Full Title

Decreased Peak Expiratory Flow Associated with Muscle Fiber-Type Switching in Spinal and Bulbar Muscular Atrophy

Short Title

Respiratory Function in SBMA

Shinichiro Yamada[1], Atsushi Hashizume[1], Yasuhiro Hijikata[1], Tomonori Inagaki[1], Keisuke Suzuki[2], Naohide Kondo[1], Kaori Kawai[1], Seiya Noda[1], Hirotaka Nakanishi[1], Haruhiko Banno[1,3], Akihiro Hirakawa[4], Haruki Koike[1], Katherine Halievski[5], Cynthia L. Jordan[5], Masahisa Katsuno[1]*, and Gen Sobue[1,6]*,

Author affiliations

[1] Department of Neurology, Nagoya University Graduate School of Medicine, Nagoya, Japan

[2] Innovation Center for Clinical Research, National Center for Geriatrics and Gerontology, Obu, Japan

[3] Institute for Advanced Research, Nagoya University, Nagoya, Japan

[4] Biostatistics Section, Center for Advanced Medicine and Clinical Research, Nagoya University Graduate School of Medicine, Nagoya, Japan

[5] Neuroscience Program, Michigan State University, East Lansing, Michigan, USA

22 [6] Research Division of Dementia and Neurodegenerative Disease, Nagoya University

23 Graduate School of Medicine, Nagoya, Japan

24

25 *** Corresponding authors**

26 E-mail: ka2no@med.nagoya-u.ac.jp (MK)

27 E-mail: sobueg@med.nagoya-u.ac.jp (GS)

Abstract

The aim of this study was to characterize the respiratory function profile of subjects with spinal and bulbar muscular atrophy (SBMA), and to explore the underlying pathological mechanism by comparing the clinical and biochemical indices of this disease with those of amyotrophic lateral sclerosis (ALS). We enrolled male subjects with SBMA (n = 40) and ALS (n = 25) along with 15 healthy control subjects, and assessed their respiratory function, motor function, and muscle strength. Predicted values of peak expiratory flow (%PEF) and forced vital capacity were decreased in subjects with SBMA compared with controls. In SBMA, both values were strongly correlated with the trunk subscores of the motor function tests and showed deterioration relative to disease duration. Compared with activities of daily living (ADL)-matched ALS subjects, %PEF, tongue pressure, and grip power were substantially decreased in subjects with SBMA. Both immunofluorescence and RT-PCR demonstrated a selective decrease in the expression levels of the genes encoding the myosin heavy chains specific to fast-twitch fibers in SBMA subjects. The mRNA levels of peroxisome proliferator-activated receptor gamma coactivator 1-alpha and peroxisome proliferator-activated receptor delta were up-regulated in SBMA compared with ALS and controls. In conclusion, %PEF is a disease-specific respiratory marker for the severity and progression of SBMA. Explosive muscle strength, including %PEF, was selectively

47 affected in subjects with SBMA and was associated with activation of the mitochondrial

48 biogenesis-related molecular pathway in skeletal muscles.

Introduction

Spinal and bulbar muscular atrophy (SBMA), or Kennedy's disease, is a slowly progressive lower motor neuron and muscular disease characterized by bulbar and limb muscle weakness and elevated levels of serum creatine kinase [1–3]. SBMA is caused by the expansion of a CAG repeat within the first exon of the androgen receptor (*AR*) gene [4]. Muscular weakness generally appears between 30 and 60 years of age, and affected individuals typically require a wheelchair 15 to 20 years after the onset of symptoms [2]. Patients occasionally experience laryngospasm, a sudden sensation of dyspnea [5,6], and often develop dysphagia at advanced stages, eventually resulting in aspiration or choking. Pneumonia and/or respiratory failure may occur at advanced stages of the disease [7], indicating that the management of swallowing and respiratory function is indispensable for the long-term care of patients with SBMA. However, in contrast to amyotrophic lateral sclerosis (ALS), another motor neuron disease for which the clinical features of dyspnea have been well documented, respiratory impairment in SBMA has not been well characterized. For instance, forced vital capacity (FVC) is the most important respiratory marker in ALS and is critical for both the respiratory and nutritional management of patients [8,9], whereas such markers for SBMA management have yet to be identified. The aim of this study was to characterize respiratory function in subjects with SBMA both cross-sectionally and longitudinally,

and to explore the potential underlying pathological mechanisms of SBMA by comparing the clinical and biochemical indices of this disease with those of ALS.

Materials and Methods

Standard protocol approvals, registration, and participant consent

This study conformed to 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. The Ethics Committee of Nagoya University Graduate School of Medicine approved this study, and all participants provided written informed consent prior to study participation.

Study population

We studied 40 consecutive male subjects diagnosed with SBMA via genetic testing and 25 consecutive male subjects with a clinical diagnosis of definite to probable ALS based on the revised El Escorial Criteria [10]. We also evaluated 15 healthy, age-matched male subjects with no diagnosed neurological disorders. The inclusion criteria were as follows: (i) subjects were 30–80 years old at the time of informed consent, and (ii)

subjects were able to stand upright for 6 min without assistance. The exclusion criteria were as follows: (i) severe complications, such as malignancy; (ii) other neurological complications; (iii) zero kg grip power in the dominant hand; or (iv) participation in any other clinical trial before providing informed consent. All subjects were Japanese males and were observed at Nagoya University Hospital between June 2013 and March 2016.

Pulmonary function test

A pulmonary function test was performed for all participants using a spirometer (FUDAC-77; FUKUDA DENSHI, Tokyo, Japan), which calculated and recorded FVC, forced expiratory volume in 1 s ($FEV_{1.0}$), the ratio of $FEV_{1.0}$ to FVC, and peak expiratory flow (PEF). The predicted values of FVC and $FEV_{1.0}$ were calculated using Baldwin's equation [11] and Berglund's equation [12], respectively. PEF is defined as the maximum expiratory flow per minute, which can be used to measure how fast a subject can exhale as well as to judge the strength of the expiratory muscles and the condition of the large airways. %PEF was calculated from regression equations for predicting PEF in the Japanese population. The subjects sat in a chair with a backrest and were instructed to inhale as deeply as possible, and then exhale through a mouthpiece as quickly as possible, with their noses occluded.

Longitudinal analysis of pulmonary function tests

Pulmonary function was measured every 6 months. To clarify the chronological changes in respiratory function in subjects with SBMA, we analyzed the longitudinal data of subjects who were evaluated for 1 year or longer during the follow-up period.

Motor function

We assessed disease severity in the subjects using the following functional parameters: the revised Amyotrophic Lateral Sclerosis Functional Rating Scale (ALSFRS-R), Spinal and Bulbar Muscular Atrophy Functional Rating Scale (SBMAFRS), modified quantitative myasthenia gravis (mQMG) score, tongue pressure, and grip power. SBMAFRS is a validated disease-specific functional scale for SBMA that demonstrates a high sensitivity for monitoring disease progression [13]. The validity of the motor functional measurements we used for SBMA is described in the S1 Methods.

Immunohistochemistry of muscle biopsy specimens

Bicep muscle specimens for immunohistochemistry were obtained from male subjects with SBMA (n = 2; 44 and 56 years old) or with ALS (n = 2; 51 and 63 years) by open

biopsy. The specimens were snap-frozen in isopentane, chilled in dry ice, and preserved at −80°C until analysis. Samples were cut on a cryostat using standard methods into 10-μm sections, as described previously [14]. Analysis of myosin heavy chain (MHC) expression was performed with primary antibodies against MHC type I (BAF-8; 1:50), MHC type IIa+IIx (SC-71; 1:600), and MHC type IIx (6H1; 1:50) (Developmental Studies Hybridoma Bank, University of Iowa) [15]. Immunoreactivity was detected using the following secondary antibodies: Alexa Fluor 647 IgG_{2b} (1:500), Alexa Fluor 488 IgG_1 (1:500), and Alexa Fluor 555IgM (1:500) (Invitrogen, Carlsbad, CA, USA). Slides were visualized with an LSM710 laser-scanning confocal microscope (Carl Zeiss, Oberkochen, Germany).

Quantitative RT-PCR

We analyzed the intramuscular mRNA expression levels of *MYH* genes in intercostal muscle specimens from 5 subjects with SBMA (mean ± SD age, 67.6 ± 10.6 years) and 5 subjects with ALS (68.4 ± 2.3 years). We also analyzed the mRNA levels of *MYH7* (encoding MHC type I), *MYH2* (encoding MHC type IIa), *MYH1* (encoding MHC type IIx), peroxisome proliferator-activated receptor alpha (*PGC-1α*), peroxisome proliferator-activated receptor alpha (*PPARα*), *PPARγ*, *PPARδ*, and *AMPK* in iliopsoas muscle specimens from 5 subjects with SBMA (67.6 ± 10.6 years), 6 subjects with ALS

(64.8 ± 11.2 years), and 4 subjects with other diseases (70.8 ± 7.6 years), including progressive supranuclear palsy (*n* = 2), Guillain-Barré syndrome (*n* = 1), and Sjögren's syndrome (*n* = 1). There was no statistically significant difference in age at the time of examination between the SBMA subjects, ALS subjects, and disease controls. The detailed procedures are described in the S1 Methods.

Genetic analysis

Genomic DNA was extracted from peripheral blood samples from subjects with SBMA using conventional techniques. PCR amplification of the *AR* CAG repeat was performed using a fluorescein-labeled forward primer (5′-TCCAGAATCTGTTCCAGAGCGTGC-3′) and an unlabeled reverse primer (5′-TGGCCTCGCTCAGGATGTCTTTAAG-3′). The detailed PCR conditions have been described previously [16].

Statistical analysis

We used an unpaired *t*-test or Mann–Whitney U test to compare continuous variables between two groups, analysis of variance (ANOVA) with Tukey’s post-hoc test for multiple comparisons, and Pearson’s correlation coefficient for analyzing correlations

among parameters. Analysis of covariance (ANCOVA) was performed to adjust the data for a covariate. We considered *p*-values less than 0.05 to be significant, and correlation coefficients (*r*) greater than 0.3 as strong. We fitted a marginal model using the generalized estimation equation (GEE) approach under an unstructured covariance matrix to clarify the population-averaged progression of the pulmonary function tests. Calculations were performed using the statistical software packages SPSS 23.0J (IBM Japan, Tokyo, Japan) and SAS 9.4 (SAS Institute Inc., NC, US).

Results

Clinical backgrounds and blood chemistry values of the subjects

The clinical backgrounds of the control subjects and the subjects with SBMA and ALS are presented in Table 1. The mean age at examination was higher in subjects with ALS than in those with SBMA, whereas the mean disease duration was shorter in the ALS subjects than in the SBMA subjects. The proportion of non-smokers, ex-smokers, and current smokers was equivalent in both groups. Serum concentrations of creatine kinase and testosterone were higher in the SBMA subjects. The characteristics of the SBMA subjects, such as age at examination, age at onset, and *AR* CAG repeat size, were similar

178 to previously reported values [17–19].

179

180 **Table 1. Clinical background of subjects.**

181

	SBMA (*n* = 40)	**ALS (*n* = 25)**	**Control (*n* = 15)**	***p*-value[a]**
Age at examination, years	53.4 ± 10.4 (33–76)	65.2 ± 7.6 (48–78)	54.4 ± 7.7 (38–68)	<0.001
Duration from onset, years	8.7 ± 5.0 (0–17)	1.3 ± 0.9 (0.5–4)	NA	<0.001
Smoking				
Non-smoker, %	40.0	32.0	33.3	N.S.
Ex-smoker, %	45.0	52.0	26.7	N.S.
Current smoker, %	15.0	16.0	40.0	N.S.
Blood chemistry values				
Creatine kinase, IU	1057.8 ± 708.1 (202–3064)	242.0 ± 276.3 (23–1383)	119.7 ± 47.5 (43–204)	<0.001
Testosterone, ng/mL	7.8 ± 3.2 (3.6–16.6)	5.3 ± 1.6 (2.8–9.7)	6.8 ± 3.7 (3.5–19.2)	0.003
CAG repeat size in *AR* gene	47.2 ± 3.3 (42–54)	NA	NA	

182

183 SBMA, spinal and bulbar muscular atrophy; ALS, amyotrophic lateral sclerosis; NS,

184 not significant; AR, androgen receptor; NA, not applicable. Data are shown as the mean

± SD.

[a]Difference between SBMA and ALS by ANOVA with Tukey's post-hoc test.

Respiratory function in SBMA

Relative to healthy controls, the subjects with SBMA exhibited decreased values for %FVC and %PEF, but not for $FEV_{1.0}$/FVC (Fig 1). The actual values for PEF were also lower in SBMA subjects than in control subjects. When comparing SBMA and ALS patients, the %PEF, an index of explosive muscle power, was significantly decreased in SBMA subjects, whereas other indices were comparable between the two groups. The difference in %PEF between SBMA and ALS subjects was significant after adjustment for the ALSFRS-R and %FVC with ANCOVA (p = 0.002 and 0.002, respectively) (Fig 2). These findings suggest that both %PEF and %FVC are decreased in SBMA patients, as observed in ALS, but the reduction of %PEF is specific to the subjects with SBMA.

Fig 1. Respiratory function profile of subjects with SBMA. The actual and predicted values of forced vital capacity (FVC) (A, B), forced expiratory volume in 1 s ($FEV_{1.0}$) (C), the ratio of $FEV_{1.0}$ to FVC (D), and actual and predicted values of peak expiratory flow (PEF) (E, F) were compared among SBMA subjects (n = 40), ALS

subjects (n = 25), or healthy controls (n = 15). Compared with the healthy controls, patients with SBMA exhibited decreased values for %FVC, PEF, and %PEF. The actual values of PEF were also lower in SBMA than in controls. When comparing SBMA and ALS subjects, %PEF was significantly decreased in SBMA, but no differences were detected for the other indices. ** $p < 0.01$. * $p < 0.05$. Data are presented as the mean ± SE. SBMA, spinal and bulbar muscular atrophy; ALS, amyotrophic lateral sclerosis; HC, healthy controls.

Fig 2. Relationships between %PEF and ALSFRS-R or %FVC in subjects with SBMA and ALS. Comparison of the relationships between %PEF and total score of ALSFRS-R (A) or %FVC (B) in SBMA and ALS. The difference in %PEF between SBMA and ALS was significant after adjustment for the ALSFRS-R and %FVC with ANCOVA. %PEF, predicted values of peak expiratory flow; ALSFRS-R, the revised Amyotrophic Lateral Sclerosis Functional Rating Scale; %FVC, predicted forced vital capacity.

Relationship between respiratory function and motor function scores in SBMA

We next investigated the relationship between respiratory parameters and the total score and subscores on the motor functional scales SBMAFRS and ALSFRS in the SBMA

223 subjects (Table 2). %PEF strongly correlated with total score as well as with the trunk
224 and lower limb subscores of the SBMAFRS, particularly with the trunk subscore (S1
225 Fig). However, we observed no significant correlations between %PEF and bulbar,
226 upper limb, or respiratory subscores. The lack of correlation between the respiratory
227 domain of SBMAFRS and %PEF or %FVC appears to stem from the fact that most
228 subjects were at relatively early stages of the disease and reported no overt dyspnea on
229 the functional scales [13]. The total scores and subscores of SBMAFRS correlated more
230 strongly with %PEF than %FVC. Similar relationships were also observed for
231 ALSFRS-R (S2 Fig). These results indicate that %PEF is a sensitive biomarker of
232 respiratory dysfunction, reflecting in particular the truncal function of subjects with
233 SBMA.

234

235 **Table 2. Correlations between motor function scores and respiratory indices in**
236 **subjects with SBMA.**

237

SBMA (*n* = 40)	**%PEF**	**%FVC**	**$FEV_{1.0}$ / FVC**
SBMAFRS	$r = 0.460$ ($p = 0.003$)	$r = 0.342$ ($p = 0.031$)	$r = 0.100$ ($p = 0.541$)
Bulbar	$r = 0.259$ ($p = 0.107$)	$r = 0.206$ ($p = 0.202$)	$r = -0.037$ ($p = 0.819$)
Upper Limb	$r = 0.007$ ($p = 0.964$)	$r = 0.017$ ($p = 0.918$)	$r = -0.142$ ($p = 0.383$)
Trunk	$r = 0.614$ ($p < 0.001$)	$r = 0.443$ ($p = 0.004$)	$r = 0.211$ ($p = 0.192$)

Lower Limb	$r = 0.379$ ($p = 0.003$)	$r = 0.248$ ($p = 0.123$)	$r = 0.260$ ($p = 0.105$)
Respiratory	$r = 0.063$ ($p = 0.699$)	$r = 0.076$ ($p = 0.642$)	$r = -0.003$ ($p = 0.983$)
ALSFRS-R	$r = 0.398$ ($p = 0.011$)	$r = 0.288$ ($p = 0.072$)	$r = 0.103$ ($p = 0.525$)
Bulbar	$r = 0.252$ ($p = 0.117$)	$r = 0.240$ ($p = 0.136$)	$r = 0.068$ ($p = 0.679$)
Upper Limb	$r = 0.002$ ($p = 0.992$)	$r = -0.078$ ($p = 0.632$)	$r = -0.156$ ($p = 0.337$)
Trunk	$r = 0.485$ ($p = 0.002$)	$r = 0.334$ ($p = 0.035$)	$r = 0.124$ ($p = 0.446$)
Lower Limb	$r = 0.331$ ($p = 0.037$)	$r = 0.271$ ($p = 0.091$)	$r = 0.199$ ($p = 0.219$)
Respiratory	$r = -0.032$ ($p = 0.699$)	$r = -0.057$ ($p = 0.725$)	$r = -0.008$ ($p = 0.960$)

SBMA, spinal and bulbar muscular atrophy; %PEF, predicted values of peak expiratory flow; %FVC, predicted values of forced vital capacity; SBMAFRS, Spinal and Bulbar Muscular Atrophy Functional Rating Scale; ALSFRS-R, the revised Amyotrophic Lateral Sclerosis Functional Rating Scale.

Longitudinal assessment of pulmonary function tests in SBMA

To examine whether respiratory parameters reflect disease progression, we prospectively analyzed longitudinal changes in pulmonary function tests in subjects with SBMA (Fig 3). Using a linear model, we assessed the data from 32 subjects with SBMA who were assessed for longitudinal changes in pulmonary function (Fig 3A–C). Results revealed slow but steady deterioration for %PEF and %FVC relative to disease duration with the speed of decline being higher for %PEF. These results suggest

252 that %PEF, together with %FVC, are biomarkers of respiratory function in subjects with
253 SBMA and could be used to quantitatively assess disease progression.
254
255 **Fig 3. Longitudinal changes in respiratory function in SBMA.** Longitudinal
256 changes in %PEF (A), %VC (B), and $FEV_{1.0}$/FVC (C) as a function of disease duration
257 were analyzed. The solid lines indicate representative disease progression over disease
258 duration calculated using a marginal model under an unstructured covariance matrix.
259 The broken curvilinear line demonstrates the 95% confidence interval of these models.
260 We calculated the estimated values at clinical onset (intercepts) and the change values
261 per year (D). In an analysis using the marginal model, a generalized estimating equation
262 (shown by the solid lines) identified that %PEF (A) and %FVC (B) demonstrated a
263 slowly but with steady progression. PEF, peak expiratory flow; FVC, forced vital
264 capacity; $FEV_{1.0}$, forced expiratory volume in 1 s.
265

266 Fast versus slow motor function in SBMA and ALS

267 The decrease in %PEF in SBMA compared with ALS subjects led us to explore the
268 possibility of a selective loss of fast muscle power in patients with this disease,
269 considering that %PEF is an index of explosive muscle strength [20]. To test this
270 hypothesis, we compared indices of fast and slow motor function that were not based

271 on respiratory function between SBMA and ALS subjects. Both groups were matched
272 for activities of daily living (ADLs) (Table 3). The total score and subscores (bulbar,
273 upper limb, trunk, lower limb, and respiratory) on the ALSFRS-R in the SBMA subjects
274 were equivalent to the scores of the ALS subjects. The mQMG scores, which are indices
275 of muscle endurance, were also similar between the groups. Nevertheless, tongue
276 pressure and grip power, both of which reflect explosive muscle strength, were
277 significantly decreased in the SBMA subjects compared with the ALS subjects. These
278 differences remained significant after adjustment for the ALSFRS-R with ANCOVA
279 (data not shown). Taken together, our results suggest that explosive muscle power is
280 preferentially affected in SBMA patients compared with ALS patients.

281

282 **Table 3. Motor function of subjects.**

283

	SBMA (***n*** **= 40)**	**ALS** (***n*** **= 25)**	**Control** (***n*** **= 15)**	***p*-value[b]**
ALSFRS-R				
Total	40.7 ± 3.3	39.9 ± 4.4	47.8 ± 0.6	N.S.
Bulbar	10.2 ± 1.1	10.0 ± 2.1	11.8 ± 0.6	N.S.
Upper Limbs	6.6 ± 0.9	6.7 ± 1.2	8.0 ± 0	N.S.
Trunk	5.8 ± 1.3	5.8 ± 2.0	8.0 ± 0	N.S.
Lower Limbs	6.2 ± 1.2	6.0 ± 1.2	8.0 ± 0	N.S.

Respiratory	11.9 ± 0.3	11.5 ± 1.1	12.0 ± 0	N.S.
mQMG score				
Total	5.48 ± 3.19	5.24 ± 3.31	ND	N.S.
Head, lifted	1.33 ± 0.86	1.32 ± 1.03	ND	N.S.
Left arm outstretched	1.33 ± 0.86	1.32 ± 1.03	ND	N.S.
Right arm outstretched	1.13 ± 0.82	1.28 ± 1.06	ND	N.S.
Left leg outstretched	0.85 ± 0.80	0.84 ± 1.07	ND	N.S.
Right leg outstretched	0.80 ± 0.72	0.68 ± 0.99	ND	N.S.
Tongue pressure, kPa	17.39 ± 6.64 (5.3–32.0)	25.89 ± 13.85 (0–47.0)	45.5 ± 7.31 (34.4–55.1)	0.003
Grip power[a], kg	19.76 ± 5.91 (7.7–31.9)	26.58 ± 10.73 (8.4–49.3)	44.3 ± 6.23 (29.2–52.0)	0.002

SBMA, spinal and bulbar muscular atrophy; ALS, amyotrophic lateral sclerosis; ALSFRS-R, revised Amyotrophic Lateral Sclerosis Functional Rating Scale; mQMG, modified quantitative myasthenia gravis; NS, not significant; NA, not applicable; ND, not determined. ALSFRS-R normal value = 48. Data are shown as the mean ± SD.

[a]Maximum value of the dominant hand.

[b]Difference between SBMA and ALS by ANOVA with Tukey's post-hoc test.

Alteration of fast- and slow-twitch fiber composition in SBMA and ALS

Next, we hypothesized that the selective decline of fast muscle power in SBMA was

295 attributable to an alteration in fast- and slow-twitch fiber composition. To examine this
296 hypothesis, we analyzed MHC isoforms in biopsied skeletal muscle specimens using
297 immunohistochemistry (Fig 4). Type IIx fibers, which generate explosive power, were
298 substantially decreased in samples from subjects with SBMA compared with samples
299 from subjects with ALS, whereas type I fibers, which are associated with endurance,
300 were atrophied in samples from ALS subjects.

301 To verify the immunofluorescence findings, we performed qRT-PCR analysis
302 on intercostal and iliopsoas muscle specimens. In the intercostal muscles, which
303 generate expiratory flow, the mRNA expression levels of *MYH1* and *MYH2*, encoding
304 MHC type IIx and IIa, respectively, but not *MYH7*, were significantly decreased in
305 samples from SBMA subjects compared with ALS subjects, strengthening the theory
306 that fast muscle power is predominantly affected in SBMA (Fig 5A–C). A
307 corresponding reduction in *MYH1* and *MYH2* mRNAs was also observed in the
308 iliopsoas muscles (Fig 5D, E). By contrast, the expression levels of *MYH7*, which
309 encodes MHC type I, in the iliopsoas muscles were higher in samples from the SBMA
310 subjects than in samples from the ALS subjects (Fig 5F). Furthermore, we compared
311 the expression of putative regulators of muscle fiber switching among the groups (Fig
312 5G–K). Results demonstrated that the mRNA levels of *PGC-1α* and *PPARδ*, which are
313 known to regulate oxidative fiber type profile [21,22], were substantially up-regulated

314 in SBMA samples compared with ALS and disease-free control samples (Fig 5H, J).
315 Although not significant, *AMPK* mRNA levels tended to be increased in subjects with
316 SBMA (Fig 5K).
317
318 **Fig 4. Disease-specific fiber type alterations in SBMA and ALS.** (A, B)
319 Representative images of anti-MHC immunostaining in the biceps muscles of subjects
320 with SBMA (A, $n = 2$) and ALS (B, $n = 2$). Type I (blue), type IIa (strong green), and
321 type IIx (strong red and intermediate green) fibers are shown in single- and merged-
322 channel images of serial cross-sections of a human bicep incubated with an antibody
323 cocktail (BAF-8, SC-71, and 6H1). Type IIx fibers were substantially decreased in
324 SBMA compared with ALS, whereas type I fibers were atrophied in ALS compared
325 with SBMA. Scale bar, 100 μm. SBMA, spinal and bulbar muscular atrophy; ALS,
326 amyotrophic lateral sclerosis; MHC, myosin heavy chain.
327 **Fig 5. Expression levels of myosin heavy chain and AMPK-PGC-1α pathway in**
328 **the skeletal muscles.** The mRNA expression levels of *MYH1* (encoding MHC type IIx),
329 *MYH2* (encoding MHC type IIa), and *MYH7* (encoding MHC type I) normalized to β2-
330 microglobulin levels in the intercostal muscles of SBMA ($n = 5$) and ALS ($n = 5$)
331 subjects (A–C). The expression levels of *MYH1* (A) and *MYH2* (B) were significantly
332 decreased in subjects with SBMA compared with subjects with ALS. The mRNA

expression levels of *MYH1*, *MYH2*, and *MYH7* normalized to β2-microglobulin levels in the iliopsoas muscles of SBMA ($n = 5$), ALS ($n = 6$), and DC ($n = 4$) subjects (D–F). The mRNA levels of *MYH1* and *MYH2* in the iliopsoas muscle were significantly decreased in subjects with SBMA compared with ALS subjects, as observed in the intercostal muscles (D, E). The expression levels of *MYH7* were significantly higher in subjects with SBMA than in subjects with ALS (F). Expression levels of the genes known to regulate muscle fiber type switching in SBMA ($n = 5$), ALS ($n = 6$), and DC ($n = 4$) samples. The mRNA levels of *PGC-1α* and *PPAR-δ*, which regulate the oxidative fiber type profile, were significantly increased in SBMA compared with ALS and DC. The Mann-Whitney U test was performed to assess significant differences for each target gene between SBMA and ALS. ANOVA with Tukey's post-hoc test was performed to compare the significance of differences in each target gene among SBMA, ALS, and DC. $^{**}p < 0.01$. $^{*}p < 0.05$. Data are presented as the mean ± SE. SBMA, spinal and bulbar muscular atrophy; ALS, amyotrophic lateral sclerosis; DC, disease control; *PGC-1α*, proliferator-activated receptor gamma coactivator 1-alpha; *PPAR*, peroxisome proliferator-activated receptor.

Discussion

In the present study, we demonstrated that %PEF and %FVC were substantially

352 decreased in SBMA compared with controls, although there was no significant
353 difference in $FEV_{1.0}$ between the groups. Both %PEF and %FVC correlated with
354 functional disease scores, particularly truncal subscores, and were linked to decreases
355 in disease progression in subjects with SBMA. PEF is the maximum expiratory flow
356 per minute and can be used to measure how fast a subject can breathe out, providing a
357 reliable global measure of voluntary cough against the risk of aspiration pneumonia
358 [23]. PEF is based on abdominal and intercostal muscle strength as well as the elastic
359 recoil of the lung and chest wall. During forced expiration, such as peak flow, the
360 abdominal muscles are activated to increase intra-abdominal pressure [24].
361 Furthermore, lower PEF values are associated with increased mortality from respiratory
362 causes [25]. %PEF decreases during the course of neuromuscular disease [26] as well
363 as during spinal cord injuries, in which a higher cord level lesion is associated with a
364 greater decrease in %PEF [27]. As SBMA is a rare disease, clinical trials have to be
365 done with a limited sample size. Therefore, identification of sensitive biomarkers for
366 detecting benefits of tested therapies is urgently needed for SBMA [28]. Our study
367 revealed that %PEF chiefly reflects truncal muscle strength in subjects with SBMA and
368 is a reliable respiratory marker for disease severity and progression.

369 When comparing SBMA and ALS subjects, only %PEF was significantly
370 decreased in SBMA. Unlike other parameters of pulmonary function tests, PEF is

generated by explosive muscle power in an effort-dependent manner. We further revealed that, in addition to %PEF, tongue pressure and grip power, which also reflect explosive muscle strength, are also specifically decreased in subjects with SBMA compared with those with ALS. By contrast, the mQMG score, which is an index of muscle endurance, was similar between the two groups. Taken together, these findings suggest a reduction in explosive muscle strength in subjects with SBMA, consistent with the preferential loss of fast-twitch muscle fibers in these subjects. Muscular function is chiefly dependent on the specific characteristics of various muscle fiber types, and immunohistochemistry of the MHC isoforms in skeletal muscles reveals four fiber types (i.e., I, IIa, IIx, and IIb) in rodents and most other mammalian species. However, only type I, IIa, and IIx fibers are present in most human muscles [29]. These fibers differ from one another in oxidative/glycolytic metabolism: type I fibers are more oxidative and regulate endurance muscle strength; type IIx fibers are more glycolytic and regulate explosive muscle strength; type IIa fibers exhibit characteristics of both type I and IIx fibers [30]. Glycolytic fast-twitch fibers are preferentially vulnerable in a transgenic mouse model of SBMA and in humans with the disorder [31–33]. Results from the present study indicate that the decrease in explosive muscle strength in subjects with SBMA is strongly associated with a reduction in the number of fast-twitch muscle fibers.

390 In the present study, the muscles of SBMA subjects demonstrated glycolytic-
391 to-oxidative switching in association with an up-regulation of PGC-1α and PPARδ.
392 Fiber type switching is induced in adult skeletal muscle by changes in nerve activity or
393 loading. Glycolytic-to-oxidative switching can be induced by tonic low-frequency
394 electrical stimulation [30]. The AMPK-PGC-1α pathway alters the fiber type profile
395 toward oxidative metabolism by regulating *MYH* gene expression. For instance,
396 overexpressing AMPK and PGC-1α in mice has been shown to increase mitochondrial
397 content and the levels of oxidative enzymes in fast muscle fibers, increasing muscle
398 resistance against fatigue [21,34]. Similarly, PPARδ signaling induces a more oxidative
399 fiber type profile in mice, with an increased amount of mitochondrial DNA, up-
400 regulation of slow contractile protein genes, and an increased resistance to fatigue [22].
401 PGC-1α was reportedly increased in the muscles of a knock-in mouse model of SBMA,
402 consistent with our findings in humans [35]. Given that AR inhibits the AMPK-PGC-
403 1α pathway [36], the loss of AR function may underlie the up-regulation of PGC-1α in
404 SBMA. In fact, subjects with SBMA often exhibit certain symptoms of androgen
405 insensitivity syndrome, such as gynecomastia and reduced fertility, which have been
406 attributed to loss of AR function [37]. Females possess a greater number of slow-twitch
407 fibers than males, which is the molecular basis for gender differences in response to
408 fatigue or muscle tetanus. These gender differences further support our view that

409 disease-specific alterations of fiber type occur in the skeletal muscles of subjects with
410 SBMA.

411 A possible alternative explanation for the preferential deficiency of fast-twitch
412 fibers in subjects with SBMA is the adaptation of surviving muscles to endurance
413 activities during the slow progression of the disease. This view is supported by the
414 observation that chronic inactivation of muscles leads to selective atrophy of fast-twitch
415 fibers [38]. A similar loss of fast-twitch fibers was also documented in spinal muscular
416 atrophy, which is another neuromuscular disorder affecting both spinal motor neurons
417 and skeletal muscle [39]. Future studies should directly address the mechanisms
418 underlying disease-specific alterations of fiber type in SBMA, and they should focus
419 on identifying pharmacological or non-pharmacological interventions to reverse these
420 alterations.

421 In summary, we found that subjects with SBMA exhibited decreased %PEF,
422 which appears to reflect the preferential involvement of fast-twitch fibers in this disease.
423 Given that the leading causes of death in subjects with SBMA are pneumonia and
424 respiratory failure [7], particular attention should be paid to %PEF decline during the
425 clinical management of SBMA.

References

1. Kennedy WR, Alter M, Sung JH. Progressive proximal spinal and bulbar muscular atrophy of late onset. A sex-linked recessive trait. Neurology. 1968;18:671–680.

2. Katsuno M, Tanaka F, Adachi H, Banno H, Suzuki K, Watanabe H, et al. Pathogenesis and therapy of spinal and bulbar muscular atrophy (SBMA). Prog Neurobiol. 2012;99:246–256.

3. Sorarù G, D'Ascenzo C, Polo A, Palmieri A, Baggio L, Vergani L, et al. Spinal and bulbar muscular atrophy: skeletal muscle pathology in male patients and heterozygous females. J Neurol Sci. 2008;264:100–105.

4. La Spada AR, Wilson EM, Lubahn DB, Harding AE, Fischbeck KH. Androgen receptor gene mutations in X-linked spinal and bulbar muscular atrophy. Nature. 1991;352:77–79.

5. Sperfeld AD, Hanemann CO, Ludolph AC, Kassubek J. Laryngospasm: an underdiagnosed symptom of X-linked spinobulbar muscular atrophy. Neurology. 2005;64:753–-754.

6. Tanaka S, Banno H, Katsuno M, Suzuki K, Suga N, Hashizume A, et al. Distinct acoustic features in spinal and bulbar muscular atrophy patients with laryngospasm. J Neurol Sci. 2014;337:193–200.

445 7. Atsuta N, Watanabe H, Ito M, et al. Natural history of spinal and bulbar
446 muscular atrophy (SBMA): a study of 223 Japanese patients. Brain. 2006;129:1446–
447 1455.

448 8. Lo Coco D, Marchese S, Pesco MC, La Bella V, Piccoli F, Lo Coco A.
449 Noninvasive positive-pressure ventilation in ALS: predictors of tolerance and survival.
450 Neurology. 2006;67:761–765.

451 9. Miller RG, Jackson CE, Kasarskis EJ, England JD, Forshew D, Johnston W, et
452 al. Practice parameter update: the care of the patient with amyotrophic lateral sclerosis:
453 drug, nutritional, and respiratory therapies (an evidence-based review): report of the
454 Quality Standards Subcommittee of the American Academy of Neurology. Neurology.
455 2009;73:1218–1226.

456 10. Brooks BR, Miller RG, Swash M, Munsat TL. World Federation of Neurology
457 Research Group on Motor Neuron Diseases. El Escorial revisited: revised criteria for
458 the diagnosis of amyotrophic lateral sclerosis. Amyotroph Lateral Scler Other Motor
459 Neuron Disord. 2000;1:293–299.

460 11. Baldwin ED, Cournand A, Richards DW Jr. Pulmonary insufficiency;
461 physiological classification, clinical methods of analysis, standard values in normal
462 subjects. Medicine. 1948;27:243–278.

463 12. Berglund E, Birath G, Bjure J, Grimby G, Kjellmer I, Sandqvist L, et al.
464 Spirometric studies in normal subjects. I. Forced expirograms in subjects between 7
465 and 70 years of age. Acta Med Scand. 1963;173:185–192.
466 13. Hashizume A, Katsuno M, Suzuki K, Banno H, Suga N, Mano T, et al. A
467 functional scale for spinal and bulbar muscular atrophy: Cross-sectional and
468 longitudinal study. Neuromuscul Disord. 2015;25:554–562.
469 14. Maeshima S, Koike H, Noda S, Noda T, Nakanishi H, Iijima M, et al.
470 Clinicopathological features of sarcoidosis manifesting as generalized chronic
471 myopathy. J Neurol. 2015;262:1035–1045.
472 15. Bloemberg D, Quadrilatero J. Rapid determination of myosin heavy chain
473 expression in rat, mouse, and human skeletal muscle using multicolor
474 immunofluorescence analysis. PLoS One. 2012;7:e35273.
475 16. Doyu M, Sobue G, Mukai E, Kachi T, Yasuda T, Mitsuma T, et al. Severity of
476 X-linked recessive bulbospinal neuronopathy correlates with size of the tandem CAG
477 repeat in androgen receptor gene. Ann Neurol. 1992;32:707–710.
478 17. Katsuno M, Banno H, Suzuki K, Takeuchi Y, Kawashima M, Yabe I, et al.
479 Efficacy and safety of leuprorelin in patients with spinal and bulbar muscular atrophy
480 (JASMITT study): a multicentre, randomised, double-blind, placebo-controlled trial.
481 Lancet Neurol. 2010;9:875–884.

482 18. Fernández-Rhodes LE, Kokkinis AD, White MJ, Watts CA, Auh S, Jeffries NO,
483 et al. Efficacy and safety of dutasteride in patients with spinal and bulbar muscular
484 atrophy: a randomised placebo-controlled trial. Lancet Neurol. 2011;10:140–147.
485 19. Hashizume A, Katsuno M, Banno H, Suzuki K, Suga N, Mano T, et al.
486 Longitudinal changes of outcome measures in spinal and bulbar muscular atrophy.
487 Brain. 2012;135:2838–2848.
488 20. Pedersen OF, Miller MR. The Peak Flow Working Group: the definition of
489 peak expiratory flow. Eur Respir J Suppl. 1997;24:9S–10S.
490 21. Lin J, Wu H, Tarr PT, Zhang CY, Wu Z, Boss O, et al. Transcriptional co-
491 activator PGC-1α drives the formation of slow-twitch muscle fibres. Nature.
492 2002;418:797–801.
493 22. Wang YX, Zhang CL, Yu RT, Cho HK, Nelson MC, Bayuga-Ocampo CR et al.
494 Regulation of muscle fiber type and running endurance by PPARdelta. PLoS Biol.
495 2004;2:e294.
496 23. Tzani P, Chiesa S, Aiello M, Scarascia A, Catellani C, Elia D, et al. The value
497 of cough peak flow in the assessment of cough efficacy in neuromuscular patients. A
498 cross sectional study. Eur J Phys Rehabil Med. 2014;50:427–432.
499 24. Sieck GC, Ferreira LF, Reid MB, Mantilla CB. Mechanical properties of
500 respiratory muscles. Compr Physiol. 2013;3:1553–1567.

501 25. Smith M, Zhou M, Wang L, Peto R, Yang G, Chen Z. Peak flow as a predictor
502 of cause-specific mortality in China: results from a 15-year prospective study of
503 ~170,000 men. Int J Epidemiol. 2013;42:803–815.
504 26. Mayer OH, Finkel RS, Rummey C, Benton MJ, Glanzman AM, Flickinger J,
505 et al. Characterization of pulmonary function in Duchenne Muscular Dystrophy. Pediatr
506 Pulmonol. 2015;50:487–494.
507 27. Wang AY, Jaeger RJ, Yarkony GM, Turba RM. Cough in spinal cord injured
508 patients: the relationship between motor level and peak expiratory flow. Spinal Cord.
509 1997;35:299–302.
510 28. Fratta P, Nirmalananthan N, Masset L, Skorupinska I, Collins T, Cortese A, et
511 al. Correlation of clinical and molecular features in spinal bulbar muscular atrophy.
512 Neurology. 2014;82:2077–2084.
513 29. Smerdu V, Karsch-Mizrachi I, Campione M, Leinwand L, Schiaffino S. Type
514 IIx myosin heavy chain transcripts are expressed in type IIb fibers of human skeletal
515 muscle. Am J Physiol. 1994;267(6 Pt 1):C1723–1728.
516 30. Schiaffino S, Reggiani C. Fiber types in mammalian skeletal muscles. Physiol
517 Rev. 2011;91:1447–1531.
518 31. Oki K, Wiseman RW, Breedlove SM, Jordan CL. Androgen receptors in
519 muscle fibers induce rapid loss of force but not mass: implications for spinal bulbar

520 muscular atrophy. Muscle Nerve. 2013;47:823–834.

521 32. Oki K, Halievski K, Vicente L, Xu Y, Zeolla D, Poort J, et al. Contractile
522 dysfunction in muscle may underlie androgen-dependent motor dysfunction in spinal
523 bulbar muscular atrophy. J Appl Physiol (1985). 2015;118:941–952.

524 33. Rocchi A, Milioto C, Parodi S, Armirotti A, Borgia D, Pellegrini M, et al.
525 Glycolytic-to-oxidative fiber-type switch and mTOR signaling activation are early-
526 onset features of SBMA muscle modified by high-fat diet. Acta Neuropathol.
527 2016;132:127–144.

528 34. Röckl KS, Hirshman MF, Brandauer J, Fujii N, Witters LA, Goodyear LJ.
529 Skeletal muscle adaptation to exercise training: AMP-activated protein kinase mediates
530 muscle fiber type shift. Diabetes. 2007;56:2062–2069.

531 35. Ranganathan S, Harmison GG, Meyertholen K, Pennuto M, Burnett BG,
532 Fischbeck KH. Mitochondrial abnormalities in spinal and bulbar muscular atrophy.
533 Hum Mol Genet. 2009;18:27–42.

534 36. Shen M, Zhang Z, Ratnam M, Dou QP. The interplay of AMP-activated
535 protein kinase and androgen receptor in prostate cancer cells. J Cell Physiol.
536 2014;229:688–695.

537 37. Dejager S, Bry-Gauillard H, Bruckert E, Eymard B, Salachas F, LeGuern E, et
538 al. A comprehensive endocrine description of Kennedy's disease revealing androgen

539 insensitivity linked to CAG repeat length. J Clin Endocrinol Metab. 2002;87:3893–
540 3901.
541 38. Mantilla CB, Greising SM, Zhan WZ, Seven YB, Sieck GC. Prolonged C2
542 spinal hemisection-induced inactivity reduces diaphragm muscle specific force with
543 modest, selective atrophy of type IIx and/or IIb fibers. J Appl Physiol (1985).
544 2013;114:380–386.
545 39. Stevens L, Bastide B, Maurage CA, Dupont E, Montel V, Cieniewski-Bernard
546 C, et al. Childhood spinal muscular atrophy induces alterations in contractile and
547 regulatory protein isoform expressions. Neuropathol Appl Neurobiol. 2008;34:659–670.

Supporting Information

S1 Methods.

S1 Fig. Relationships between %PEF and SBMAFRS scores in subjects with SBMA. Relationships between %PEF and the total score (A) and subscores (B–F) of SBMAFRS in SBMA. %PEF in subjects with SBMA were correlated well with the total scores (A) and trunk (D) and lower limb (E) subscores of SBMAFRS. %PEF, predicted values of peak expiratory flow; SBMAFRS, Spinal and Bulbar Muscular Atrophy Functional Rating Scale; SBMA, spinal and bulbar muscular atrophy.

S2 Fig. Relationships between %PEF and ALSFRS-R scores in subjects with SBMA. Relationships between %PEF and the total score (A) and subscores (B–F) of ALSFRS-R in SBMA. %PEF values in subjects with SBMA correlated well with the total score (A) and the trunk (D) and lower limb (E) subscores of ALSFRS-R. %PEF, predicted values of peak expiratory flow; ALSFRS-R, the revised Amyotrophic Lateral Sclerosis Functional Rating Scale; SBMAFRS, Spinal and Bulbar Muscular Atrophy Functional Rating Scale.