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ORIGINAL COMMUNICATION

Elevated serum creatine kinase in the early stage of sporadic amyotrophic lateral sclerosis

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Abstract

Objective To assess the changes of muscle-related biomarkers at the early stage of amyotrophic lateral sclerosis, and to confirm these findings in an experimental animal model.

Methods Thirty-nine subjects with sporadic amyotrophic lateral sclerosis and 20 healthy controls were enrolled and longitudinally evaluated. We evaluated serum creatine kinase and creatinine levels and appendicular lean soft-tissue mass using dual X-ray absorptiometry. The levels of biomarkers at early ALS stages were estimated using linear mixed models with unstructured correlation and random intercepts. We also analyzed the longitudinal changes of serum creatine kinase and creatinine, together with the mRNA levels of acetylcholine receptor subunit γ (*Chrng*) and muscle-associated receptor tyrosine kinase, markers of denervation, in the gastrocnemius muscle of superoxide dismutase 1 $(SOD1)^{G93A}$ transgenic mice, an animal model of amyotrophic lateral sclerosis.

Results The estimated levels of creatine kinase were higher in subjects with amyotrophic lateral sclerosis at the early stage than in healthy controls, although the estimated appendicular lean soft-tissue mass and creatinine levels were equivalent between both groups, suggesting that the elevation of creatine kinase precedes both muscular atrophy and subjective motor symptoms in sporadic amyotrophic lateral sclerosis. In $SOD1^{G93A}$ mice, the serum levels of creatine kinase were elevated at 9 weeks of age (peri-onset) when *Chrng* started to be up-regulated, and were then down-regulated at 15 weeks of age, consistent with the clinical data from patients with sporadic amyotrophic lateral sclerosis.

Interpretation Creatine kinase elevation precedes muscular atrophy and reflects muscle denervation at the early stage.

Keywords Amyotrophic lateral sclerosis · Creatine kinase · Biomarker

Introduction

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease caused by the selective loss of upper and lower motor neurons. ALS causes rapidly progressive muscle weakness and atrophy, leading to death in approximately 3–5 years from onset, chiefly due to respiratory failure [1, 2]. Mutations in genes such as chromosome 9 open reading frame 72 (*C9orf72*), TAR DNA binding protein, fused in sarcoma, and superoxide dismutase 1 (*SOD1*) have been identified in 50–70% of familial cases, as well as in a smaller population of sporadic cases, suggesting that several biological mechanisms are involved in the pathophysiology of ALS, e.g., RNA metabolism, protein homeostasis, nucleocytoplasmic trafficking, and neuroinflammation [3–5]. These insights have inspired a myriad of attempts to develop disease-modifying therapies for this devastating disease; nevertheless,

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most agents that have shown promise in animal studies have failed to demonstrate clear efficacy in clinical trials [6, 7]. This failure in translation may be attributable to various clinical and biological factors, among which disease progression before the onset and early stages of neurological symptoms is a key issue for the successful development of a cure for ALS.

It is now widely accepted that pathological changes begin long before the clinical symptoms manifest, indicating the preclinical progression of neurodegenerative processes [8–10]. This hypothesis is explicitly emphasized in Alzheimer's disease, in which amyloid β deposition, a component of senile plaques, precedes brain volume loss and cognitive decline by a couple of decades [8]. Similar changes of biomarkers have been reported in most neurodegenerative diseases, including Huntington's disease, Parkinson's disease, and spinal and bulbar muscular atrophy, another adult-onset motor neuron disease [11, 12]. These findings provide a theoretical basis for the development of preventive therapies that target the preclinical phase of neurodegenerative disorders [13, 14].

As for ALS, a few markers have been identified that show changes in gene mutation carriers at the preclinical stage. For instance, dipeptide repeat proteins are detectable in the cerebrospinal fluid of asymptomatic *C9orf72* mutation carriers [15]. In addition, alterations of microRNA expression profiles in cerebrospinal fluid have also been reported in *C9orf72* mutation carriers [16]. It is also reported that short-interval intracortical inhibition, an index of cortical hyperexcitability in electrophysiological studies, is affected in asymptomatic *SOD1* mutation carriers [17]. Furthermore, a decrease in the estimated number of motor units, another electrophysiological marker of motor neuron degeneration, is detectable in asymptomatic *SOD1* mutation carriers [18]. However, there is no report on the preclinical changes of biomarkers in sporadic ALS, except for scattered studies on altered body fat and lipid metabolism as risk factors for ALS [19, 20].

Here, we conducted a longitudinal study to estimate the changes of biomarkers at the very early stage of ALS by analyzing clinical data from sporadic patients at the early stage. Our results indicate the elevation of the serum levels of creatine kinase (CK) in the early stage of ALS, which was reproduced in G93A mutant SOD1 ($SOD1^{G93A}$) mice, an animal model of ALS.

Materials and methods

Participants

Subjects who were clinically diagnosed with the revised El Escorial Criteria of definite, probable, or possible ALS were recruited consecutively. The principal inclusion criteria were no family history and disease duration of ≤ 2 years at the time of enrollment. Subjects who had severe complications such as malignancy, heart failure, or renal failure were excluded from this study.

Age- and sex-matched healthy controls were also recruited during the same period as the ALS patients. Subjects with sporadic ALS were assessed during hospitalization at the initial evaluation and followed up every 6 months at our out-patient clinic as long as they were able to continue to attend out-patient clinic. Healthy controls were followed up every 6 or 12 months at the out-patient clinic.

Subjects with ALS who were evaluated twice or more were analyzed longitudinally. Results of DXA which were performed within 1 week after a radiological examination with contrast agents or radioisotopes were excluded as they influence the results of dual-energy X-ray absorptiometry (DXA).

We also retrospectively analyzed serum CK before the onset, which had been measured for the purpose of medical practice, in patients with or without follow-up data. All study subjects were Japanese and observed at the Nagoya University Hospital between May 2013 and March 2018.

Definition of disease onset and onset site

Disease onset was defined as the time point when the subject felt weakness of any body part. Subjects with ALS were classified into two groups, limb-onset type and bulbar-onset type, according to the site, where they first felt weakness. The limb-onset type included subjects who felt weakness in either of the four extremities first, while the bulbar-onset type included subjects who felt dysarthria or dysphagia first. There were no patients with respiratory-onset type ALS.

Outcome measures

Disease severity was assessed with the Japanese version of the revised ALS Functional Rating Scale (ALSFRS-R), a validated questionnaire-based functional rating scale for ALS [21, 22].

Venous blood samples were collected in the supine position after more than 12 h of fasting and just after waking up during hospitalization. At the out-patient clinic, they were collected, while the subjects were in the sitting or supine position after more than 12 h of fasting. We measured the following serological indices: CK, creatinine, cystatin C, albumin, blood glucose, hemoglobin A1c, low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), aspartate aminotransferase (AST), alanine aminotransferase, gamma-glutamyl transpeptidase, alkaline phosphatase (ALP), total bilirubin (T-bil), lactate dehydrogenase, uric acid, uric nitrate, white blood cell

count, hemoglobin, and platelet count. The levels of serum CK were log transformed before analysis, as they had a non-normal distribution.

Body composition was assessed with DXA using fan-beam technology (Discovery A; Hologic, Inc., Bedford, MA). DXA has been utilized frequently to evaluate body composition including bone mineral content, fat mass, and lean body mass [23]. We calculated the sum of appendicular lean soft-tissue (ALST) mass as an index of skeletal muscle mass [24].

ALS model animal

Transgenic mice overexpressing the human *SOD1* gene carrying the G93A mutation, $SOD1^{G93A}$ mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and maintained as hemizygotes by mating transgenic males with B6/SJLF1 females [25]. Mutant male mice and their male littermate were sacrificed, and their venous blood was collected by puncturing the right ventricle using a 26G needle under pentobarbital anesthesia at the age of 5, 9, and 15 weeks: the pre-onset, peri-onset, and symptomatic stage, respectively [26]. Serum CK and creatinine levels were measured with L-Type CK (Wako, Japan) and an enzymatic method, respectively, at Oriental Yeast Co., Ltd. (Tokyo, Japan). Hemolyzed samples were excluded from the analysis.

Quantitative real-time PCR of murine muscle

At the age of 5, 9, and 15 weeks, gastrocnemius muscles from mutant $SOD1^{G93A}$ mice and their littermates were dissected and snap-frozen with powdered CO_2 in acetone. Total RNA was extracted from mouse skeletal muscles using TRIzol and a PureLink™ RNA Mini Kit (Invitrogen, Carlsbad, CA). The extracted RNA was reverse-transcribed into first-strand cDNA with ReverTra Ace reverse transcriptase (TOYOBO Co., Osaka, Japan). Quantitative real-time PCR was performed using KOD SYBR® qPCR Mix (TOYOBO Co., Osaka, Japan), and the product was detected using the CFX96™ real-time system (Bio-Rad Laboratories, Hercules, CA). The reaction conditions were 98 °C for 2 min, followed by 40 cycles of 10 s at 98 °C, 10 s at 60 °C, and 30 s at 68 °C. The expression levels of acetylcholine receptor subunit γ (*Chrng*) and muscle-associated receptor tyrosine kinase (*Musk*) in gastrocnemius muscles were measured as markers of muscle denervation [27]. The expression of glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) was also quantified and used as an internal standard control. The primers used were 5′-AGCCTCCCCAGCCATCCAGG-3′ and 5′-AGCCTCCCCAGCCATCCAGG-3′ for *Chrng*, 5′-ATCACCACGCCTCTTGAAAC-3′ and 5′-TGTCTTCCACGCTCAGAATG-3′ for *Musk* [27], and 5′-GAATTTGCCGTGAGTGGAGT-3′ and 5′-CGTCCCGTAGACAAAATGGT-3′ for *Gapdh*.

Statistical analysis

Linear mixed models with unstructured correlation and random intercepts were applied to estimate the average trajectories of each biomarker [28]. Polynomial basis functions were included to incorporate quadratic smoothing. Estimated values and 95% confidence intervals are shown from 0 to + 50 months relative to symptom onset at 5-month intervals in subjects with ALS.

The Chi-square test, unpaired *t* test, and Mann–Whitney *U* test were used for the comparison of variables between two groups, and Pearson's correlation coefficient was used for analyzing correlations among parameters. A partial correlation was performed to determine the relationship between ALSFRS-R and log CK while controlling for age, disease duration, and ALST mass. $p < 0.05$ was considered to be significant, and correlation coefficients (r) were interpreted as follows; greater than 0.8 was very strong, 0.6–0.8 was moderately strong and 0.3–0.5 was fair [29]. All data are presented as the mean ± standard deviation unless stated otherwise.

Statistical Package for the Social Sciences 25.0 J software (IBM Japan, Tokyo, Japan) and SAS version 9.4 (SAS Institute, Inc., Cary, NC) were used to perform all statistical analyses.

Results

Participants

A total of 81 subjects with ALS and 20 age- and sex-matched healthy controls were recruited. As a result, we analyzed the longitudinal data from 39 patients with sporadic ALS and 20 healthy controls. Four subjects with ALS could not undergo longitudinal evaluation of DXA, although they underwent other longitudinal evaluation including blood test. The data prior to the initial evaluation from 8 subjects with ALS were also analyzed retrospectively (Fig. 1).

Baseline characteristics

Mean age at the initial evaluation, sex ratio, and body mass index was equivalent between both groups (Table 1). The mean disease duration was 12.6 ± 5.3 months, indicating that the subjects with ALS in our study were early cases. The clinical backgrounds of the limb-onset and bulbar-onset groups were equivalent.

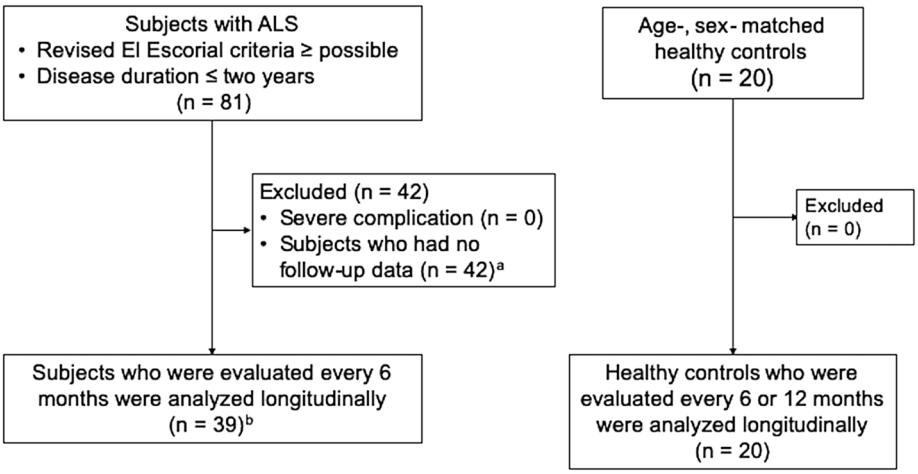

Fig. 1 Flowchart of study population enrollment. Flowchart describing the enrollment and exclusion of the study population. Data before the onset were retrospectively analyzed in 4 subjects without follow-up data[a] and 4 subjects on whom longitudinally analysis was performed[b]. Longitudinal data of body compositions was not available in 4 subjects with ALS. *ALS* amyotrophic lateral sclerosis, *DXA* dual X-ray absorptiometry

Table 1 Baseline characteristics

	Total ALS ($n=39$)	HC ($n=20$)	p value	Bulbar ($n=12$)	Limb ($n=27$)	p value
Sex (male%)	64.1	65	0.946	66.7	63.0	0.824
Age at the first evaluation (years)	66.4 ± 7.3	64.4 ± 6.9	0.314	69.6 ± 5.8	65.0 ± 7.6	0.071
Disease duration (months)	12.6 ± 5.3	N.A	N.A	12.0 ± 5.8	12.9 ± 5.2	0.788
Height (cm)	160.4 ± 7.9	162.9 ± 8.9	0.293	158.5 ± 7.3	161.3 ± 8.2	0.320
Body weight (kg)	56.7 ± 10.9	62.2 ± 11.0	0.071	54.6 ± 9.3	57.6 ± 11.6	0.428
Body mass index	21.9 ± 3.0	23.3 ± 2.8	0.081	21.6 ± 2.6	22.0 ± 3.1	0.685
ALSFRS-R	40.9 ± 4.7	47.8 ± 0.3	< 0.001	40.5 ± 4.8	41.1 ± 4.8	0.730

Data represent mean ± standard deviation

ALS amyotrophic lateral sclerosis, *ALSFRS-R* revised amyotrophic lateral sclerosis functional rating scale, *Bulbar* bulbar-onset ALS, *HC* healthy control, *Limb* limb-onset ALS, *NA* not available

Blood tests and body composition

The baseline levels of serum creatinine and ALST mass, which reportedly correlate with whole muscle mass [24, 30], were lower in subjects with ALS than in healthy controls. In contrast, the baseline levels of serum CK were higher in subjects with ALS (Table 2). Both male and female subjects had similar results (Supplemental Table 1). As for the onset sites of ALS, the baseline levels of CK tended to be higher and those of creatinine were lower in limb-onset type ALS compared to the bulbar-onset group (CK, 262.5 ± 257.2 vs. 135.6 ± 79.7 U/L, respectively, $p=0.105$; creatinine, 0.62 ± 0.15 vs. 0.80 ± 0.27 mg/dL, respectively, $p=0.013$).

We followed up the subjects with ALS and healthy controls for 8.2 ± 4.2 months and 11.4 ± 3.8 months, respectively. The annualized differences of those parameters between the initial and final evaluation are shown in Table 2. The levels of CK, creatinine, and ALST mass decreased longitudinally in subjects with ALS, although they rarely changed in healthy controls. These changes were also common in both male and female subjects (Supplemental Table 1). These results suggest that ALST mass in DXA could be a biomarker reflecting muscular atrophy in ALS patients. In addition, the levels of LDL-C, AST, ALP, and T-bil increased longitudinally in subjects with ALS, although they rarely changed in healthy controls (Table 2).

Correlation between muscle-related biomarkers and clinical indices

ALSFRS-R was weakly correlated with log serum CK, but not with ALST mass or serum creatinine, at the initial evaluation (Fig. 2a–c, Supplementary Table 2). The correlation between log CK and ALSFRS-R at baseline was still significant after being adjusted for age, disease duration, and ALST mass (partial correlation coefficient = 0.453, $p=0.006$). Furthermore, the annualized differences (shown as delta) of all of these muscle-related markers were correlated with those of ALSFRS-R (Fig. 2d–f). However, log serum CK, ALST mass, and serum creatinine levels at the initial evaluation were not correlated with the longitudinal change of motor function (data not shown). These results suggest that serum CK levels reflect the motor function of each individual, although they do not indicate the prognosis of ALS.

Table 2 Baseline and changes of blood tests and body composition

	Baseline			Change at 48 weeks		
	ALS	HC	*p* value	ALS	HC	*p* value
Blood test	ALS ($n=39$)	HC ($n=20$)		ALS ($n=39$)	HC ($n=20$)	
CK (U/L)	223.5 ± 225.0	107.4 ± 53.5	0.004	− 77.0 ± 136.9	2.2 ± 49.7	0.002
Log CK	2.21 ± 0.34	1.99 ± 0.20	0.008	− 0.19 ± 0.31	0.004 ± 0.14	0.001
Cr (mg/dL)	0.68 ± 0.21	0.77 ± 0.16	0.100	− 0.09 ± 0.14	0.02 ± 0.08	< 0.001
CysC (mg/L)	0.96 ± 0.20	0.90 ± 0.14	0.221	0.09 ± 0.16	0.01 ± 0.08	0.017
UN (mg/dL)	15.8 ± 4.6	13.4 ± 2.2	0.010	0.36 ± 8.31	0.26 ± 2.60	0.941
UA (mg/dL)	5.1 ± 1.3	5.5 ± 1.0	0.328	− 0.69 ± 1.53	0.28 ± 1.28	0.012
AST (U/L)	24.3 ± 7.3	25.6 ± 6.3	0.502	7.4 ± 24.7	− 1.0 ± 4.3	0.044
ALT (U/L)	22.1 ± 10.8	21.1 ± 7.8	0.695	5.4 ± 27.7	− 1.0 ± 6.4	0.178
T-Bil (mg/dL)	0.87 ± 0.36	0.92 ± 0.42	0.688	0.25 ± 0.49	− 0.31 ± 0.22	0.001
ALP (U/L)	209.2 ± 61.6	242.1 ± 59.8	0.055	29.6 ± 80.9	− 30.8 ± 45.9	0.003
γ-GTP (U/L)	34.2 ± 26.6	56.2 ± 57.8	0.120	17.7 ± -10.6	− 10.6 ± 71.0	0.159
TP (g/dL)	7.0 ± 0.4	7.2 ± 0.3	0.065	0.17 ± 0.96	− 0.09 ± 0.76	0.291
Alb (g/dL)	4.2 ± 0.3	4.4 ± 0.3	0.077	0.10 ± 0.81	− 0.09 ± 0.43	0.354
LDH (U/L)	210.7 ± 42.8	185.4 ± 34.7	0.026	− 19.8 ± 49.5	− 15.0 ± 33.9	0.699
LDL-C (mg/dL)	119.4 ± 36.6	117.3 ± 27.9	0.822	23.0 ± 57.9	1.2 ± 19.7	0.043
HDL-C (mg/dL)	54.6 ± 15.9	59.7 ± 18.2	0.282	4.0 ± 18.3	0.85 ± 7.9	0.365
HbA1c (%)	5.9 ± 0.8	5.9 ± 0.4	0.935	− 1.4 ± 1.9	0.10 ± 0.25	< 0.001
WBC (10^3/μL)	5.7 ± 2.1	5.8 ± 1.7	0.910	0.9 ± 3.0	− 0.18 ± 1.36	0.072
Hb (g/dL)	14.6 ± 4.9	14.6 ± 1.4	0.970	− 1.6 ± 10.1	− 0.3 ± 1.1	0.571
Plt (10^3/μL)	232.8 ± 69.5	222.3 ± 57.0	0.561	8.0 ± 75.6	− 5.7 ± 26.1	0.313
Body composition	ALS ($n=39$)[a]	HC ($n=20$)		ALS ($n=35$)	HC ($n=20$)	
ALST mass (kg)	16.65 ± 4.01	19.20 ± 4.5	0.030	− 2.56 ± 2.27	− 0.42 ± 1.22	< 0.001
BMC (kg)	1.90 ± 0.39	1.94 ± 0.42	0.716	− 0.09 ± 0.12	0.02 ± 0.12	0.002
Total fat mass(kg)	14.25 ± 4.12	15.21 ± 3.79	0.385	− 0.97 ± 4.49	0.46 ± 1.65	0.094

Change at 48 weeks shows the annualized difference of each parameter between the initial and final evaluation

Data represent mean ± standard deviation

Alb albumin, *ALP* alkaline phosphatase, *ALS* amyotrophic lateral sclerosis, *ALST* appendicular lean soft tissue, *ALT* alanine aminotransferase, *AST* aspartate aminotransferase, *BMC* bone mineral content, *CK* creatine kinase, *Cr* creatinine, *CysC* cystatin C, *γ-GTP* gamma glutamyl transpeptidase, *Hb* haemoglobin, *HbA1c* hemoglobin A1c, *HC* healthy control, *HDL-C* high-density lipoprotein cholesterol, *LDH* lactate dehydrogenase, *LDL* low-density lipoprotein cholesterol, *Plt* platelet count, *T-Bil* total bilirubin, *TP* total protein, *UA* uric acid, *UN* uric nitrate, *WBC* white blood cell count.

[a]Longitudinal data of body compositions was not available in 4 subjects with ALS

Estimation of muscle-related biomarkers at the early stage of ALS

Muscle-related biomarkers at early ALS stages and average trajectories were estimated using a linear mixed model with quadratic smoothing (Fig. 3, Table 3). The estimated values of serum CK at early ALS stages were higher in ALS subjects than in controls, though estimated serum creatinine and ALST mass were equivalent between both groups in this statistical model. These results suggest that, at the early stage of ALS, serum CK levels are elevated, while there appears to be little muscular atrophy.

Retrospective analysis of serum CK before disease onset

For 8 male subjects with sporadic ALS, their serum CK levels before clinical onset had been recorded as a part of medical practice independent of ALS. In these subjects, the levels of serum CK started to be elevated before onset, reached their maximum approximately around onset, and then decreased after onset (Fig. 4).

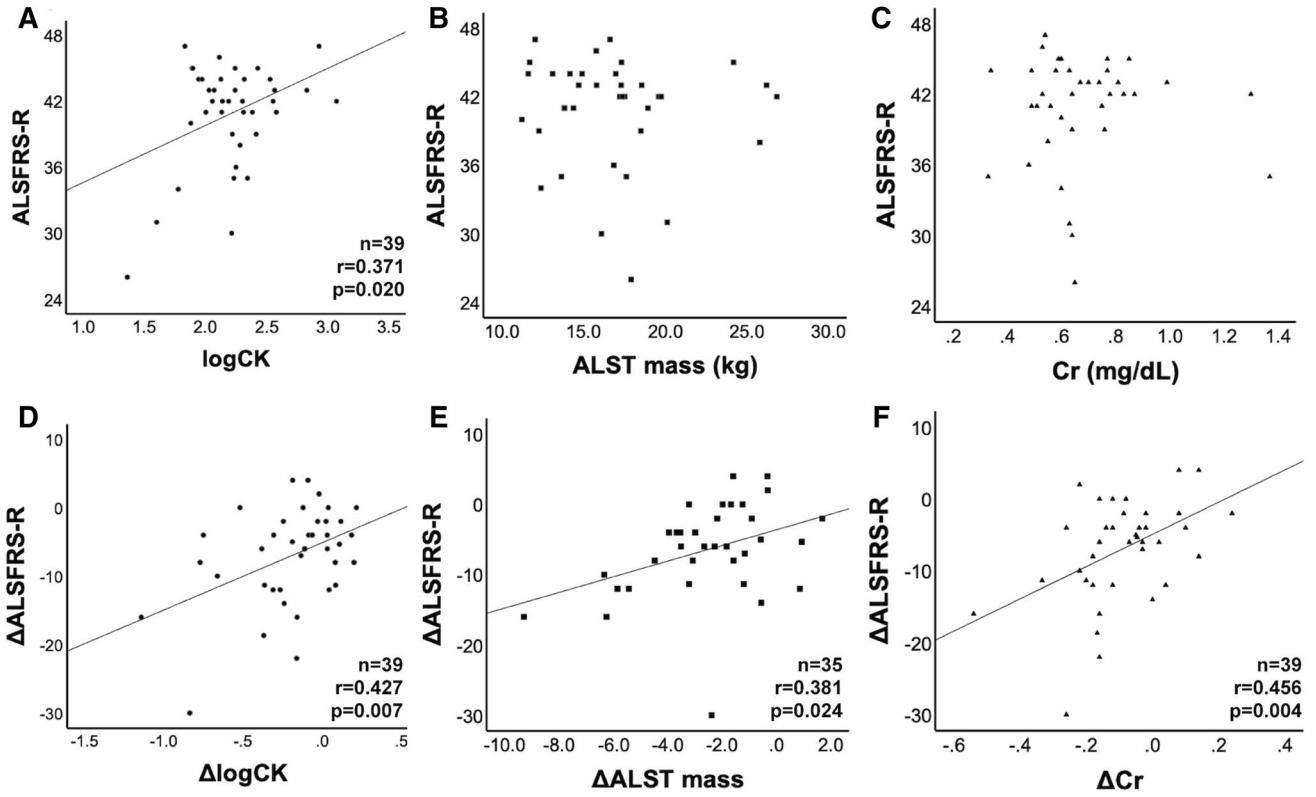

Fig. 2 Correlation between ALSFRS-R and muscle-related biomarkers. **a**–**c** Scatter plots of ALSFRS-R and muscle-related biomarkers [**a**, logCK ($n=39$); **b**, ALST mass ($n=35$); **c**, Cr ($n=39$)] at the initial evaluation. **d**–**f** Scatter plots of the annualized differences of ALSFRS-R and muscle-related biomarkers [**d**, ΔlogCK ($n=39$); **e**, ΔALST mass ($n=35$); **f**, ΔCr ($n=39$)]. Significant correlation coefficients and *p* values are annotated. *ALS* amyotrophic lateral sclerosis, *ALSFRS-R* revised ALS Functional Rating Scale, *ALST* appendicular lean soft tissue, *CK* creatine kinase, *Cr* creatinine, *Δ(delta)* is the annualized difference of each parameter between the initial and final evaluation

Muscle-related biomarkers in an animal model of ALS

The results of the clinical part of the present study led us to conduct biomarker analysis in an animal model of ALS. We examined the changes of muscle-related serum markers, CK and creatinine, in SOD1^{G93A} transgenic mice. We also measured the mRNA levels of *Chrng* and *Musk* in the gastrocnemius muscles of the mice, as they are reportedly elevated with denervation [27]. At week 5, the levels of all indices were equivalent between mutant SOD1 mice and their wild-type littermates (Fig. 5a–d). At week 9, the timing of onset, serum CK levels were elevated and *Chrng* mRNA expression was up-regulated significantly in mutant SOD1 mice compared with wild-type mice (Fig. 5a, c). Although not statistically significant, *Musk* was also up-regulated in SOD1^{G93A} transgenic mice at week 9 (Fig. 5d). At the symptomatic stage, week 15, serum CK levels were decreased in mutant SOD1 mice, despite the continued up-regulation of *Chrng* mRNA (Fig. 5a, c). These results suggest that serum CK starts to be elevated at a very early stage when muscle denervation emerges, and declines with disease progression, consistent with the results of our clinical study.

Discussion

In the present study, we found that the estimated level of serum CK was elevated at the onset of motor symptoms in patients with sporadic ALS. Our study also indicated that other muscle-related markers, i.e., serum creatinine and ALST mass in DXA, were not substantially altered at the early stage, although they declined with disease progression during the symptomatic phase of the disease. These results indicate that serum CK is a potential biomarker that reflects the progression of ALS at the early stage.

Several studies have documented the elevation of serum CK in ALS [31–37]. As for the pathophysiology underlying this phenomenon, an association between active denervation and the elevation of serum CK was demonstrated in a previous electromyographic study [38]. Increased serum CK levels are associated with muscle cramp in patients with

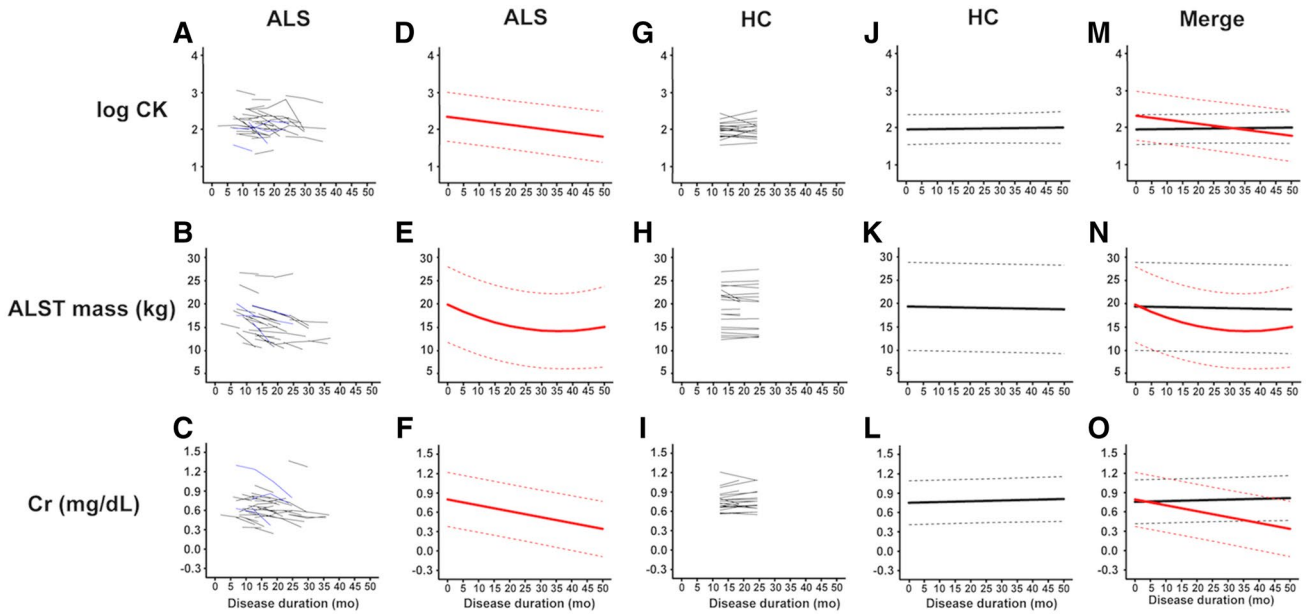

Fig. 3 Raw data and estimated average trajectories of muscle-related biomarkers**. a–c**. Raw data of muscle-related biomarker in subjects with ALS [**a** LogCK ($n=39$); **b** ALST mass ($n=35$); **c** Cr ($n=39$)]. **d–f**. Estimated average trajectories and their confidence intervals in subjects with ALS. **g–l** Raw data and average trajectories in HCs ($n=20$). The date of the initial evaluation of HCs was set at the mean disease duration (12.6 months) of subjects with ALS. **m–o** Merged average trajectories of both groups. Blue lines indicate the data of the subjects for whom retrospective data of serum CK before onset was also analyzed (Patients ALS1, 2, 3, and 4 in Fig. 4). Red lines and red dotted lines demonstrate average trajectories and their 95% confidence intervals in subjects with ALS. Black trajectories and black dotted lines demonstrate average trajectories and their 95% confidence intervals of HCs. *ALS* amyotrophic lateral sclerosis, *ALST* appendicular lean soft tissue, *CK* creatine kinase, *Cr* creatinine, *HC* healthy control, *mo* months

Table 3 Estimated values of muscle-related biomarkers at onset

	ALS		HC ($n=20$)	
	Estimation	95% CI	Estimation	95% CI
Log CK	2.34 ($n=39$)	1.68–3.00	1.97	1.57–2.37
ALST mass (kg)	19.91 ($n=35$)[a]	11.73–28.09	19.32	9.76–28.89
Cr (mg/dL)	0.80 ($n=39$)	0.38–1.22	0.75	0.41–1.09

The values were calculated using a linear mixed model with unstructured correlation and random intercepts

ALS amyotrophic lateral sclerosis, *ALST* appendicular lean soft tissue, *CI* confidence interval, *CK* creatine kinase, *Cr* creatinine, *HC* healthy controls

[a]Longitudinal data of body compositions was not available in 4 subjects with ALS

ALS [33] and Guillain–Barre syndrome [39]. Furthermore, denervation reportedly induces membrane instability in muscle tissue and leakage of CK into blood in vivo [40]. Taken together, the elevation of serum CK at the early stage in ALS appears to be caused by membrane instability or the destruction of muscle tissue due to the denervation and hyperexcitability of motor neurons.

We also revealed that mutant SOD1 mice had increased serum CK levels, together with up-regulation of *Chrng*, at the onset of motor dysfunction. *Chrng* encodes the gamma subunit of the acetylcholine receptor protein, which is up-regulated upon muscle denervation, together with *Musk*. Given a previous report on denervation and compensative reinnervation in skeletal muscle of mutant SOD1 mice at their preclinical stage [41], our findings suggest that the elevated levels of CK likely reflect denervation at the preclinical stage of ALS.

However, it is also possible that other factors are involved in the elevation of serum CK. Several reports have suggested that serum CK can be elevated by energetic compensatory changes of muscle metabolism, as CK is an enzyme that phosphorylates creatine for the contraction of muscle fibers [30, 36]. The level of serum CK is also influenced by physical activity [42]. Although ALS is classified as a motor neuron disease, a primary change of muscle is also suggested [43]. For example, microRNA-206, which regulates histone deacetylase 4 (HDAC4), is up-regulated in skeletal muscle and has a protective effect on disease progression in a mouse model of ALS [44]. The relative mRNA expression level of HDAC4 in skeletal muscle of ALS patients reportedly correlates with disease progression [45]. Alternatively, the elevation of CK might reflect the difference in the proportion of type 1 and 2 muscle fibers affected. In mutant SOD1 mice, the selective vulnerability of fast-fatigable motor neurons,

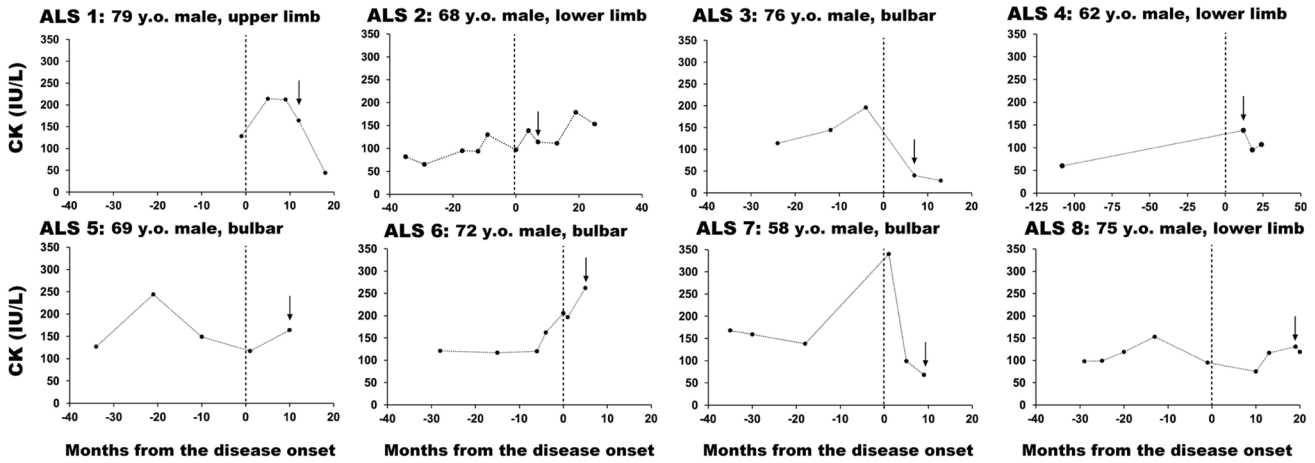

Fig. 4 Retrospective data of serum CK levels in 8 subjects with ALS. Serum CK levels in 8 subjects with sporadic ALS, for whom serum CK levels had been measured for the purpose of medical practice other than ALS. Age, sex, and the site of onset were described above each graph. Vertical dotted lines demonstrate the timing of the first symptom of weakness and arrow heads demonstrate the timing of the initial evaluation. *ALS* amyotrophic lateral sclerosis, *CK* creatine kinase

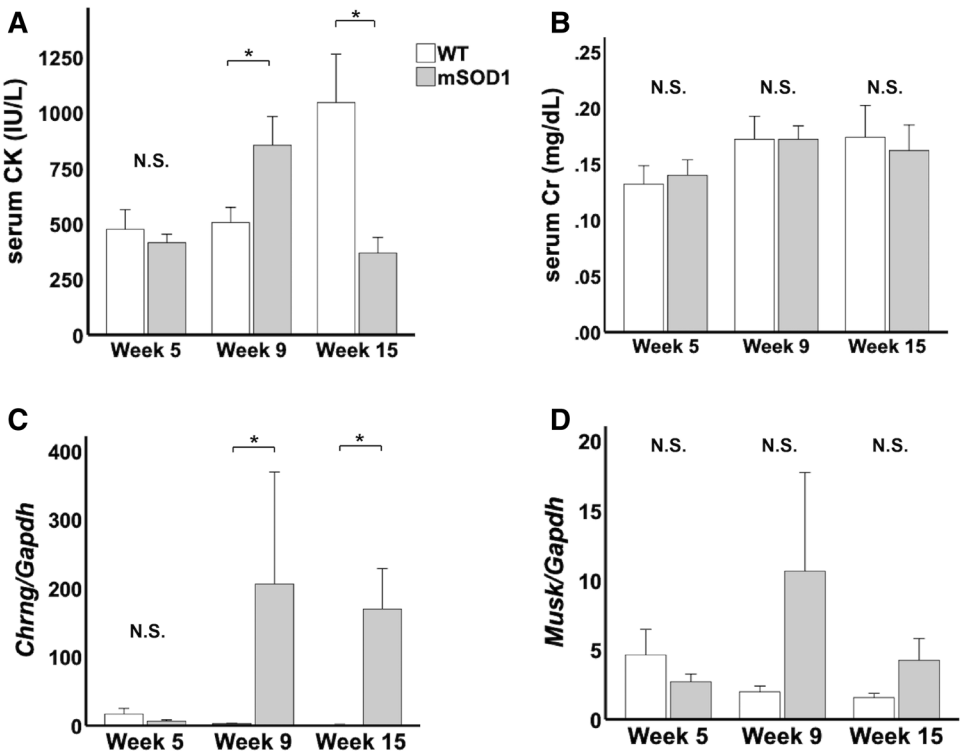

Fig. 5 Serum CK, creatinine, and mRNA expression of denervation markers in SOD1^{G93A} transgenic mice and their littermates. Serum CK (**a**), serum creatinine (**b**), and *Chrng* (**c**) and *Musk* (**d**) mRNA levels in the gastrocnemius muscles (wild-type, male, $n=5$; SOD1^{G93A}, male, $n=5$). $^{*}p<0.05$. Data are presented as the mean ± standard error. *CK* creatine kinase, *Cr* creatinine, *Chrng* acetylcholine receptor subunit γ, *Gapdh* glyceraldehyde-3-phosphate dehydrogenase, *mSOD1* SOD1^{G93A} transgenic mice, *Musk* muscle-associated receptor tyrosine kinase, *NS* not significant, *WT* wild-type mice

which innervate type 2b muscle fibers, is observed in the pre-symptomatic stage [41]. Given that the enzyme activity of CK is higher in type 2 fibers [46], the proportion of affected myofiber types might be another determinant of CK elevation and motor function at the early stage of ALS.

The decrease of serum CK levels with disease progression both in patients and animals in the present study appears to be associated with the loss of muscle volume, given the progressive decline in serum creatinine levels and ALST mass in DXA of sporadic ALS patients. Serum creatinine levels and ALST in DXA are both regarded as clinical biomarkers for the skeletal muscle mass of patients with neuromuscular disorders including ALS [24]. However, it is of note that the estimated values of these indices were not decreased at the onset of sporadic ALS, suggesting that muscle volume is not an appropriate biomarker

at the early stage, although they can be used to monitor disease progression at the symptomatic stage of ALS. This view is in accordance with a previous observation that there is little muscle atrophy at disease onset when motor neuron loss is supposed to have already begun [43].

There are some reports describing a change of lipid metabolism in ALS [20, 47, 48]. A preclinical study of Swedish ALS patients demonstrated elevation of LDL-C and HDL-C 10 years before the disease onset, being more conspicuous in female [20]. However, our cohort study did not show a change of HDL-C or LDL-C in ALS patients at the baseline, although prospective evaluation showed a longitudinal increase in LDL-C levels. The disagreement between our study and previous report may be explained by several factors. Our study population was relatively male dominant. The level of LDL-C in our study tended to be higher in 14 female patients than in the male patients (data not shown), and this might have influenced the results. Furthermore, a difference in genetic background might be another explanation for the discrepancy of cholesterol, considering that the proportion of sporadic ALS patients who carry the hexanucleotide repeat expansion of C9orf72 is 0.4% in Japan, which is rather small compared to patients in Western countries (3–21%) [49]. As for the longitudinal elevation of LDL-C, it was accompanied by longitudinal elevations of AST, ALP, and T-bil. This might reflect dyslipidemia and steatosis of liver, both of which have been observed during progression of ALS [47, 48]. The present study has several limitations. First, as the number of subjects with ALS in the present study was small, selection bias could not be excluded. Second, the estimation of values was based on a statistical model, even though we confirmed that estimation using retrospective data from patients was in agreement with the animal experimental data. Moreover, we did not analyze comprehensive changes of metabolism in ALS patients. More detailed analysis should be performed focused on creatine and its related metabolism to discuss the changes of creatinine and CK. A further large-scale study is required to assess the preclinical changes in subjects with ALS.

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Author Contributions DI, AH, and MK conceived and designed the study. DI, AH, YH, SY, YK, and HM contributed to the acquisition of clinical data. DI, MI, and YI performed animal experiments. DI and MK performed analysis and interpretation of the data. AH performed statistical analysis. DI drafted the manuscript, and AH and MK revised it for intellectual content.

Compliance with ethical standards

Conflicts of interest The authors have no relevant conflicts of interest to report.

Ethical standards This study was performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments, the Ethics Guidelines for Human Genome/Gene Analysis Research, and the Ethical Guidelines for Medical and Health Research Involving Human Subjects endorsed by the Japanese government. This study was approved by the Ethics Review Committee of Nagoya University Graduate School of Medicine (Nos. 2013–0035 and 2015–0041), and all participants gave written informed consent before participation. All of the animal experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and under the approval of the Nagoya University Animal Experiment Committee (No. 29170).

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