

RESEARCH ARTICLE

Impaired muscle uptake of creatine in spinal and bulbar muscular atrophy

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Abstract

Objective: The aim of this study was to explore the pathomechanism underlying the reduction of serum creatinine (Cr) concentrations in spinal and bulbar muscular atrophy (SBMA). **Methods:** We evaluated blood chemistries, motor function, and muscle mass measured by dual-energy X-ray absorptiometry in male subjects with SBMA ($n = 65$), amyotrophic lateral sclerosis (ALS; $n = 27$), and healthy controls ($n = 25$). We also examined the intramuscular concentrations of creatine, a precursor of Cr, as well as the protein and mRNA expression levels of the creatine transporter (SLC6A8) in autopsy specimens derived from subjects who had SBMA and ALS and disease controls. Furthermore, we measured the mRNA expression levels of *SLC6A8* in cultured muscle cells (C2C12) transfected with the polyglutamine-expanded androgen receptor (AR-97Q). **Results:** Serum Cr concentrations were significantly lower in subjects with SBMA than in those with ALS ($P < 0.001$), despite similar muscle mass values. Intramuscular creatine concentrations were also lower in with the autopsied specimen of SBMA subjects than in those with ALS subjects ($P = 0.018$). Moreover, the protein and mRNA expression levels of muscle *SLC6A8* were suppressed in subjects with SBMA. The mRNA levels of *SLC6A8* were also suppressed in C2C12 cells bearing AR-97Q. **Interpretation:** These results suggest that low serum Cr concentration in subjects with SBMA is caused by impaired muscle uptake of creatine in addition to being caused by neurogenic atrophy. Given that creatine serves as an energy source in skeletal muscle, increasing muscle creatine uptake is a possible therapeutic approach for treating SBMA.

Introduction

Spinal and bulbar muscular atrophy (SBMA), or Kennedy's disease, is an adult-onset, slowly progressive neuromuscular disease characterized by bulbar and limb muscle weakness.^{1–5} SBMA is caused by the expansion of a CAG repeat, encoding a polyglutamine tract, within the first exon of the androgen receptor (AR) gene.⁶ Ligand-dependent nuclear accumulation of pathogenic AR proteins is central to the molecular pathogenesis of SBMA, which provides a potential target for therapeutic intervention.^{7,8} Several studies have indicated the involvement of skeletal muscle in the pathogenesis of SBMA. Patients with SBMA have elevated serum concentrations of creatine kinase (CK), and their skeletal muscle biopsies have shown both neurogenic and myopathic changes.^{2,9} In addition, an investigation using a knock-in mouse model of SBMA demonstrated that skeletal muscle pathology precedes neurodegeneration.¹⁰

We previously reported that the degree of decrease in serum creatinine (Cr) levels reflects the severity of motor dysfunction in subjects with SBMA.¹¹ Creatine, the precursor of Cr, is mostly present in the skeletal muscle tissue. It is absorbed into the muscle tissue from the blood by a creatine transporter (SLC6A8) against a large concentration gradient. Intramuscular creatine and phosphocreatine are then nonenzymatically converted to Cr at a near steady rate. Subsequently, Cr is excreted by the kidneys into the urine.¹² Serum Cr concentrations are, therefore, construed as an index of skeletal muscle mass. However, it remains unclear whether the serum Cr decrease observed in subjects with SBMA simply results from the loss of muscle mass or whether it is associated with the molecular pathology of the disease.

Herein, we investigate the pathophysiology underlying decreased serum Cr concentrations in SBMA. To address this issue, we analyzed the relationship between serum Cr concentration and markers of muscle mass in subjects with SBMA and amyotrophic lateral sclerosis (ALS) and healthy controls. Furthermore, we examined the mRNA expression levels of SLC6A8 in human autopsied muscle and in a cultured muscle cell model of SBMA.

Methods

Standard protocol approvals, registrations, and participant consent

This study was conducted according to the Ethics Guidelines for Human Genome/Gene Analysis Research and according to the Epidemiological Studies endorsed by the Japanese government. This study was approved by the Ethics Committee of Nagoya University Graduate School of Medicine, and all participants gave their written informed consent before participation.

Study population

We studied 65 consecutive subjects with SBMA. All subjects were genetically confirmed to have SBMA. Subjects were excluded from the study if they fulfilled any of the following criteria: (1) severe complications such as malignancy, heart failure, and renal failure; (2) other neurological complications; (3) use of a hormonal agents or creatine monohydrate supplements within 48 weeks before providing informed consent; or (4) participation in any other clinical trial. Study results were compared with findings from subjects with ALS ($n = 27$), which was clinically diagnosed using the El Escorial Criteria of definite to probable ALS, and with healthy controls ($n = 25$). All subjects with SBMA, ALS, and healthy controls were Japanese males and were observed at the Nagoya University Hospital between June 2013 and April 2015.

Biochemical parameters

The measured biochemical parameters included serum Cr, creatine, and cystatin C (CysC). Although serum Cr concentration is known to correlate well with lean body mass in healthy individuals,¹³ it is also commonly used as an index of renal function. CysC is another marker of renal function, but it is less affected by the muscle mass than Cr.¹⁴ We, therefore, calculated the serum Cr/CysC ratio to minimize the effect of renal function on serum Cr concentrations.¹⁵

Motor function

We assessed SBMA disease severity using the following functional parameters: (1) the revised Amyotrophic Lateral Sclerosis Functional Rating Scale (ALSFRS-R), a validated questionnaire-based functional scale that is also generally applicable to SBMA;¹⁶ (2) the SBMA Functional Rating Scale (SBMAFRS), a validated disease-specific scale that has a high sensitivity to disease progression in SBMA¹⁷; and (3) the quantitative myasthenia gravis (QMG) score, a quantitative measurement used to detect the fatigue of enduring muscle power.¹⁸ We used a protocol from the QMG score that evaluates the muscle power of the limb and neck flexion as a "modified QMG score."⁵ Grip power was measured using an electronic hand dynamometer. A timed walking test was also used to measure the time required to walk a distance of 15 feet.

Evaluation of skeletal muscle mass

We evaluated skeletal muscle mass using dual-energy X-ray absorptiometry (DXA). DXA is a widely used quantitative method for the evaluation of body composition.¹⁹ The body composition of all subjects was measured with

DXA using fan-beam technology (Discovery A; Hologic Inc., Bedford, MA). We calculated the sum of appendicular lean soft tissue (ALST) mass, assuming that skeletal muscle mass and lean soft tissue in the extremities are approximately equal.^{20,21} In addition, ALST mass measured with DXA has been previously validated by skeletal muscle mass measurement using magnetic resonance imaging and computed tomography.^{22,23} Moreover, ALST mass has been highly correlated with total body skeletal muscle in healthy subjects.²¹ Therefore, in this study, we used ALST mass as an index of skeletal muscle mass.

Measurement of intramuscular creatine concentrations

We analyzed intramuscular creatine concentrations in autopsied skeletal muscle specimens derived from five subjects who had SBMA (age: mean \pm standard deviation [SD] = 74.0 \pm 5.3 years), five subjects who had ALS (67.8 \pm 3.2 years), and five subjects who had other diseases (73.8 \pm 6.1 years), including progressive supranuclear palsy ($n = 2$), Parkinson's disease ($n = 1$), Lewy body dementia ($n = 1$), and multiple system atrophy ($n = 1$). There were no statistically significant differences in patient's age at the time of examination among SBMA, ALS, and disease controls (DCs). Neither were there significant differences in the post-mortem intervals from death to autopsy among the groups (SBMA, 512.6 \pm 374.8 min; ALS, 648.4 \pm 494.9 min; and DC, 403.2 \pm 339.5 min [mean \pm SD]). Individual 100 mg muscle samples were homogenized in 1 mL of CellLytic lysis buffer (Sigma-Aldrich, St. Louis, MO) containing a protease inhibitor cocktail (Thermo Scientific, Waltham, MA). The samples were then centrifuged at 2500g for 15 min. The resultant supernatant was used for analyzing the concentrations of creatine using an enzymatic method at LSI Medience Co. (Tokyo, Japan). Moreover, using the same method, we also analyzed intramuscular creatine concentrations in skeletal muscle specimens derived from the wild-type mice ($n = 8$) and AR-97Q mice ($n = 8$), a transgenic mouse model of SBMA.⁷

Immunoblotting

We analyzed SLC6A8 expression in autopsied iliopsoas muscle specimens from subjects who had SBMA ($n = 3$; 74.0 \pm 6.9 years), ALS ($n = 3$; 68.6 \pm 2.5 years), and non-neuromuscular diseases ($n = 3$; 71.6 \pm 7.5 years) using immunoblotting technique as described in Methods section in Data S1. The DC group included subjects with Lewy body dementia, Parkinson's disease, or multiple system atrophy. We also analyzed SLC6A8 expression in cultured muscle cells (mouse C2C12 myoblast cells; DS Pharma Biomedical, Osaka, Japan) stably expressing a

full-length human AR (AR-24Q or AR-97Q), as indicated in the Methods section in Data S1.

Immunohistochemistry

SLC6A8 expression was examined by immunohistochemistry of the iliopsoas muscle samples derived from the subjects who had SBMA, ALS, and other diseases ($n = 3$ for each group) as described in the Methods section in Data S1. The DC group included subjects with Lewy body dementia, progressive supranuclear palsy, and Sjogren's syndrome.

Quantitative real-time polymerase chain reaction (RT-PCR)

Individual 50 mg frozen autopsied samples of muscle (the same as those for immunoblotting) and the C2C12 cells stably expressing a full-length human AR (AR-24Q or AR-97Q) were homogenized. The total RNA was extracted using the TRIzol method (Invitrogen, Carlsbad, CA).^{24,25} For details on RT-PCR, see the Methods section in Data S1.

Genetic analysis

Genomic DNA was extracted from peripheral blood samples of subjects with SBMA using conventional techniques. The PCR amplification of the CAG repeat of AR was performed using a fluorescein-labeled forward primer (5'-TCCAGAATCTGTTCCAGAGCGTGC-3') and an unlabeled reverse primer (5'-TGGCCTCGCTCAGGATGTCTTTAA G-3'). The detailed PCR conditions have been previously described.²⁶

Statistical analysis

We used unpaired Student's *t*-test for the comparison of continuous variables between two groups, analysis of variance with Tukey's post hoc tests for multiple comparisons, and Pearson correlation coefficient for analyzing correlations among parameters. Analysis of covariance (ANCOVA) was performed to adjust the data for a covariate. We considered $P < 0.05$ as significant and correlation coefficients (r) greater than 0.3 as strong. The Statistical Package for the Social Sciences (SPSS) 23.0J software (IBM Japan, Tokyo, Japan) was used to perform statistical analysis.

Results

Clinical backgrounds and biochemical parameter values

The clinical features and biochemical parameter values of subjects with SBMA, ALS, and those of healthy controls

are presented in Tables 1 and 2, respectively. The mean age at examination was higher in subjects with ALS than in those with SBMA, and the mean disease duration was shorter in subjects with ALS than in those with SBMA. However, motor function was similar between the two groups. Serum concentrations of CK and liver enzymes were higher in subjects with SBMA than in the other groups. The characteristics of subjects with SBMA, such as age at examination, age at onset, and AR CAG repeat size, were similar to those in previous reports.^{27,28}

Correlation of serum Cr concentration with motor function parameters and markers of muscle mass in SBMA

To verify whether serum Cr concentration reflects the clinical severity of SBMA, we evaluated possible correlations between serum Cr concentration and various parameters. The results demonstrated strong correlations between serum Cr concentration and functional indices in subjects with SBMA (Fig. S1A–E). Furthermore, the

serum Cr/CysC ratio, which corresponds to serum Cr adjusted for renal function,¹⁵ was also strongly correlated with motor function parameters, although no correlations were found between functional parameters and CysC (Table S1). In contrast, there were no strong correlations between serum Cr concentration and clinical features such as CAG repeat length ($r = -0.132$; $P = 0.296$), body mass index ($r = -0.037$; $P = 0.769$), or age at examination ($r = -0.030$; $P = 0.812$). Although we found that ALST mass was correlated with both grip power and the timed walking test in subjects with SBMA (Fig. S2D and E), the muscle mass did not correlate with the other functional parameters such as ALSFRS-R, SBMAFRS, or with the modified QMG score (see Fig. S2A–C).

Comparison of serum Cr concentration and markers of muscle mass between SBMA and other groups

Next, we evaluated possible relationships between serum Cr concentration and ALST mass in patients with SBMA

Table 1. Clinical parameters of subjects with SBMA, ALS, and healthy controls.

| | SBMA ($n = 65$) | ALS ($n = 27$) | Healthy controls ($n = 25$) |
|-----------------------------------------|---------------------------|---------------------------|-------------------------------|
| CAG repeat size in AR gene | 47.7 ± 3.6 (42–55) | NA | NA |
| Age at examination, year | 54.1 ± 9.8 (33–76) | 63.9 ± 8.5 (41–78) | 55.9 ± 11.6 (34–76) |
| Duration from onset, year | 9.4 ± 6.2 (0–29) | 1.7 ± 1.3 (0–5) | NA |
| Age at onset, year | 44.5 ± 10.4 (26–68) | 62.0 ± 8.6 (36–75) | NA |
| Height, cm | 169.8 ± 6.1 (152.9–183.5) | 165.1 ± 8.2 (154.3–182.9) | 165.6 ± 5.9 (154.3–177.3) |
| Weight, kg | 65.5 ± 10.3 (43.0–100.5) | 60.7 ± 10.0 (38.2–79.5) | 62.9 ± 8.4 (46.7–79.3) |
| Body mass index | 22.7 ± 3.5 (12.8–35.6) | 22.1 ± 2.7 (15.6–27.0) | 22.8 ± 2.6 (17.4–27.6) |
| ALSFRS-R | 40.4 ± 3.7 (28–47) | 38.4 ± 7.3 (11–47) | 48.0 ± 0.0 (48) |
| SBMAFRS | 40.6 ± 6.4 (19–53) | 41.4 ± 10.3 (12–60) | 56.0 ± 0.0 (56) |
| Modified QMG score | 5.9 ± 3.1 (0–15) | 6.1 ± 3.5 (0–13) | NC |
| Grip power, ¹ kg | 20.5 ± 5.4 (7.5–33.2) | 20.7 ± 11.4 (0.0–48.7) | 39.9 ± 7.7 (25.1–52.4) |
| Timed walking (15 ft), ² sec | 4.0 ± 1.7 (1.77–9.08) | 4.9 ± 4.9 (1.25–21.90) | 2.1 ± 0.3 (1.51–3.06) |

Data are shown as mean ± SD. AR, androgen receptor; ALS, amyotrophic lateral sclerosis; ALSFRS-R, revised amyotrophic lateral sclerosis functional rating scale; NA, not applicable; NC, not calculated; QMG, quantitative myasthenia gravis; SBMA, spinal and bulbar muscular atrophy; SBMAFRS, spinal and bulbar muscular atrophy functional rating scale; ALSFRS-R normal value = 48, SBMAFRS normal value = 56.

¹The mean value for both hands.

²One subject with SBMA and two subjects with ALS were not able to perform the test.

Table 2. Blood chemistry values of subjects with SBMA, ALS, and healthy controls.

| | SBMA ($n = 65$) | ALS ($n = 27$) | Healthy controls ($n = 25$) |
|------------------------------|--------------------------|------------------------|-------------------------------|
| Total protein, g/dL | 7.3 ± 0.3 (6.4–8.2) | 6.9 ± 0.4 (6.0–7.8) | 7.2 ± 0.3 (6.7–8.1) |
| Albumin, g/dL | 4.5 ± 0.2 (3.8–5.4) | 4.2 ± 0.4 (3.0–5.4) | 4.5 ± 0.3 (3.5–5.3) |
| Total cholesterol, mg/dL | 211.9 ± 30.0 (140–277) | 202.6 ± 26.8 (159–252) | 201.5 ± 44.0 (120–316) |
| Triglyceride, mg/dL | 155.8 ± 81.1 (42–406) | 135.5 ± 71.8 (53–396) | 148.6 ± 117.2 (43–571) |
| Aspartate transaminase, IU/L | 44.4 ± 18.2 (16–106) | 23.6 ± 6.0 (14–36) | 22.1 ± 7.2 (9–46) |
| Alanine transaminase, IU/L | 51.6 ± 30.2 (8–156) | 24.5 ± 12.6 (9–60) | 20.0 ± 7.0 (9–42) |
| Creatine kinase, IU | 1021.5 ± 680.2 (84–3064) | 200.7 ± 141.1 (23–675) | 122.2 ± 40.4 (43–216) |

Data are shown as mean ± SD. ALS, amyotrophic lateral sclerosis; SBMA, spinal and bulbar muscular atrophy.

and ALS, and in healthy controls. In all these groups, strong correlations were found between serum Cr concentration and muscle mass (Fig. 1A and B); however, ALST mass-adjusted serum Cr concentration was significantly reduced in subjects with SBMA when compared with healthy controls ($P < 0.001$ by ANCOVA). In contrast, no significant difference in ALST mass-adjusted serum Cr concentration was observed between ALS and healthy controls ($P = 0.127$). Thus, these findings indicate that there might be other underlying causes for the markedly low concentrations of serum Cr in subjects with SBMA, which needs further exploration.

Creatine–Cr metabolism in subjects with SBMA and other diseases

To clarify the basis for decreased serum Cr in subjects with SBMA, we compared creatine–Cr metabolism in subjects with SBMA with that in ALS and healthy controls. Both muscle mass measured with ALST and serum Cr concentration were significantly lower in subjects with SBMA and ALS than in healthy controls (Fig. 2A and B). Serum Cr concentration was significantly lower in subjects with SBMA than in subjects with ALS, despite there being no significant difference in muscle mass between these two groups (Fig. 2A and B). To further clarify the difference in creatine–Cr metabolism between SBMA and other groups, we evaluated serum creatine concentration. The concentrations of serum creatine in subjects with SBMA were significantly higher than those in subjects

with ALS and in healthy controls (Fig. 2C). Next, we investigated whether perturbation of intramuscular creatine–Cr metabolism contributes to the observed changes in serum levels of Cr and creatine in SBMA. Intramuscular creatine was significantly decreased in SBMA compared with ALS (Fig. 2D). We further confirmed the decreased intramuscular creatine in AR-97Q mice, a transgenic mouse model of SBMA, compared with the wild-type mice (Fig. 2E). Together, these findings suggest that muscle creatine uptake is hampered in case of SBMA.

Impaired muscle uptake of creatine in SBMA

To elucidate the mechanisms of decreased muscle creatine concentrations in SBMA, we analyzed SLC6A8 expression in the skeletal muscle of subjects with SBMA, ALS, and non-neuromuscular diseases. Immunohistochemistry revealed that the expression of SLC6A8 in the skeletal muscle was attenuated in the skeletal muscle of subjects with SBMA compared with that in subjects with ALS and DCs (Fig. 3A and B). This reduction in skeletal muscle SLC6A8 in SBMA was also confirmed by immunoblotting (Fig. 3C and D). The protein expression levels of SLC6A8 correlated with intramuscular creatine concentrations among the examined subjects (Fig. S3). Furthermore, quantitative RT-PCR analyses showed that the mRNA levels of *SLC6A8* were lower in subjects with SBMA than in those with ALS and in DCs, suggesting that a transcriptional deficit may contribute to the reduction of

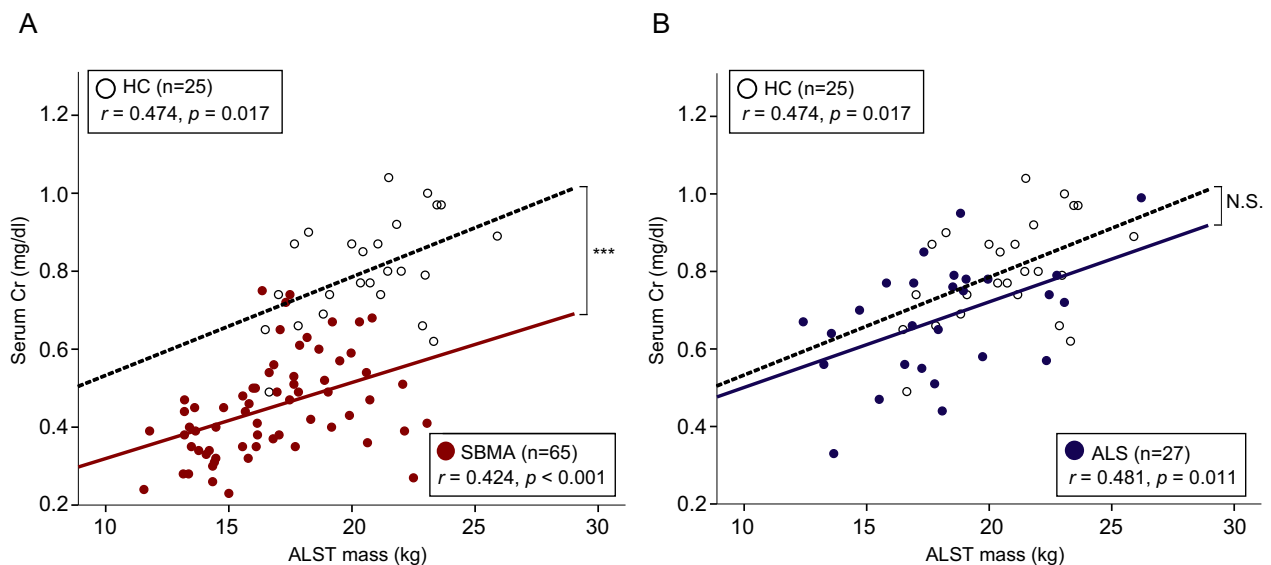


Figure 1. Relationship between serum creatinine and muscle mass. (A, B) Comparison of the relationship between serum Cr concentration and ALST mass in SBMA, ALS, and healthy controls. N.S., not significant, $***P < 0.001$ by analysis of covariance. ALS, amyotrophic lateral sclerosis; ALST, appendicular lean soft tissue; Cr, creatinine; SBMA, spinal and bulbar muscular atrophy; HC, healthy controls.

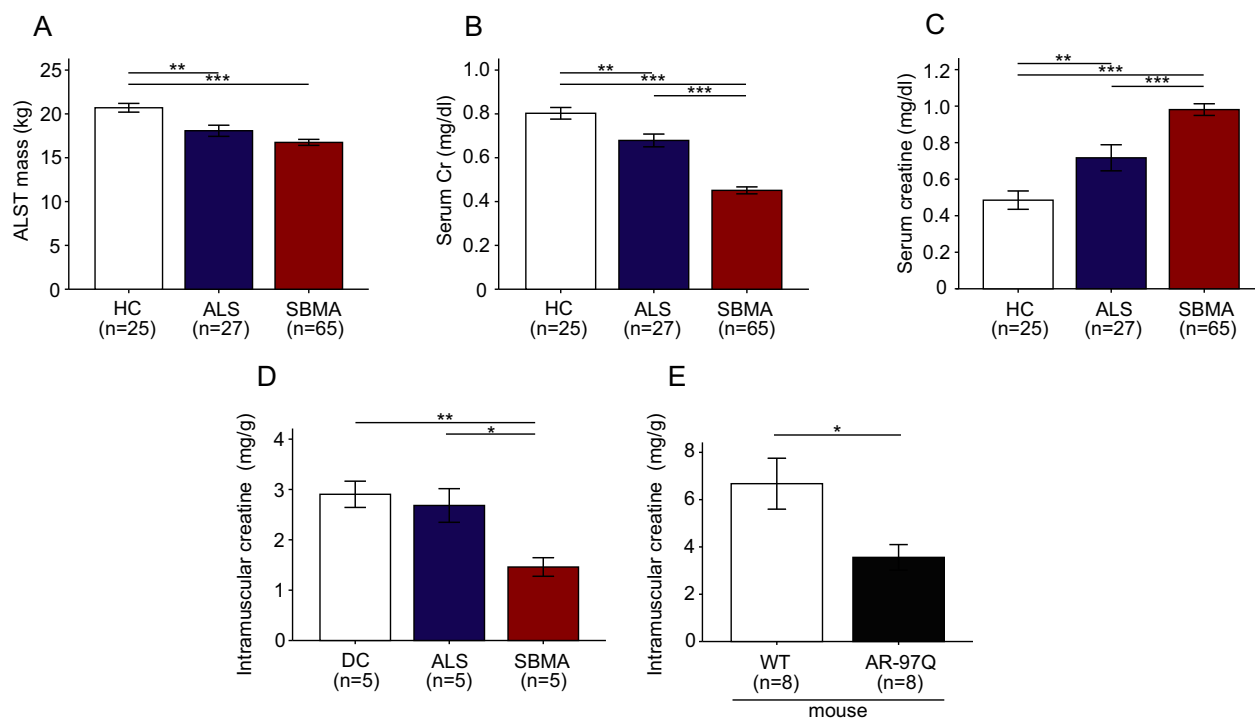


Figure 2. Muscle mass and creatine–creatinine metabolism in subjects with SBMA, ALS, and controls. The mean value of ALST mass (A), serum Cr (B), and serum creatine (C) in subjects with SBMA, ALS, and in healthy controls. (D) The intramuscular creatine in subjects with SBMA, ALS, and in disease controls. (E) The intramuscular creatine in the wild-type mice and AR-97Q mice, a transgenic mouse model of SBMA. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ by Tukey's multiple comparison test. Data depict the mean \pm SE values. ALS, amyotrophic lateral sclerosis; AR, androgen receptor; ALST, appendicular lean soft tissue; Cr, creatinine; DC, disease controls; SBMA, spinal and bulbar muscular atrophy; HC, healthy controls.

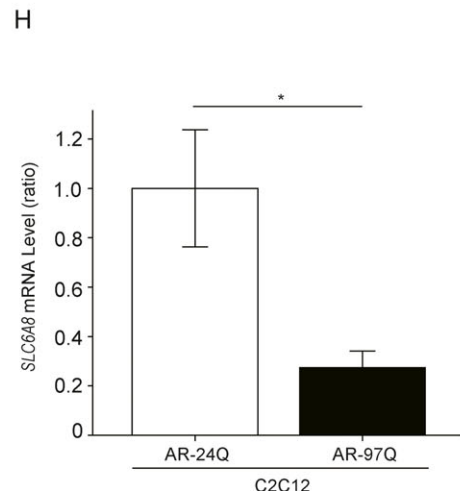
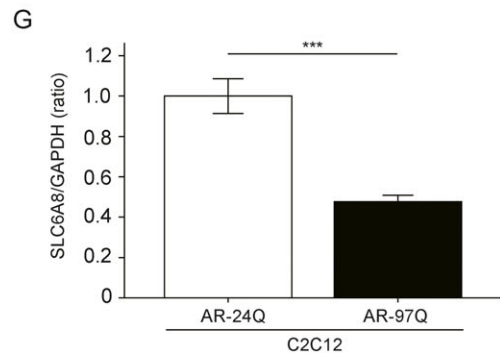
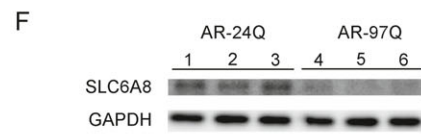
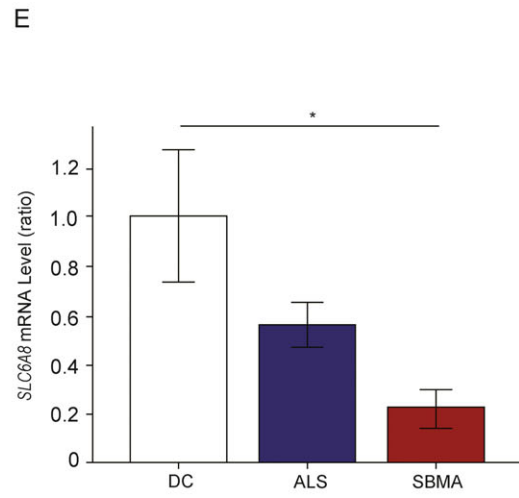
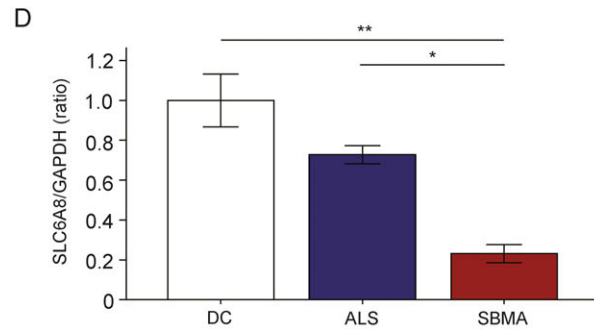
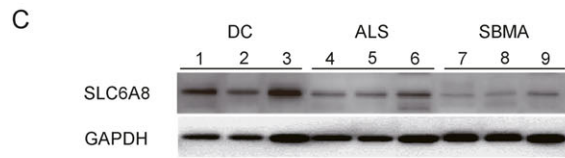
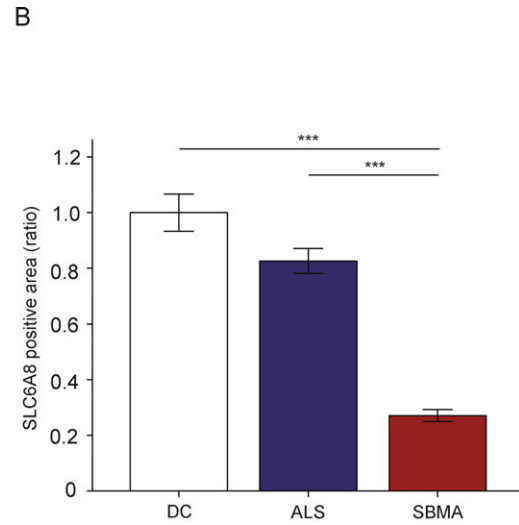
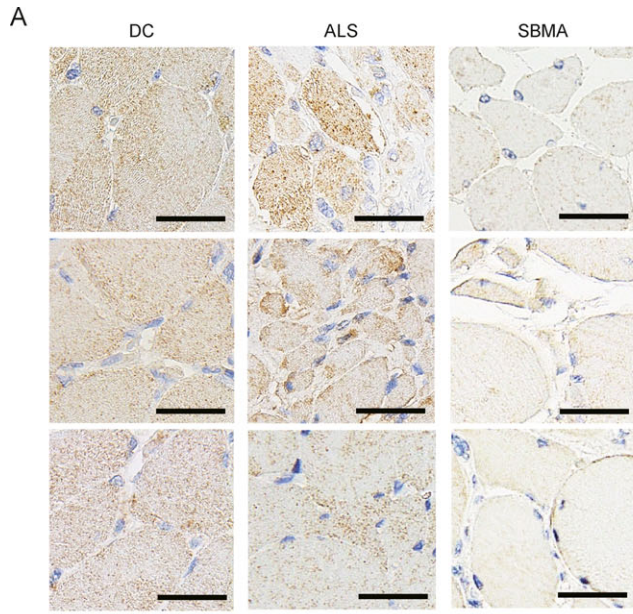
SLC6A8 protein expression levels and to an eventual intramuscular depletion of creatine in SBMA (Fig. 3E). To examine whether the toxicity of pathogenic AR is related to the decrease in *SLC6A8* expression in skeletal muscle, we performed immunoblotting of cultured muscle cells (C2C12) stably expressing human AR with or without the polyglutamine expansion (AR-97Q or AR-24Q, respectively) treated with dihydrotestosterone. The results showed that the SLC6A8 protein levels were lower in the C2C12 stably expressing AR-97Q than in the cells expressing AR-24Q (Fig. 3F and G). This difference was

undetectable when the cells were not treated with testosterone (data not shown). We confirmed that mRNA expression levels of *SLC6A8* were decreased in the C2C12 cells expressing AR-97Q by performing quantitative RT-PCR (Fig. 3H).

Discussion

In this study, we found that the uptake of creatine in the muscle is attenuated in SBMA because of the downregulation of SLC6A8, providing a molecular basis for the

Figure 3. SLC6A8 expression in human subjects and muscular cells. (A) Representative microphotographs of autopsied iliopsoas muscle specimens immunostained for SLC6A8. (B) Quantitative analysis of anti-SLC6A8 immunohistochemistry in SBMA, ALS, and other disease controls ($n = 3$ in each group). (C, D) Immunoblotting with quantitative analysis of SLC6A8 protein expression obtained from autopsied muscle specimens of controls (lanes 1, 2, and 3), ALS (lanes 4, 5 and 6), and SBMA (lanes 7, 8, and 9). (E) Quantitative RT-PCR analysis of muscle *SLC6A8* gene expression levels in subjects with SBMA, ALS, and DC ($n = 3$ in each group). (F) Immunoblotting of SLC6A8 protein expression in C2C12 cells stably expressing full-length AR-24Q (lanes 1, 2, and 3) or AR-97Q (lanes 4, 5 and 6). (G) Quantitative analysis of SLC6A8 protein expression in C2C12 cells ($n = 6$). (H) *SLC6A8* gene expression levels of full-length AR-24Q or AR-97Q-expressing C2C12 cells ($n = 3$ in each group). Data depict the mean \pm SE values. ALS, amyotrophic lateral sclerosis; ALST, appendicular lean soft tissue; AR, androgen receptor; Cr, creatinine; DC, disease controls; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; SBMA, spinal and bulbar muscular atrophy. Scale bar: 25 μ m. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ by Tukey's multiple comparison test.



decreased levels of serum Cr observed in the subjects with this disease.

Our analysis on the relationship between biomarker and clinical phenotype confirmed the strong association of serum Cr concentration with the severity of motor dysfunction, as was reported in our previous study.¹¹ Furthermore, we found that serum Cr concentrations in subjects with SBMA correlated well with muscle mass as measured by DXA. One of the major advantages of using serum Cr as a biomarker is that the measurement is simple, inexpensive, and noninvasive, enabling repeated examination and longitudinal follow-up. In addition, our previous longitudinal study demonstrated a steady decrease in serum Cr concentrations along with SBMA progression.¹¹ The results from the present study indicated that serum Cr is a marker of muscle mass not only in SBMA, but also in ALS and healthy controls. In support of these findings, several studies have demonstrated that serum Cr concentrations correlate with lean body mass in healthy individuals and patients with various diseases.^{13,29,30} Similarly, it has been reported that serum Cr concentrations correlate with both the severity of motor impairment and the fat-free mass in patients with ALS.³¹ To verify the reliability of the biomarker, test–retest reproducibility of serum Cr needs to be confirmed in future analysis.

Our results further indicated that low serum Cr concentrations in subjects with SBMA could not be explained simply by neurogenic amyotrophy, given that serum Cr concentrations were lower in subjects with SBMA than in those with ALS, although there was no significant difference in muscle mass between these groups. The serum Cr concentrations after adjustment for muscle mass in subjects with SBMA were lower than in healthy controls. In contrast, this finding was not observed in subjects with ALS, although the number of examined subjects was smaller than that in the SBMA group. In addition, we found that the intramuscular concentrations of creatine were lower in subjects with SBMA than those in subjects with ALS. These differences are probably due to impaired creatine uptake in the skeletal muscle of subjects with SBMA. The results of this study showed that reduction in the gene expression of *SLC6A8* to be a potential molecular basis for the decreased intramuscular creatine in SBMA.

Moreover, our study demonstrated that the expression of *SLC6A8* mRNA is suppressed in the cultured muscular cells stably expressing polyglutamine-expanded human AR, indicating that the deficiency of the transporter stems from the toxicity of the pathogenic AR protein in muscle. Previous reports have demonstrated that the function of transcriptional coactivators is impeded by abnormal polyglutamine-elongated protein which accumulates in

the nucleus of affected cells.³² Given that the pathogenic polyglutamine-expanded proteins are shown to perturb transcriptional machinery at least partially via this aberrant protein–protein interaction,^{33,34} our results suggest that the pathogenic AR induces downregulation of *SLC6A8* in the skeletal muscle of subjects with SBMA, leading to impaired creatine uptake by the muscle cells.

The impairment of creatine uptake may contribute to a motor function deficit seen in subjects with SBMA, given that the decrease in intramuscular creatine leads to defects in the control of energetic transfer producing ATP and calcium homeostasis, which is needed to regulate muscle excitation–contraction coupling.³⁵ Furthermore, dysregulation of creatine metabolism can result in the suppression of mitochondrial ADP-stimulated respiration, which further contributes toward a decreased energy production in skeletal muscle cells.³⁶

At present, there is no treatment available for counteracting muscle weakness in patients with SBMA. Our findings support the idea that restoration of muscle creatine uptake may be an important therapeutic strategy for SBMA management. Several studies have demonstrated that creatine supplementation ameliorates motor function deficits in subjects with myopathies, in which muscle *SLC6A8* expression and serum Cr concentrations are decreased.^{37,38} For example, creatine supplementation increases intramuscular phosphocreatine concentrations in myopathies.^{38,39} Moreover, a systematic review of randomized controlled trials has demonstrated that creatine treatment substantially increases muscle strength and functional performance in muscular dystrophies.⁴⁰ One particular clinical trial (UMIN No.000012503), currently in progress, may clarify whether creatine supplementation is an effective therapeutic strategy for SBMA management.

In conclusion, our study identifies serum Cr concentration as a potential biomarker reflecting both the loss of skeletal muscle mass and the impaired muscle uptake of creatine in SBMA. Furthermore, we showed that polyglutamine-expanded AR-induced impairment of muscular creatine uptake underlies the decreased serum Cr concentration and motor dysfunction in SBMA. These findings support the development of muscle-targeted therapies for SBMA such as creatine supplementation.

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Author Contributions

Drs. Hijikata, Katsuno, and Sobue conceived and designed the study. All authors contributed to the acquisition, analysis, or interpretation of the data. Dr. Hijikata drafted the manuscript. Drs. Katsuno and Sobue did the critical revision of the manuscript for important intellectual content. Drs. Katsuno, Hashizume, and Hirakawa did statistical analysis. Administrative, technical, or material support was provided by Dr. Noda, Nakanishi, Adachi, Watanabe, and Yamamoto. Drs. Katsuno and Sobue had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

Conflict of Interest

The authors have no relevant conflicts of interest to report.

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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Data S1. Methods.

Table S1. Correlation of clinical parameters with serum parameters in subjects with SBMA.

Figure S1. Correlation of serum Cr with clinical parameters in SBMA.

Figure S2. Correlation of skeletal muscle mass index with clinical parameters in subjects with SBMA.

Figure S3. Correlation of intramuscular creatine concentrations and the SLC6A8/GAPDH ratio.