes to clarify the representative progression of spinal and bulbar muscular atrophy. To this end, linear multivariate regression analyses using random effects (random coefficient regression models) were utilized to model our longitudinal data since this model is robust to inter- and intra-individual variation and allowed the analysis of the repeated data of each subject (Deschaintre *et al.*, 2009; Nandhagopal *et al.*, 2009). In addition to the linear relationship, we also assessed non-linear models by adding a quadratic term of disease duration as an explanatory variable, and used exponentially decreasing models as an alternative description of the relationship. We evaluated the *P*-values of the quadratic term of estimate and Akaike's information criterion (Akaike, 1973) for

Table 2 Haematological profiles at baseline (n = 34)

Haematological test	Mean ± SD (range)	Reference range	Out of reference range (%)	
			Low	High
Total lymphocytes ($\times 10^3/\mu l$)	2.6 ± 0.7 (1.2–4.2)	1.5–3.5	2.9	11.8
Total protein (g/dl)	7.5 ± 0.5 (6.6–8.9)	6.7–8.3	2.9	2.9
Albumin (g/dl)	4.2 ± 0.3 (3.7–5.0)	4.0–5.0	20.6	0
HbA1c (l)	5.4 ± 0.8 (4.7–8.8)	4.3–5.8	0	14.7
Creatine kinase (IU)	969 ± 573 (144–2050)	62–287	0	91.2
Aspartate transaminase (IU/l)	46.2 ± 27.3 (23–159)	13–33	0	64.7
Alanine transaminase (IU/l)	57.7 ± 47.6 (17–272)	6–30	0	79.4
Uric acid (mg/dl)	5.5 ± 1.5 (2.5–9.3)	3.6–7.0	5.9	17.6
Testosterone (μg/dl)	7.4 ± 3.1 (3.7–15.0)	1.66–8.11	0	23.5
Creatinine (mg/dl)	0.45 ± 0.09 (0.22–0.66)	0.60–1.10	94.1	0
Prothrombin time (%)	101.4 ± 9.3 (85.0–129.2)	80–120	0	2.9
Activated partial thromboplastin time (%)	33.6 ± 2.8 (10.4–29.0)	80–120	14.7	32.4

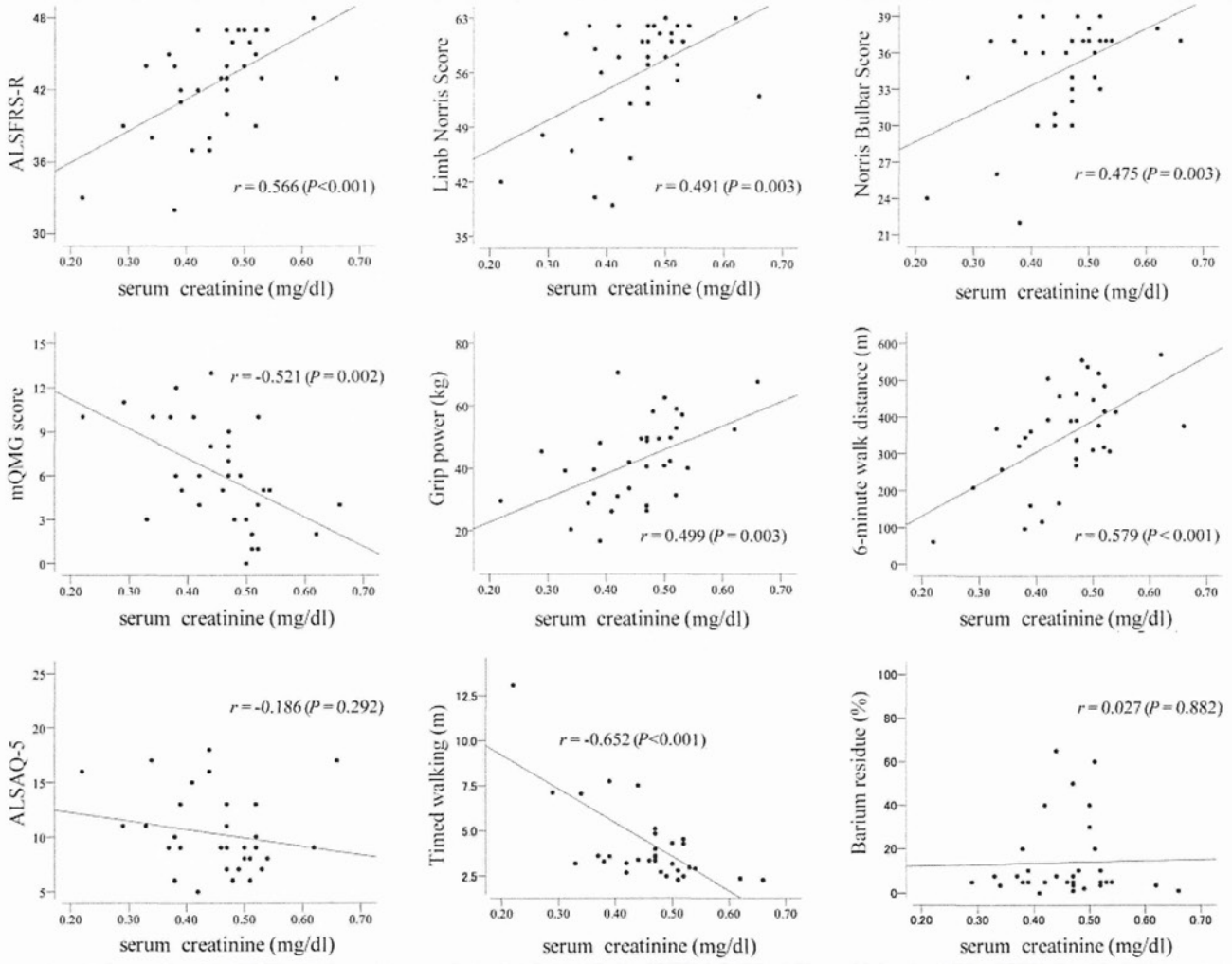

Figure 1 Simple correlations of serum creatinine levels with the outcome measures. Each outcome measure, other than barium residue, correlates well with the serum creatinine levels. mQMG = modified quantitative myasthenia gravis score.

Table 3 Longitudinal change of outcome measures

Clinical outcomes	Baseline, mean ± SD (range)	Follow-up[b], mean ± SD (range)	*P*-value[c]	Change per year[d], mean ± SD
ALSFRS-R (*n* = 33)[a]	43.0 ± 3.7 (33–48)	39.8 ± 4.0 (26–46)	<0.001	−1.1 ± 0.9
Limb Norris score (*n* = 33)	56.4 ± 6.4 (39–63)	49.7 ± 8.9 (27–62)	<0.001	−2.2 ± 1.6
Norris Bulbar score (*n* = 33)	34.9 ± 3.7 (24–39)	31.9 ± 4.4 (21–38)	<0.001	−1.0 ± 0.9
Modified quantitative myasthenia gravis score (*n* = 32)	5.9 ± 3.3 (0–13)	7.1 ± 3.4 (1–13)	<0.001	0.4 ± 0.5
Grip power (*n* = 32)	42.6 ± 13.8 (16.6–70.6)	36.5 ± 13.8 (14.0–66.0)	<0.001	−1.7 ± 3.0
6-min walking distance (*n* = 32)	360 ± 126 (60–569)	315 ± 136 (1–515)	<0.001	−20.3 ± 26.0
ALSAQ-5 (*n* = 32)	10.2 ± 3.7 (5–18)	11.2 ± 3.6 (5–16)	0.051	0.2 ± 1.2
Timed walking (15 ft) (*n* = 32)	4.36 ± 2.44 (2.29–13.08)	5.46 ± 4.26 (2.06–22.53)	0.004	1.00 ± 3.37
Barium residue (%) (*n* = 29)	13.1 ± 17.1 (1–65)	13.8 ± 16.9 (1–75)	0.796	0.2 ± 5.1
Creatinine (*n* = 32)	0.45 ± 0.09 (0.22–0.66)	0.41 ± 0.10 (0.19–0.58)	<0.001	−0.013 ± 0.030

a The numbers of patients whose data were analysed are shown. The data of patients who were evaluated once during follow-up were eliminated from the analysis.
b Follow-up data were defined as the value of the last evaluation.
c *P*-value for paired *t*-test.
d Change per year was defined as follows. [(Follow-up data) − (Baseline data)/(observational period (years)].
Barium residue = barium residue after initial swallowing.

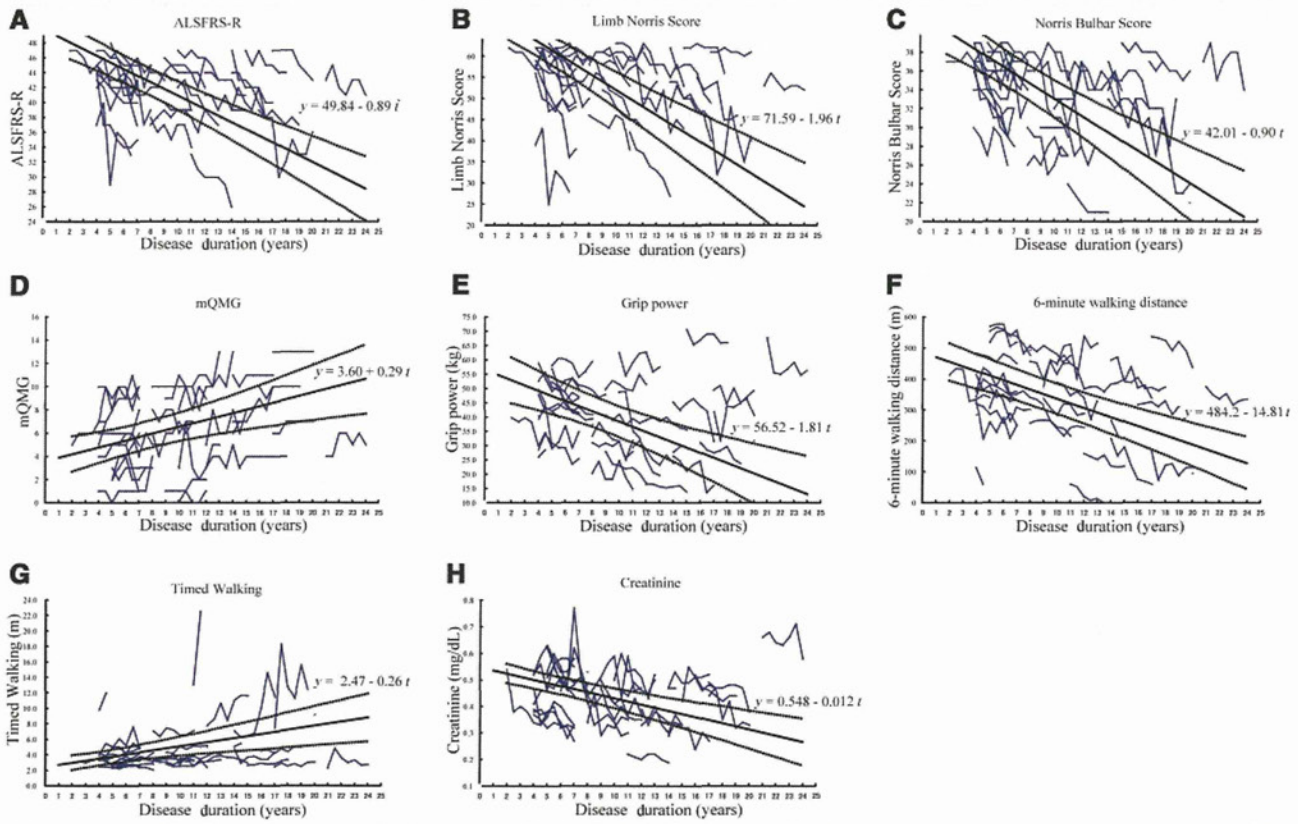

Figure 2 Longitudinal changes of outcome measures in consideration of disease duration. Neurological outcome measures and laboratory data obtained in each patient followed, according to disease duration, in years. The solid lines indicate representative disease progression over disease duration calculated by using random coefficient linear regression models. The broken curvilinear lines show the 95% confidence interval of these models. (**A**) ALSFRS-R; (**B**) Limb Norris score; (**C**) Norris bulbar score; (**D**) modified quantitative myasthenia gravis score (mQMG) (**E**) grip power; (**F**) 6-min walk distance; (**G**) timed walking; and (**H**) serum creatinine.

assessing fitness of these models. However, neither non-linear nor exponential models provided a substantially better fit than the linear models for the present data (Supplementary Table 5). In the analyses using the linear models, a fitted line was identified for all of the outcome measures, indicating a relentless deterioration of motor function in patients with spinal and bulbar muscular atrophy (Fig. 2). The linear models, shown by the solid lines in Fig. 2, were of the form $F(t) = a + bt$, where a, b and t were regression parameters to be estimated: t represented the disease duration; a represented the intercept at $t = 0$ (onset of symptoms); and b represented the disease progression rate. The broken curvilinear lines in each figure indicate the 95% confidence intervals of these models. In order to confirm that disease progression was not significantly affected by the patients' backgrounds, we split the patient population into two groups according to the level of their background variables, thereby generating two linear models. The results of this subgroup analysis considering CAG repeat length are shown in Supplementary Table 6. For all of the outcome measures, except for timed walking and creatinine, the progression rate was not significantly different, suggesting that disease progression is not strongly affected by CAG repeat length. Similarly, subgroup analyses according to the median value of age at onset and serum testosterone level also showed no substantial differences between the subgroups, except for the deterioration of grip power, which was faster than in patients with lower serum levels of testosterone (Supplementary Tables 7 and 8).

Figure 2 also showed that the individual behaviour of the chronological change had a large variation. Not only intra-individual but also interindividual variation was notably detected in each outcome measure. Thus we next investigated the characteristics of the patients whose baseline data of ALSFRS-R was below (Severe group) or above (Mild group) the curvilinear line of 95% confidence intervals (Fig. 2A). The results of this comparison showed that the patients of the mild group had better motor function despite longer disease durations compared with patients of the severe group, although there was no difference of the CAG repeat length in the androgen receptor gene or the age at the first examination between the groups. This indicates that factors other than CAG repeat size, such as physical capacity before the onset, might contribute to the variability of phenotypes in patients with spinal and bulbar muscular atrophy (Supplementary Table 9).

We next investigated the intercepts of the regression lines that corresponded to the estimated severity at clinical onset to identify clinical markers that are sensitive to the clinical changes during the early stage of the disease (Fig. 2). The intercepts of these regression lines were almost equal to or beyond the full score regarding

the subjective functional parameters, such as the ALSFRS-R and modified Norris score, indicating that motor functional deficits at an early stage of the disease may not be detected using these measures. By contrast, the estimated values at onset were far more or less than the normal level for the objective outcome measures, such as the 6-min walk distance, grip power and serum creatine kinase and creatinine levels, implying that disease severity may be evaluated by using these objective measures. Thus, our findings suggest that the objective and quantitative assessments, but not functional scales, are sensitive measures to detect subtle clinical deficits at an early or preclinical stage of spinal and bulbar muscular atrophy.

Clinical phenotypes and onset site distribution of spinal and bulbar muscular atrophy

In the analyses of the baseline data, we noticed that the degree of bulbar symptoms do not necessary correspond to that of limb involvement. For instance, pharyngeal barium residue, a clinical measure of dysphagia, was relatively little in certain patients who showed a decreased 6-min walk distance (Patients 9 and 11; Supplementary Table 10). Conversely, walking capacity was relatively preserved in Patients 28 and 32 who demonstrated increased barium residue in videofluorography. These findings prompted us to categorize the clinical symptoms of spinal and bulbar muscular atrophy with respect to the site of involvement using factor analysis (Fig. 3 and Supplementary material). This result suggests that upper limb function is closely related to bulbar function, but not to lower limb function. To confirm this view, the relationship among each domain of ALSFRS-R and that of the modified Norris score were investigated. The results showed that upper limb function is closely related to bulbar function compared with lower limb function, supporting the findings of our factor analysis (Supplementary Table 11). These observations suggest that the phenotypes of spinal and bulbar muscular atrophy may take a bulbar/upper limb-dominant or lower limb-dominant form. However, subgroup analyses according to the initially affected site showed no substantial differences between the patients whose initial symptom were bulbar or upper limb weakness and those who first noticed lower limb symptoms (Supplementary Table 12).

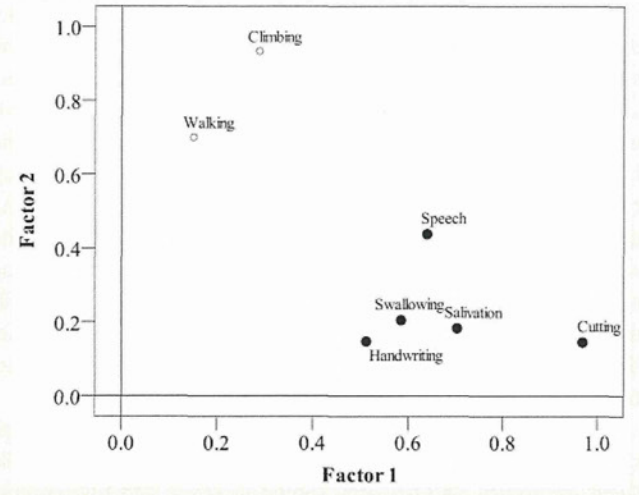

Figure 3 Factor analysis of ALSFRS-R subscores. Plot of variables for Factors 1 and 2 derived from factor analyses. Each variable indicates the items of the ALSFRS-R.
Climbing = climbing stairs; Cutting = cutting food and handling utensils.

Discussion

Spinal and bulbar muscular atrophy is a relatively rare neurodegenerative disease, for which the data regarding longitudinal analyses of clinical measures are limited (Katsuno *et al.* 2010; Fernández-Rhodes *et al.* 2011). The 3-year natural history data of quantitative outcome measures in spinal and bulbar muscular atrophy obtained from the present study will be useful for the design of future therapeutic trials, including the choice of outcome measures, determination of the observation period, stratification of patients and calculation of the sample size. In our longitudinal analyses, all of the outcome measures, except for the ALSAQ-5 and barium residue, showed a statistically significant progression, suggesting a slow but steady deterioration of symptoms in patients with spinal and bulbar muscular atrophy. The lack of significant longitudinal changes of pharyngeal barium residue may result from unequivocal variation among patients and piecemeal deglutition, a possible compensatory mechanism against slowly progressive bulbar palsy, which may hinder the measurement of the residue in patients with spinal and bulbar muscular atrophy (Katsuno *et al.*, 2010).

The results of sample size calculation indicated that the employment of functional rating scales as the primary endpoint may reduce the sample size. However, even with these functional outcome measures, clinical trials of disease-modifying therapies that suppress the exacerbation of symptoms appear to be less practical than those testing symptomatic therapies. Furthermore, these scales are shown to be more susceptible to placebo effects than objective measures (Hashizume *et al.*, 2012). This issue should also be taken into account to design clinical trials of spinal and bulbar muscular atrophy using subjective outcome measures. In addition, the effect of ageing on outcome measures is an alternative factor that may compromise the sample size estimation. Since motor function declines with age, the longitudinal changes of outcome measures in the present study might contain both disease-specific and age-related deterioration of function. This issue appears to be particularly critical when using objective measures, whereas the effects of ageing appear to be less problematic for subjective measures, the score of which is expected to be full even in aged subjects with normal activity. For instance, previous studies suggest that the 6-min walk distance test shows an age-dependent decline at ~5 m/year that may lead to the overestimation of disease progression in patients with spinal and bulbar muscular atrophy (Enright *et al.*, 1998; Takeuchi *et al.*, 2008).

In this longitudinal study, we also analysed the individual raw data in consideration of disease duration because it influences the severity of the neurological symptoms of spinal and bulbar muscular atrophy (Takeuchi *et al.*, 2008; Rhodes *et al.*, 2009). To this end, we summarized the individual data into a representative line by using random coefficient regression models. The results indicated that the disease progression is relatively linear, rather than quadratic or exponential, in the population studied (Fig. 2). The slope of the line was equivalent to the actual disease progression calculated for each parameter, indicating the plausibility of this modelling process (Table 3). This result raised the possibility that the data from the present study can be used as comparative historical control data in future clinical studies.

In the present study, we also confirmed that CAG repeat length correlated well with the age of onset and other activity of daily living milestones, as previously shown in spinal and bulbar muscular atrophy and other polyglutamine diseases (Abe *et al.*, 1998; Stevanin *et al.*, 2000; Atsuta *et al.*, 2006; Walker, 2007; Reetz *et al.*, 2011). In contrast to the strong correlation of CAG repeat size with the age at onset, the disease progression of spinal and bulbar muscular atrophy was not affected by CAG repeat length in androgen receptor. This result may suggest that the size of the CAG repeat influences the timing of the onset of clinical symptoms, but not the progression of neurological deficits, and that different mechanisms underlie disease initiation and progression in spinal and bulbar muscular atrophy (Atsuta *et al.*, 2006). In support of this view, the onset of motor dysfunction is reportedly determined by the expression of causative proteins in neurons, but disease progression is largely dependent on glial pathology, in a mutant super oxide dismutase 1 mouse model of amyotrophic lateral sclerosis (Boille *et al.*, 2006). Alternatively, the length of the CAG repeat may determine the nucleation speed of pathogenic androgen receptor proteins and the eventual onset of disease, but not the rate of aggregation that is likely to influence progression (Zhou *et al.*, 2011). It can also be inferred that the older age at onset in patients with a shorter CAG repeat may lead to accelerated progression, which overwhelms the direct effects of genotype on the post-onset course of the disease. This may underlie the faster deterioration of timed walking and serum creatinine levels in the patients with a shorter CAG repeat (Supplementary Table 6). In our subgroup analyses considering age at onset, patients with an older age of onset tended to show a more rapid deterioration of timed walking and serum creatinine levels, although the intergroup differences were not significant (Supplementary Table 7).

Laboratory tests often detect high serum levels of creatine kinase in patients with spinal and bulbar muscular atrophy, a possible clue to early diagnosis (Sorarù *et al.*, 2008; Chahin and Sorenson, 2009; Rhodes *et al.*, 2009). Our results of the baseline analysis suggested that the elevation of creatine kinase and the decrease of creatinine levels in serum were the most characteristic blood findings in patients with spinal and bulbar muscular atrophy. Since there are no established blood markers for spinal and bulbar muscular atrophy, it is important to determine if each blood index can be used as a biomarker to evaluate the effects of tested therapies in future clinical trials. In the present study, multiple regression analyses using baseline data raised the possibility that the serum level of creatinine is a reliable biomarker of disease severity. Creatinine is a biosysthetic product of creatine phosphate, which is a key molecule for energy production in muscle. Creatine is converted to creatinine and transported from muscle through the circulation to the kidneys (Viollet *et al.*, 2009). Because the serum creatinine level is associated with the whole muscle mass, it may be a useful marker for monitoring disease progression in spinal and bulbar muscular atrophy. The correlation between the serum levels of creatinine and clinical severity also suggested that the precise measurement of the whole muscle mass is essential to develop new biomarkers. Conversely, the serum level of creatine kinase was not correlated with most of the outcome measures, possibly because it is vulnerable to the patient's activity before blood sampling. Therefore, careful management of the patient's activity before sampling appears to be necessary when the serum levels of creatine kinase are used as a biomarker of spinal and bulbar muscular atrophy (Banno *et al.*, 2009).

Preventive or early intervention is construed as a key factor for successful translational research on disease-modifying therapies for neurodegenerative diseases (Holtzman, 2008). With regard to spinal and bulbar muscular atrophy, the results of phase III trials suggest that leuprorelin might be more effective in patients whose disease duration is <10 years (Katsuno *et al.*, 2010). These observations imply the need to evaluate disease severity at an early stage using sensitive clinical markers to facilitate clinical trials of disease-modifying therapies. In the longitudinal analyses of the present study, it was suggested that the biological or neurological deficit at a preclinical or early stage of the disease might be detectable using objective functional or blood parameters, but not using subjective outcome measures. In support of these findings, the reduction of brain volume and the decline of quantitative motor function were demonstrated in pre-manifest carriers in a prospective analysis of the natural history of Huntington's disease (Tabrizi *et al.*, 2009). These results might suggest the need to adopt appropriate objective measures for designing clinical trials of early interventions, and to reconsider the conventional definition of the onset of neurodegenerative diseases, including spinal and bulbar muscular atrophy, on the basis of the patients' perception of subjective symptoms for the development of disease-modifying therapies. The variability of onset age with a similar CAG repeat length may also suggest the limit of clinical definition of disease onset (Supplementary Table 9).

On the basis of the observation that the degree of bulbar involvement is not necessarily similar to that of limb impairment, we analysed the clinical phenotype of spinal and bulbar muscular atrophy using baseline data. The results indicated that upper limb function is closely related to bulbar function, but not to lower limb function and that patients with spinal and bulbar muscular atrophy appear to be diverse in terms of the preferentially affected site. These observations suggest that the severity of neurodegeneration may be associated with neuroanatomical closeness in spinal and bulbar muscular atrophy. In support of this view, the degeneration of neurons is shown to affect the dynamics of cell death in neighbouring cells (Friedlander, 2003). Additionally, disease-specific patterns of the topographical expansion of pathology have been suggested for several neurodegenerative diseases (Goedert *et al.*, 2010).

In summary, the results of the present study demonstrated the slow but steady progression of motor impairment in spinal and bulbar muscular atrophy. Analyses using random coefficient models did not indicate that the disease progression of spinal and bulbar muscular atrophy is substantially affected by the CAG repeat length, the age of onset, or serum levels of testosterone, suggesting that these variables may not be critical factors for the stratification of patients in clinical trials. Biological and neurological deficits were detectable using objective functional or blood parameters, even during the early or preclinical stage of spinal and bulbar muscular atrophy, suggesting that these indices may be used as endpoints in clinical trials of disease-modifying therapies for spinal and bulbar muscular atrophy.

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Supplementary material

Supplementary material is available at *Brain* online.

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Oxidative stress induced by glutathione depletion reproduces pathological modifications of TDP-43 linked to TDP-43 proteinopathies

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ABSTRACT

TAR DNA-binding protein 43 (TDP-43) is a major component of ubiquitin-positive inclusion of TDP-43 proteinopathies including amyotrophic lateral sclerosis and frontotemporal lobar degeneration with ubiquitinated inclusions, which is now referred to as FTLD-TDP. TDP-43 in the aberrant inclusion is known to be hyperphosphorylated at C-terminal sites, to be truncated at the N-terminal region, and to re-distribute from nucleus to cytoplasm or neurite. The pathogenic role of these modifications, however, has not been clarified. Furthermore, there is no evidence about the initial cause of these modifications. Herein we show that ethacrynic acid (EA), which is able to increase cellular oxidative stress through glutathione depletion, induces TDP-43 C-terminal phosphorylation at serine 403/404 and 409/410, insolubilization, C-terminal fragmentation, and cytoplasmic distribution in NSC34 cells and primary cortical neurons. In the investigation using a nonphosphorylable mutant of TDP-43, there was no evidence that C-terminal phosphorylation of TDP-43 contributes to its solubility or distribution under EA induction. Our findings suggest that oxidative stress induced by glutathione depletion is associated with the process of the pathological TDP-43 modifications and provide new insight for TDP-43 proteinopathies.

Introduction

TAR DNA-binding protein 43 (TDP-43) is a major component of ubiquitin-positive inclusion, a pathological hallmark of TDP-43 proteinopathies including amyotrophic lateral sclerosis (ALS) and frontotemporal lobar degeneration with ubiquitinated inclusions, which is now referred to as FTLD-TDP (Arai et al., 2006; Neumann et al., 2006). Both diseases occur in sporadic or familial forms, and are characterized by late-onset progressive deterioration of motor and/or cognitive function. TDP-43 is a heterogeneous nuclear ribonucleoprotein (hnRNP), which is known to regulate gene transcription and exon splicing through interactions with RNA, hnRNPs, and nuclear bodies (Ayala et al., 2005; Buratti et al., 2005; Wang et al., 2002, 2004). In addition, this protein has also been reported to stabilize human low molecular weight neurofilament (hNFL) mRNA through direct interaction with its 3'UTR (Strong et al., 2007), regulate retinoblastoma protein phosphorylation through the repression of cyclin-dependent kinase 6 (Cdk6) expression (Ayala et al., 2008), regulate activity of Rho family GTPases (Iguchi et al., 2009), and alter the expression of selected microRNAs, such as let-7b and miR-663 (Buratti et al., 2010). Furthermore, very recent works using cross-linking immunoprecipitation sequencing show that multiple RNAs interact with TDP-43 (Polymenidou et al., 2011; Sephton et al., 2011; Tollervey et al., 2011).

Although it mostly localizes in the nucleus under normal conditions, TDP-43 is distributed from nucleus to cytoplasm or neurite, and forms aggregates consisting mainly of C-terminal fragments in affected neurons of patients with TDP-43 proteinopathies. In addition, TDP-43 in the aberrant aggregation is hyperphosphorylated at multiple C-terminal sites (Hasegawa et al., 2008). However, neither the pathogenic role nor the initial cause of these abnormal modifications of TDP-43 has been elucidated. The fact that the majority of patients with TDP-43 proteinopathies are sporadic suggests that exogenous factors induce post-translational modifications of TDP-43 that are seen in the disease. Furthermore, TDP-43 inclusions have also been observed in Alzheimer disease (AD), Parkinson disease (PD),

Abbreviations: TDP-43, TAR DNA-binding protein of 43 kDa; ALS, amyotrophic lateral sclerosis; hnRNP, heterogeneous nuclear ribonucleoprotein; hNFL, human low molecular weight neurofilament; Cdk6, cyclin-dependent kinase 6; ROS, reactive oxygen species; EA, ethacrynic acid; NAC, N-acetylcysteine; CK1, casein kinase 1; CK2, casein kinase 2; WT-TDP-43, wild type TDP-43; SA-TDP-43, nonphosphorylable TDP-43.

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dementia with Lewy bodies (DLB), and Huntington disease (HD), argyrophilic grain disease, suggesting that the aggregation of this protein may be a secondary feature of neurodegeneration (Amador-Ortiz et al., 2007; Arai et al., 2009, 2010; Geser et al., 2008; Hasegawa et al., 2007). These findings complicate understanding of the pathogenic role of TDP-43. On the other hand, there is considerable evidence that reactive oxygen species (ROS) and oxidative stress are associated with many neurodegenerative conditions including ALS (Abe et al., 1995, 1997; Beal et al., 1997; Butterfield et al., 2007; Ferrante et al., 1997; Lovell and Markesbery, 2007; Nunomura et al., 2002; Shaw et al., 1995). Herein we show that oxidative stress induced by glutathione depletion reproduces the pathological modifications of TDP-43, that are seen in TDP-43 proteinopathies, in motor neuron-like cells and primary cortical neurons.

Materials and methods

Cell culture and treatment

Mouse NSC34 motor neuron-like cells (a kind gift of N.R. Cashman, University of British Columbia, Vancouver, Canada) were cultured in a humidified atmosphere of 95% air-5% CO2 in a 37 °C incubator in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS). To differentiate the cells, the medium was changed to DMEM containing 1% FBS and 1% NEAA, and was cultured for 24 h. For the interventions, the cells were then incubated with ethacrynic acid (EA) (Sigma-Aldrich, St. Louis, MO), with or without N-acetylcysteine (NAC) (Sigma-Aldrich), casein kinase 1 (CK1) inhibitor (D4476), or casein kinase 2 (CK2) inhibitor (TBCA) (Sigma-Aldrich). Primary cultures of mouse embryonic cortical neurons that were dissociated from embryonic cortex of embryonic day 15 (E15) C57BL/6 J pregnant mice were plated onto poly-L-lysine-coated plates or glass bottom dishes, and maintained in neuron culture medium (Sumilon, Osaka, Japan). Five days after the incubation, the indicated interventions were performed. In both NSC34 cells and primary cortical neurons, the transfections of the intended plasmids were performed using Lipofectamine 2000 (Invitrogen, Eugene, OR), according to the manufacturer's instructions.

DNA constructs

Human wild type TDP-43 (WT-TDP-43) (accession number NM 007375) cDNA was amplified by PCR from cDNA of human spinal cord using the following primers: 5′-CACCATGTCTGAATATATTCGGGTAAC-3′ and 5′-CTACATTCCCCAGCCAGAAGACTTAGAAT-3′. The PCR product was cloned into the pENTR/D-TOPO vector (Invitrogen). For nonphosphorylable TDP-43 (SA-TDP-43) vector, primers containing the mutant substitution of TDP-43 serine 403/404 and 409/410 to alanine were used to mutagenize WT-TDP-43 (KOD-Plus-Mutagenesis kit; Toyobo, Osaka, Japan). The entry vector of WT- or SA-TDP-43 was transferred into pcDNA6.2/N-EmGFP-DEST Vector or pcDNA3.1/nV5-DEST using Gateway LR Clonase II enzyme mix (Invitrogen). The sequences of all constructs were verified using CEQ 8000 genetic analysis system (Beckman Coulter, Brea, CA).

Immunoblot analysis

For whole lysate analysis, NSC34 cells and primary cortical neurons were lysed in 2% SDS sample buffer. For analysis of protein solubility, cells cultured in 6-well plates were lysed in 100 μl of Tris (TS) buffer (50 mM Tris–HCl buffer, pH 7.5, 0.15 M NaCl, 5 mM EDTA, 5 mM EGTA, protein phosphatase inhibitors, and protease inhibitor cocktail). Lysates were sonicated and centrifuged at 100,000 ×*g* for 15 min. To prevent carryover, the pellets were washed with TS buffer, followed by sonication and centrifugation. TS-insoluble pellets were lysed in 50 μl of Triton-X100 (TX) buffer (TS buffer containing 1% Triton X-100), sonicated, and centrifuged at 100,000 *g* for 15 min. The pellets were washed with TX buffer, followed by sonication and centrifuge. TX-insoluble pellets were lysed in 50 μl of Sarkosyl (Sar) buffer (TS buffer containing 1% Sarkosyl), sonicated and centrifuged at 100,000 × g for 15 min. Sar-insoluble pellets were lysed in 25 μl of SDS sample buffer. After denaturation, 3 μl of each cell lysate was separated by SDS-PAGE (5%–20% gradient gel) and analyzed by western blotting with ECL Plus detection reagents (GE Healthcare, Buckinghamshire, UK). Primary antibodies used were as follows: anti-TDP-43 rabbit polyclonal antibody (1:1000, ProteinTech, Chicago, IL), anti-TDP-43 (405–414) rabbit polyclonal antibody (1:1000, Cosmo Bio Co. Ltd., Tokyo, Japan), anti-TDP-43 (phospho Ser403/404, Cosmo Bio) rabbit polyclonal antibody (1:1000, Cosmo Bio), anti-TDP-43 (phospho Ser409/410, Cosmo Bio) rabbit polyclonal antibody (1:1000, Cosmo Bio), anti-GAPDH mouse monoclonal antibody (1:2000, Temecula, CA), anti-GFP mouse monoclonal antibody (1:2000, MBL, Nagoya, Japan), and anti-V5 mouse monoclonal antibody (1:2000, Invitrogen).

Assay of ROS production

NSC34 cells to be treated with intended agents were incubated in 96-well plates with 5-(and-6)-chloromethyl-2′,7′-dichlorodihydro fluoresceindiacetate acetyl ester (CM-H2DCFDA) (Molecular Probes, Eugene, OR, USA) for 1 h. Oxidation in the cells was then measured in a multiple-plate reader (PowerscanHT, Dainippon Pharmaceutical, Japan) at excitation and emission wavelengths of 485 nm and 530 nm, respectively. The assays were carried out in 6 wells for each condition.

Immunocytochemistry

NSC34 cells and primary cortical neurons were fixed with 4% paraformaldehyde, incubated with PBS containing 0.2% Triton X-100 for 5 min, blocked, and incubated overnight with anti-TDP-43 rabbit polyclonal antibody (1:1000, ProteinTech), anti-TDP-43 (phospho Ser409/410) mouse monoclonal antibody (1:2000, Cosmo Bio) and anti-TIAR mouse monoclonal antibody (1:1000, BD Transduction Laboratories, Milan, Italy). After washing, samples were incubated with Alexa-488-conjugated goat anti-rabbit IgG (1:1000, Invitrogen) and Alexa-564-conjugated goat anti-mouse IgG (1:1000, Invitrogen) for 30 min, mounted with (Vector Laboratories, Inc. Burlingame, CA), then imaged with a laser conforcal microscope (Nikon A1, Nikon, Tokyo, Japan).

Time lapse analysis

NSC34 cells or mouse primary cortical neurons were grown on glass base dishes, transfected with GFP-WT-TDP-43, and treated with EA. GFP and phase contrast imaging was done every 10 min using a 40X objective lens on a laser scanning confocal microscope.

Cell viability analysis

The 3-(4,5-dimethylthiazol-2-yl)-5-(3-caboxymethoxyphenyl)-2-(4-sulfophenyl)-2H -tetrazolium (MTS)-based cell proliferation assay (MTS assay) was carried out using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI), according to the manufacturer's instructions. Absorbance at 490 nm was measured in a multiple-plate reader (PowerscanHT, Dainippon Pharmaceutical, Japan). The assays were carried out in 6 wells for each condition.

Statistical analysis

Statistical differences were analyzed by ANOVA and Bonferroni post hoc analyses for three group comparisons (SPSS version 15.0, SPSS Inc., Chicago, IL). Two-tailed $p<0.05$ was regarded as statistically significant.