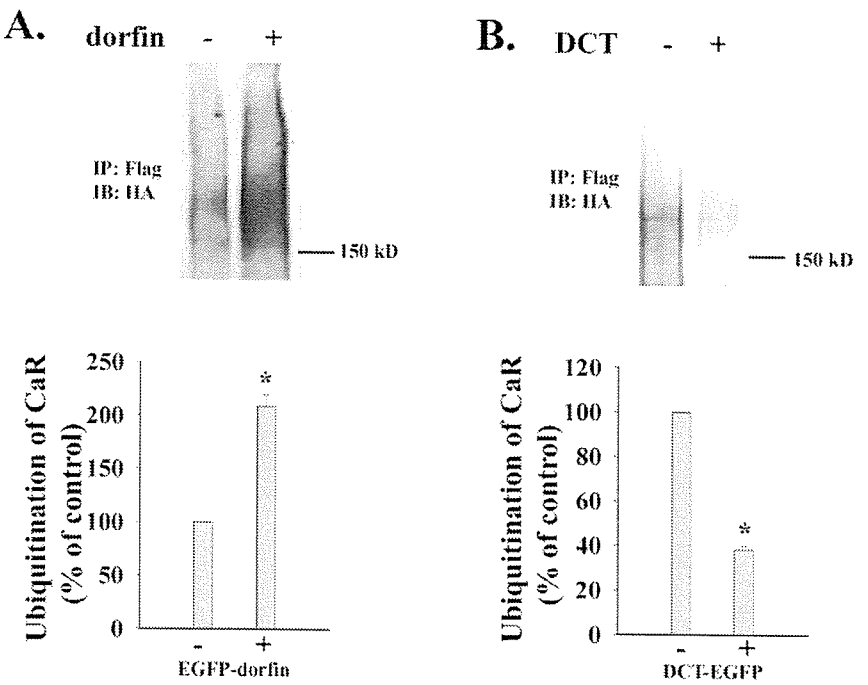
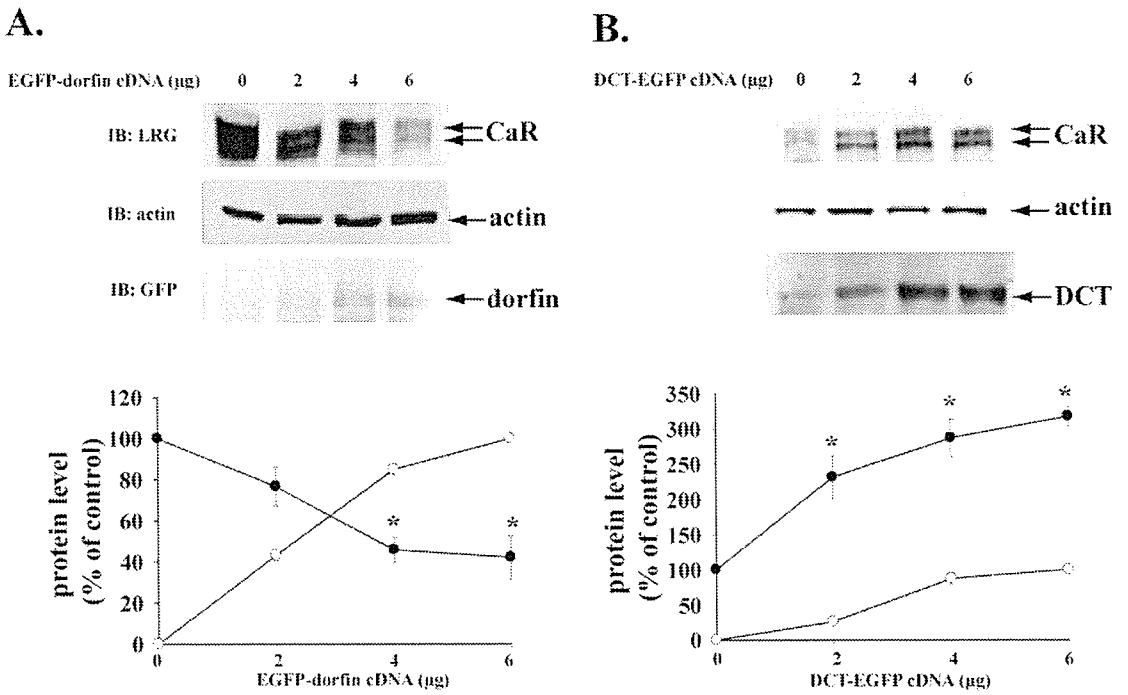

A.
dorfin - +
IP: Flag
IB: HA
150 kD
Ubiquitination of CaR
(% of control)
250
200
150
100
50
0
*
- +
EGFP-dorfin
B.
DCT - +
IP: Flag
IB: HA
150 kD
Ubiquitination of CaR
(% of control)
120
100
80
60
40
20
0
*
- +
DCT-EGFP

Figure 4


A.
EGFP-dorfin cDNA (μg) 0 2 4 6
IB: LRG
CaR
IB: actin
actin
IB: GFP
dorfin
protein level (% of control)
EGFP-dorfin cDNA (μg)
B.
DCT-EGFP cDNA (μg) 0 2 4 6
CaR
actin
DCT
protein level (% of control)
DCT-EGFP cDNA (μg)

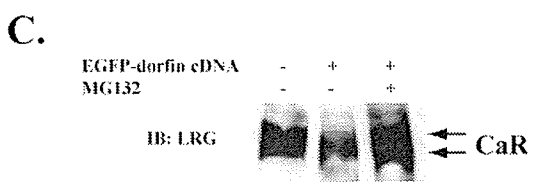

C.
EGFP-dorfin cDNA - + +
MG132 - - +
IB: LRG
CaR

Figure 5

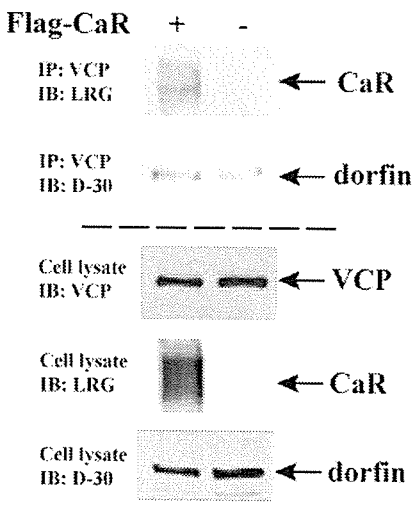

Flag-CaR + -
IP: VCP
IB: LRG
CaR
IP: VCP
IB: D-30
dorfin
Cell lysate
IB: VCP
VCP
Cell lysate
IB: LRG
CaR
Cell lysate
IB: D-30
dorfin

Figure 6

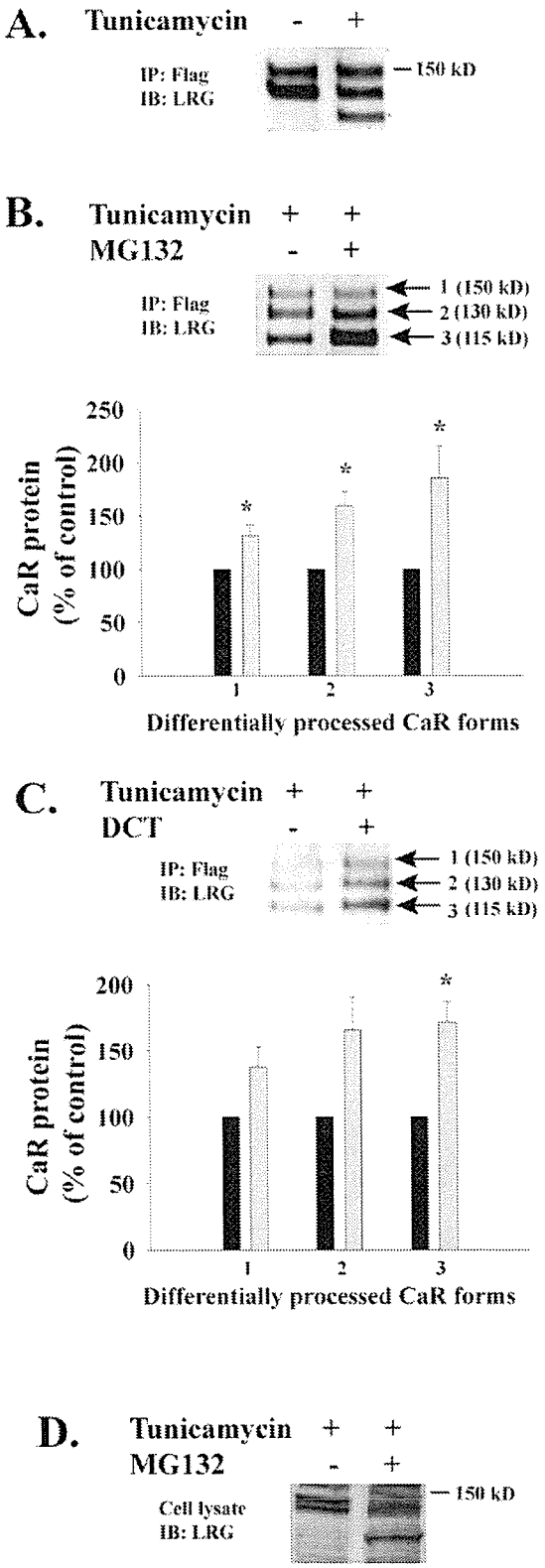

A.
Tunicamycin - +
IP: Flag
IB: LRG
150 kD
B.
Tunicamycin + +
MG132 - +
IP: Flag
IB: LRG
1 (150 kD)
2 (130 kD)
3 (115 kD)
CaR protein (% of control)
250
200
150
100
50
0
1
2
3
Differentially processed CaR forms
C.
Tunicamycin + +
DCT - +
IP: Flag
IB: LRG
1 (150 kD)
2 (130 kD)
3 (115 kD)
CaR protein (% of control)
200
150
100
50
0
1
2
3
Differentially processed CaR forms
D.
Tunicamycin + +
MG132 - +
Cell lysate
IB: LRG
150 kD

Figure 7

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Underediting of GluR2 mRNA, a neuronal death inducing molecular change in sporadic ALS, does not occur in motor neurons in ALS1 or SBMA

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Abstract

Deficient RNA editing of the AMPA receptor subunit GluR2 at the Q/R site is a primary cause of neuronal death and recently has been reported to be a tightly linked etiological cause of motor neuron death in sporadic amyotrophic lateral sclerosis (ALS). We quantified the RNA editing efficiency of the GluR2 Q/R site in single motor neurons of rats transgenic for mutant human Cu/Zn-superoxide dismutase (SOD1) as well as patients with spinal and bulbar muscular atrophy (SBMA), and found that GluR2 mRNA was completely edited in all the motor neurons examined. It seems likely that the death cascade is different among the dying motor neurons in sporadic ALS, familial ALS with mutant SOD1 and SBMA.

Keywords: ALS; SOD1; Spinal and bulbar muscular atrophy; Motor neuron; RNA editing; GluR2; AMPA receptor; Neuronal death

1. Introduction

Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disease with selective loss of both upper and lower motor neurons, and familial cases are rare. The etiology of sporadic ALS remains elusive but recently deficient RNA editing of AMPA receptor subunit GluR2 at the Q/R site is reported in motor neurons in ALS that occurs in a disease-specific and motor neuron-selective manner (Kawahara et al., 2004; Kwak and Kawahara, 2005). Moreover, underediting of the GluR2 Q/R site greatly increases the Ca^{2+} permeability of AMPA receptors (Hume et al., 1991; Verdoorn et al., 1991; Burnashev et al., 1992), which may cause neuronal death due to increased Ca^{2+} influx through the receptor channel, hence mice with RNA editing deficiencies at the GluR2 Q/R site die young (Brusa et al., 1995) and mice transgenic for an artificial Ca^{2+}-permeable GluR2 develop motor neuron disease 12 months after birth (Kuner et al., 2005). Such evidence lends strong support to the close relevance of deficient RNA editing of the GluR2 at the Q/R site to death of motor neurons in sporadic ALS. However, although we and other researchers have demonstrated that dying neurons in several neurodegenerative diseases exhibit edited GluR2 (Kwak and Kawahara, 2005), it has not yet been demonstrated whether the underediting of GluR2 occurs in dying motor neurons in motor neuron diseases other than ALS. Such investigation is of particular importance since it will help clarify whether the molecular mechanism of motor neurons death is common among various subtypes of motor neurons.

ALS associated with the SOD1 mutation (ALS1) is the most frequent familial ALS (Rosen et al., 1993), and mutated human SOD1 transgenic animals have been studied extensively as a disease model of ALS1, yet the etiology of neuronal death in the animals has not been elucidated. Another example of non-ALS motor neuron disease is spinal and bulbar muscular atrophy (SBMA), which predominantly affects lower motor neurons with a relatively slow clinical course. Since the CAG

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Table 1
RNA editing efficiency of single motor neurons in SBMA

Case	Age at death (year)	Sex	No. of CAG repeats[a]	Postmortem delay (h)	GluR2(+) MN[b]	MN with 100% editing efficiency (% of GluR2(+) MN)
SBMA, case 1	71	M	48	2.5	12	12 (100)
SBMA, case 2	78	M	42	2.5	16	16 (100)
SBMA, case 3	60	M	44	1	16	16 (100)

[a] Number of CAG repeats in the androgen receptor gene.
[b] Motor neurons in which GluR2 RT-PCR amplifying product was detected.

repeat expansion in the androgen receptor gene has been demonstrated in SBMA (La Spada et al., 1991), and pharmacological castration is therapeutically effective in animal models (Katsuno et al., 2002, 2003), the death cascade responsible for SBMA is likely different from sporadic ALS. In this paper, an investigation is carried out into whether or not the dying mechanism underlying sporadic ALS is the same as ALS1 and SBMA by determining the editing status of the GluR2 Q/R site in single motor neurons.

2. Materials and methods

The animals used in this study were $SOD1^{G93A}$ and $SOD1^{H46R}$ transgenic male rats (Nagai et al., 2001) ($n = 3$ each) that had exhibited progressive neuromuscular weakness with their littermates as the control ($n = 3$ each) (Table 2). The first sign of disease in these rats was weakness of their hindlimbs, mostly exhibited by the dragging of one limb. Onset of motor neuron disease was scored as the first observation of abnormal gait or evidence of limb weakness. The mean age of onset of clinical weakness for the $SOD1^{G93A}$ and $SOD1^{H46R}$ lines was 122.9 ± 14.1 and 144.7 ± 6.4 days, respectively. As the disease progressed, the rats exhibited marked muscle wasting in their hindlimbs, and then in the forelimbs. The mean duration after the clinical expression of the disease in the $SOD1^{G93A}$ and $SOD1^{H46R}$ lines was 8.3 ± 0.7 and 24.2 ± 2.9 days, respectively (Nagai et al., 2001). The rats were killed 3 days and 2 weeks after the onset for the $SOD1^{G93A}$ and $SOD1^{H46R}$ lines, respectively, and we examined their fifth lumbar cord. Animals were handled according to Institutional Animal Care and Use Committee approved protocols that are in line with the Guideline for Animal Care and Use by the National Institute of Health. Spinal cords were isolated after deep pentobarbiturate anesthesia. In addition, spinal cords were obtained at autopsy from three genetically confirmed patients with SBMA (Table 1). Written informed consent was obtained from all subjects prior to death or from their relatives, and the Ethics Committees of Graduate School of Medicine, the University of Nagoya and the University of Tokyo approved the experimental procedures used. Spinal cords were rapidly frozen on dry ice and maintained at −80 °C until use.

Table 2
RNA editing efficiency of single motor neurons in mutated human SOD1 transgenic rats

Case (n)	GluR2(+) MN[a]	MN with 100% editing efficiency (% of GluR2(+) MN)
$SOD1^{G93A}$-1	13	13 (100)
$SOD1^{G93A}$-2	21	21 (100)
$SOD1^{G93A}$-3	21	21 (100)
$SOD1^{H46R}$-1	19	19 (100)
$SOD1^{H46R}$-2	23	23 (100)
$SOD1^{H46R}$-3	20	20 (100)
$SOD1^{G93A}$, littermates (3)	22	22 (100)
$SOD1^{H46R}$, littermates (3)	20	20 (100)

[a] Motor neurons in which GluR2 RT-PCR amplifying product was detected.

Single motor neurons were isolated and collected into respective single test tubes that contained 200 μl of TRIZOL Reagent (Invitrogen Corp., Carlsbad, CA, USA) using a laser microdissection system as previously described (Kawahara et al., 2003b, 2004) (LMD, Leica Microsystems Ltd., Germany) (Fig. 1a). After extracting total RNA from single neuron tissue, we analyzed the RNA editing efficiency at the GluR2 Q/R site by means of RT-PCR coupled with digestion of the PCR amplified products with a restriction enzyme Bbv-1 (New England BioLabs, Beverly, MA, USA) (Takuma et al., 1999; Kawahara et al., 2003a, 2004), and the editing efficiency was calculated by quantitatively analyzing the digests with a 2100 Bioanalyser (Agilent Technologies, Palo Alto, CA, USA), as previously described (Kawahara et al., 2003a). Briefly, after gel purification using Zymoclean Gel DNA Recovery Kit according to the manufacturer's protocol (Zymo Research, Orange, CA, USA), PCR products were quantified using a 2100 Bioanalyser. An aliquot (0.5 μg) was then incubated at 37 °C for 12 h with 10 × restriction buffer and 2 U of *Bbv*I in a total volume of 20 μl and inactivated at 65 °C for 30 min. The PCR products had one intrinsic *Bbv*I recognition sites, whereas the products originating from unedited GluR2 mRNA had an additional recognition site. Thus, restriction digestion of the PCR products originating from edited rat (278 bp) and human (182 bp) GluR2 mRNA should produce two bands (human GluR2 in parenthesis) at 219 (116) and 59 (66) bp, whereas those originating from unedited GluR2 mRNA should produce three bands at 140 (81), 79 (35), and 59 (66) bp. As the 59 (66) bp band would originate from both edited and unedited mRNA, but the 219 (116) bp band would originate from only edited mRNA, we quantified the molarity of the 219 (116) and 59 (66) bp bands using the 2100 Bioanalyser and calculated the editing efficiency as the ratio of the former to the latter for each sample.

The following primers were used for PCR for rat and human GluR2 (amplified product lengths are also indicated): for rat GluR2 (278 bp): rF (5′-AGCAGATTTAGCCCCTACGAG-3′) and rR (5′-CAGCACTTTCGATGGGAGACAC-3′). for human GluR2, the first PCR (187 bp): hG2F1 (5′-TCTGGTTTTCCTTGGGTGCC-3′) and hG2R1 (5′-AGATCCTCAGCACTTTCG-3′); for the nested PCR (182 bp): hG2F2 (5′-GGTTTTCCTTGGGTGCCTTTAT-3′) and hG2R2 (5′-ATCCTCAGCACTTTCGATGG-3′). We confirmed that these primer pairs were situated in two distinct exons with an intron between them and did not amplify products originating from other GluR subunits (data not shown). PCR amplification for rat GluR2 was initiated with a denaturation step that was carried out at 95 °C for 2 min, followed by 40 cycles of 95 °C for 30 s, 62 °C for 30 s, and 72 °C for 1 min. PCR amplification for human GluR2 began with a 1 min denaturation step at 95 °C, followed by 35 cycles of denaturation at 95 °C for 10 s, annealing at 64 °C for 30 s and extension at 68 °C for 60 s. Nested PCR was conducted on 2 μl of the first PCR product under the same conditions with the exception of the annealing temperature (66 °C).

3. Results

The number of motor neurons was severely decreased in the spinal cord of SBMA patients, and we analyzed 44 neurons dissected from three cases (12 from case 1, 16 from cases 2 and 3). Restriction digestion of the PCR products yielded only 116 and 66 bp fragments but no 81 or 35 bp fragments as seen in ALS motor neurons in all the SBMA motor neurons examined. Likewise, restriction digestion of the PCR products from motor

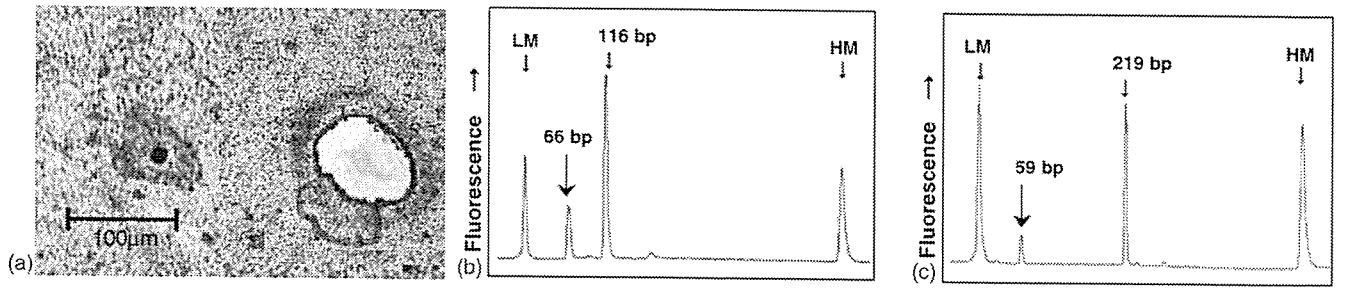

Fig. 1. (a) A single motor neuron from an SBMA patient before (left) and after (right) the dissection with a laser-microdissector. (b and c) An example of electropherogram by a 2100 Bioanalyser. Samples are the Bbv-1-digest of PCR product from tissues of a single motor neuron from an SBMA patient (b) and from a mutated human $SOD1^{G93A}$ transgenic mouse (c). LM: lower marker (15 bp), HM: higher marker (600 bp).

neurons of mutated human SOD1 transgenic rats yielded only 219 and 59 bp fragments (Fig. 1). Therefore, the values of RNA editing efficiency at the Q/R site of GluR2 were 100% in 44 motor neurons from three SBMA cases (Table 1), 55 single motor neurons from three $SOD1^{G93A}$ transgenic rats, 62 neurons from three $SOD1^{H46R}$ transgenic rats, as well as in 42 neurons from three littermate rats of each group (Table 2). The consistent finding that the GluR2 Q/R site is 100% edited in motor neurons of SBMA patients and transgenic rats for mutated human SOD1 is in marked contrast to the finding in ALS motor neurons that the editing efficiency widely varied among neurons ranging from 0% to 100% (Kawahara et al., 2004).

4. Discussion

Compared to the significant underediting reported for the GluR2 Q/R site in motor neurons of sporadic ALS (Kawahara et al., 2004), GluR2 mRNA in all the examined motor neurons of the mutated human SOD1 transgenic rats with two different mutation sites and SBMA patients was completely edited at the Q/R site. We have confirmed that postmortem delay hardly influenced the editing efficiency at the GluR2 Q/R site (Kawahara et al., 2003b), hence the significant difference in the postmortem delay between the SBMA patients in this study and ALS patients in the previous report (Kawahara et al., 2004) would not have affected these results. We examined the motor neurons in the spinal cord segment corresponding to the hindlimb of mutated human SOD1 transgenic rats after their hindlimbs had become weak, indicating that the motor neurons examined were already pathologically affected. Likewise, we found that only a small number of motor neurons remained in the spinal cord of SBMA patients. Thus our results indicate that GluR2 RNA editing was complete in the dying motor neurons in both the mutated human SOD1 transgenic rats and SBMA patients, implying that the neuronal death mechanism is not due to the underediting of GluR2 mRNA seen in sporadic ALS. Since the pathogenic mechanism underlying ALS1 is considered to be the same as in mutant human SOD1 transgenic animals, motor neurons in affected ALS1 patients would be expected to have only edited GluR2 mRNA. Indeed, an association study of the SOD1 gene in a considerable number of patients with sporadic ALS reported no significant association with mutations of the SOD1 gene (Jackson et al., 1997). Due to the lack of appropriate animal model for sporadic ALS, mutant human SOD1 transgenic animals have been used as a model for ALS in general, particularly in studies searching for therapeutically effective drugs. However, it should be kept in mind that mutated human SOD1 transgenic animals are merely a suggestive model for sporadic ALS and a gain of toxic function in mutated SOD1 kills motor neurons via mechanisms other than the demise of RNA editing. There are likely multiple different death pathways in motor neurons, and motor neurons in sporadic ALS, ALS1 and SBMA die by different death cascades.

Acknowledgements

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References

Brusa, R., Zimmermann, F., Koh, D., Feldmeyer, D., Gass, P., Seeburg, P., Sprengel, R., 1995. Early-onset epilepsy and postnatal lethality associated with an editing-deficient GluR-B allele in mice. Science 270, 1677–1680.

Burnashev, N., Monyer, H., Seeburg, P., Sakmann, B., 1992. Divalent ion permeability of AMPA receptor channels is dominated by the edited form of a single subunit. Neuron 8, 189–198.

Hume, R.I., Dingledine, R., Heinemann, S.F., 1991. Identification of a site in glutamate receptor subunits that controls calcium permeability. Science 253, 1028–1031.

Jackson, M., Al-Chalabi, A., Enayat, Z.E., Chioza, B., Leigh, P.N., Morrison, K.E., 1997. Copper/zinc superoxide dismutase 1 and sporadic amyotrophic lateral sclerosis: analysis of 155 cases and identification of a novel insertion mutation. Ann. Neurol. 42, 803–807.

Katsuno, M., Adachi, H., Doyu, M., Minamiyama, M., Sang, C., Kobayashi, Y., Inukai, A., Sobue, G., 2003. Leuprorelin rescues polyglutamine-dependent phenotypes in a transgenic mouse model of spinal and bulbar muscular atrophy. Nat. Med. 9, 768–773.

Katsuno, M., Adachi, H., Kume, A., Li, M., Nakagomi, Y., Niwa, H., Sang, C., Kobayashi, Y., Doyu, M., Sobue, G., 2002. Testosterone reduction prevents phenotypic expression in a transgenic mouse model of spinal and bulbar muscular atrophy. Neuron 35, 843–854.

Kawahara, Y., Ito, K., Sun, H., Aizawa, H., Kanazawa, I., Kwak, S., 2004. RNA editing and death of motor neurons. Nature 427, 801.

Kawahara, Y., Ito, K., Sun, H., Kanazawa, I., Kwak, S., 2003a. Low editing efficiency of GluR2 mRNA is associated with a low relative abundance of

ADAR2 mRNA in white matter of normal human brain. Eur. J. Neurosci. 18, 23–33.

Kawahara, Y., Kwak, S., Sun, H., Ito, K., Hashida, H., Aizawa, H., Jeong, S.-Y., Kanazawa, I., 2003b. Human spinal motoneurons express low relative abundance of GluR2 mRNA: an implication for excitotoxicity in ALS. J. Neurochem. 85, 680–689.

Kuner, R., Groom, A.J., Bresink, I., Kornau, H.C., Stefovska, V., Muller, G., Hartmann, B., Tschauner, K., Waibel, S., Ludolph, A.C., Ikonomidou, C., Seeburg, P.H., Turski, L., 2005. Late-onset motoneuron disease caused by a functionally modified AMPA receptor subunit. Proc. Natl. Acad. Sci. U.S.A. 102, 5826–5831.

Kwak, S., Kawahara, Y., 2005. Deficient RNA editing of GluR2 and neuronal death in amyotropic lateral sclerosis. J. Mol. Med. 83, 110–120.

La Spada, A.R., Wilson, E.M., Lubahn, D.B., Harding, A.E., Fischbeck, K.H., 1991. Androgen receptor gene mutations in X-linked spinal and bulbar muscular atrophy. Nature 352, 77–79.

Nagai, M., Aoki, M., Miyoshi, I., Kato, M., Pasinelli, P., Kasai, N., Brown Jr., R.H., Itoyama, Y., 2001. Rats expressing human cytosolic copper–zinc superoxide dismutase transgenes with amyotrophic lateral sclerosis: associated mutations develop motor neuron disease. J. Neurosci. 21, 9246–9254.

Rosen, D.R., Siddique, T., Patterson, D., Figlewicz, D.A., Sapp, P., Hentati, A., Donaldson, D., Goto, J., O'Regan, J.P., Deng, H.X., et al., 1993. Mutations in Cu/Zn superoxide dismutase gene are associated with familial amyotrophic lateral sclerosis. Nature 362, 59–62.

Takuma, H., Kwak, S., Yoshizawa, T., Kanazawa, I., 1999. Reduction of GluR2 RNA editing, a molecular change that increases calcium influx through AMPA receptors, selective in the spinal ventral gray of patients with amyotrophic lateral sclerosis. Ann. Neurol. 46, 806–815.

Verdoorn, T., Burnashev, N., Monye, R.H., Seeburg, P., Sakmann, B., 1991. Structural determinants of ion flow through recombinant glutamate receptor channels. Science 252, 1715–1718.

Mutant Androgen Receptor Accumulation in Spinal and Bulbar Muscular Atrophy Scrotal Skin: A Pathogenic Marker

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Objective: **Spinal and bulbar muscular atrophy (SBMA) is a hereditary motor neuron disease caused by the expansion of a polyglutamine tract in the androgen receptor (AR). The nuclear accumulation of mutant AR is central to the pathogenesis of SBMA. Androgen deprivation with leuprorelin inhibits mutant AR accumulation, resulting in rescue of neuronal dysfunction in a mouse model of SBMA. This study aimed to investigate whether mutant AR accumulation in the scrotal skin is an appropriate biomarker of SBMA.** ***Methods:*** **Immunohistochemistry of both scrotal skin and the spinal cord was performed on five autopsied SBMA cases. Neurological severity and scrotal skin findings were studied in another 13 patients. Five other patients received subcutaneous injections of leuprorelin and underwent scrotal skin biopsy.** ***Results:*** **The degree of mutant AR accumulation in scrotal skin epithelial cells tended to be correlated with that in the spinal motor neurons in autopsy specimens, and it was well correlated with CAG repeat length and inversely correlated with the amyotrophic lateral sclerosis functional scale. Leuprorelin treatment inhibited mutant AR protein accumulation in the scrotal skin of SBMA patients.** ***Interpretation:*** **These observations suggest that scrotal skin biopsy findings are a potent pathogenic marker of SBMA and can be a surrogate end point in therapeutic trials.**

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Spinal and bulbar muscular atrophy (SBMA), also known as Kennedy's disease, is an adult-onset motor neuron disease characterized by muscle atrophy, weakness, contraction fasciculations, and bulbar involvement.[1–4] SBMA exclusively affects men in their 30s or 40s, and disease progression is slow.[1,5] The molecular basis of SBMA is the expansion of a trinucleotide CAG repeat, which encodes a polyglutamine (polyQ) tract, in the androgen receptor (AR) gene.[6] The CAG repeat numbers range from 38 to 62 in SBMA patients, whereas healthy individuals have 10 to 36 CAGs.[6,7] The number of CAGs is correlated with disease severity and is inversely correlated with age of onset,[8,9] as observed in other polyQ-related neurodegenerative diseases including Huntington's disease and several forms of spinocerebellar ataxia.[10]

Histopathologically, lower motor neurons are markedly depleted in the spinal cord and brainstem, and nuclear inclusions (NIs) containing the mutant and truncated AR with expanded polyQ are present in the residual motor neurons, as well as in cells of the scrotal skin and other visceral organs.[3,11,12] Although NIs are a disease-specific pathological marker, they may reflect a cellular protective response against the toxicity of abnormal polyQ-containing protein.[13] In contrast, the therapeutic effect of testosterone deprivation in our SBMA transgenic mouse model suggested that diffuse nuclear accumulation of mutant AR is a cardinal pathogenic process underlying neurological manifestations.[14,15] This hypothesis has also been clearly illustrated by the observation that the extent of diffuse nuclear accumulation of mutant AR, but not NIs, in the motor neurons of the spinal cord was closely related to CAG repeat length in autopsied SBMA cases.[16] Nuclear localization of the mutant protein has now been considered essential for inducing neuronal cell dysfunction and degeneration in the majority of polyQ diseases.[10]

A characteristic clinical feature of SBMA is that the disease occurs in male but not female individuals, even when they are homozygous for the mutation.[17,18] Several studies have clarified that the sex dependency of disease manifestation in SBMA arises from testosterone-dependent nuclear accumulation of mu-

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tant AR.[14,15,19,20] Leuprorelin, a leuteinizing hormone–releasing hormone agonist that reduces testosterone release from the testis and inhibits nuclear accumulation of mutant AR, rescued motor dysfunction in male transgenic mice carrying the full-length human AR with expanded polyQ.[15]

Although data from transgenic mice studies indicated that androgen deprivation from leuprorelin treatment is a potent therapeutic agent for SBMA,[14,15] clinical experience using this drug for SBMA patients is limited.[21] Because long-term clinical trials are needed to establish the efficacy of therapeutics ameliorating disease progression in slowly progressive neurodegenerative diseases such as SBMA, an appropriate biomarker reflecting pathogenic processes of the disease is necessary. The aim of this study was to test the hypothesis that peripheral accumulation of mutant AR in the scrotal skin represents a suitable biomarker of SBMA that can be applicable as a surrogate end point in therapeutic trials.

Patients and Methods

Patients

Twenty-three patients with clinically and genetically confirmed SBMA were examined. Patient characteristics are shown in the Table. Five of the 23 patients underwent autopsy, and both the scrotal skin and the spinal cord were examined; another 13 patients underwent biopsy of the scrotal skin. The remaining five patients were enrolled in a leuprorelin study and also underwent biopsy of the scrotal skin. All patients were hospitalized and underwent follow-up examinations at Nagoya University Hospital (Nagoya, Japan) and its affiliated hospitals.

For each of the 18 patients who underwent biopsy of the scrotal skin, three scrotal skin specimens were made by punch biopsy using a 3mm diameter Dermapunch (Nipro, Tokyo, Japan) under 10ml lidocaine acetate local anesthesia. All patients who underwent biopsy sterilized the wound for several days by themselves and received 4 days of cefaclor (250mg three times a day) antibiotic therapy after the procedure. The 13 patients who underwent biopsy who were not enrolled in the leuprorelin trial were also assessed on the amyotrophic lateral sclerosis functional scale (limb Norris score), as described previously.[22]

Five other male subjects (age, 60–74 years; mean, 67.3 years) who died of nonneurological diseases served as control subjects. The Nagoya University Hospital Institutional Review Board approved the collection of data and specimens, and all patients gave their written, informed consent to participate.

Leuprorelin Administration

Five patients received subcutaneous injections of 3.75mg leuprorelin once every 4 weeks. The patients, aged 43 to 68 years, were capable of walking with or without a cane and expressed no desire to father a child. They were observed for 6 months (24 weeks), and scrotal skin biopsies were taken from each patient at 0, 4, and 12 weeks after initial leuprorelin administration. Serum creatine kinase (CK) was determined by ultraviolet measurement using hexokinase and glucose-6-phosphate. Serum testosterone levels were measured by radioimmunoassay using the DPC total testosterone kit (Diagnostic Products Corporation, Los Angeles, CA).

Immunohistochemical Detection of the Mutant Androgen Receptor in the Scrotal Skin and Spinal Cord

Immunohistochemistry of scrotal skin specimens and the spinal cord were conducted as described previously.[16] In brief, we prepared 5μm-thick, formalin-fixed, paraffin-embedded sections of scrotal skin and spinal cord from SBMA patients. Sections were deparaffinized and rehydrated through a graded series of alcohol-water solutions. For the mutant AR immunohistochemical study, sections were pretreated with immersion in 98% formic acid for 5 minutes, and then with microwave oven heating for 15 minutes in 10mM citrate buffer at pH 6.0. Sections were incubated with a mouse anti-expanded polyQ antibody (1:20,000; 1C2; Chemicon, Temecula, CA)[23] to evaluate the nuclear accumulation of mutant AR.[14–16] Immune complexes were visualized using the Envision-plus kit (Dako, Glostrup, Denmark). Sections were counterstained with Mayer's hematoxylin. For electron microscopic immunohistochemistry, the sections were processed

Table. Patient Characteristics

Characteristics	Autopsy Study (N = 5)	Biopsy Alone Study (N = 13)	Leuprorelin + Biopsy Study (N = 5)
Age (mean ± SD), yr	64.8 ± 10.8	54.8 ± 9.6	50.2 ± 10.8
Duration of weakness (mean ± SD), yr	38.4 ± 14.7	11.0 ± 7.4	8.8 ± 4.9
(CAG)n (mean ± SD)	47.4 ± 4.9	48.2 ± 3.0	49.2 ± 4.9
ADL (cane/independent ratio)	NA	4/9	2/3
Limb Norris score (mean ± SD)	NA	53.4 ± 6.9	52.0 ± 6.8
Norris bulbar score (mean ± SD)	NA	32.2 ± 3.4	32.8 ± 6.2
ALSFRS-R (Japanese edition) (mean ± SD)	NA	40.3 ± 3.2	39.2 ± 3.8
Cause of death	Pneumonia (n = 4); Lung cancer (n = 1)	NA	NA

The amyotrophic lateral sclerosis functional rating scale-revised.
SD = standard deviation; (CAG)n = number of expanded CAG repeats in the SBMA allele; NA = not applicable; ADL = activities of daily living.

as described for light microscopic immunohistochemistry, and then fixed with 2% osmium tetroxide in 0.1M phosphate buffer at pH 7.4, dehydrated in graded alcohol-water solutions, and embedded in epoxy resin. Ultrathin sections were cut for observation under an electron microscope (H-7100; Hitachi High-Technologies Corporation, Tokyo, Japan).

Quantification of Cell Population with Diffuse Nuclear Staining

For quantitative assessment of scrotal skin cells, the frequency of diffuse nuclear staining was calculated from counts of more than 500 nuclei in 5 randomly selected fields of each section photographed at 400× magnification (BX51TF; Olympus, Tokyo, Japan). To assess the nuclear accumulation of mutant AR in spinal cord motor neurons, we prepared at least 100 transverse sections each from the cervical, thoracic, and lumber spinal cord for anti-expanded polyQ antibody staining with 1C2. The numbers of 1C2-positive cells in the ventral horn on both the right and left sides were counted on every 10th section under the light microscope with a computer-assisted image analyzer (BX51TF; Olympus), as described previously.[16,24] Populations of 1C2-positive cells were expressed as percentages of the total skin cell or neuronal count.

Statistical Analysis

We analyzed the data by Pearson's coefficient, Spearman's rank correlation, and Student's paired t test as appropriate using StatView software (version 5; Hulinks, Tokyo, Japan) and considering p values less than 0.05 to be indicative of significance.

Results

Mutant Androgen Receptor Nuclear Accumulation in the Scrotal Skin and Spinal Motor Neuron

In the five autopsied cases, mutant AR nuclear accumulations were clearly visualized with anti-expanded polyQ immunostaining with 1C2 in the scrotal skin and spinal cord specimens (Fig 1A). Pathological accumulation of mutant AR was distributed in all layers of the epithelium. Diffuse nuclear accumulations were predominantly observed, and the occurrence of NIs was less frequent. This was also the case in the spinal cord specimens. Electron microscopic immunohistochemistry with the 1C2 antibody demonstrated granular dense and amorphous aggregates corresponding to diffuse nuclear staining in both spinal motor neurons and epithelial cells of scrotal skin (see Figs 1B, C). Filamentous structures such as those reported in Huntington's disease,[25] dentatorubal-pallidoluysian atrophy (DRPLA),[26] and Machado–Joseph disease[27] were not seen. No diffuse nuclear staining was seen in the control subjects. The extent of mutant AR accumulation in the scrotal skin epithelial cells showed a tendency to correlate with that in the anterior horn cells ($r = 0.84$; $p = 0.08$; see Fig 1D). Mutant AR accumulation was remarkable in both the spinal motor neurons and the scrotal skin of Patient 1, but was far less remarkable in Patient 2 (see Figs 1A, D).

Correlations of the Mutant Androgen Receptor Accumulation in the Scrotal Skin to CAG Repeat Length and Amyotrophic Lateral Sclerosis Score

Mutant AR nuclear accumulations in scrotal skin biopsies from the 13 SBMA patients who did not receive leuprorelin were assessed by 1C2 antibody staining of expanded PolyQ. The 1C2-positive cell population in the scrotal skin biopsies was significantly correlated with CAG repeat length ($r = 0.61$; $p = 0.03$; Fig 2A) and was inversely correlated with the functional scale assessed by the Norris score on limbs ($r = -0.63$; $p = 0.02$; see Fig 2B).

Leuprorelin Treatment Depletes Mutant Androgen Receptor Accumulation in the Scrotal Skin

In all five patients in which leuprorelin was administered (see the Table), both the intensity and the frequency of diffuse nuclear 1C2 staining in the scrotal epithelial cells was decreased after the first 4 weeks of administration compared with the preadministration values, and this effect was markedly enhanced after 12 weeks of treatment (Figs 3A, B). Quantitative analysis demonstrated a significant decrease in the frequency of 1C2-positive cells both 4 and 12 weeks after the initiation of leuprorelin treatment ($p < 0.01$) (see Fig 3C). Serum testosterone levels decreased to the castration level after 1 to 2 months of treatment (see Fig 3D), and serum CK values were also significantly decreased in all patients (see Fig 3D).

None of the patients showed the hot flush or obesity often reported in leuprorelin trials for prostate cancer. Although a loss of sexual function including erectile disorder was observed in all patients, no patients experienced depression. No marked exacerbations were observed in total cholesterol, triglyceride, fasting blood sugar, or HbA1c (data not shown). We could not find significant motor function changes assessed by amyotrophic lateral sclerosis functional scores in 24 weeks, but three of the five enrolled patients expressed apparent subjective improvement.

Discussion

This study demonstrated that scrotal skin biopsy with anti-expanded polyQ staining is a strong candidate for an appropriate biomarker with which to monitor SBMA pathogenic processes. Previous studies showed that the severity and progression of motor dysfunction and abatement of abnormalities in mice that were castrated or given leuprorelin paralleled the extent of diffuse nuclear mutant AR accumulation in their spinal motor neurons.[14,15] Furthermore, we demonstrated previously a significant, close correlation between the length of CAG repeat expansion and frequency of dif-

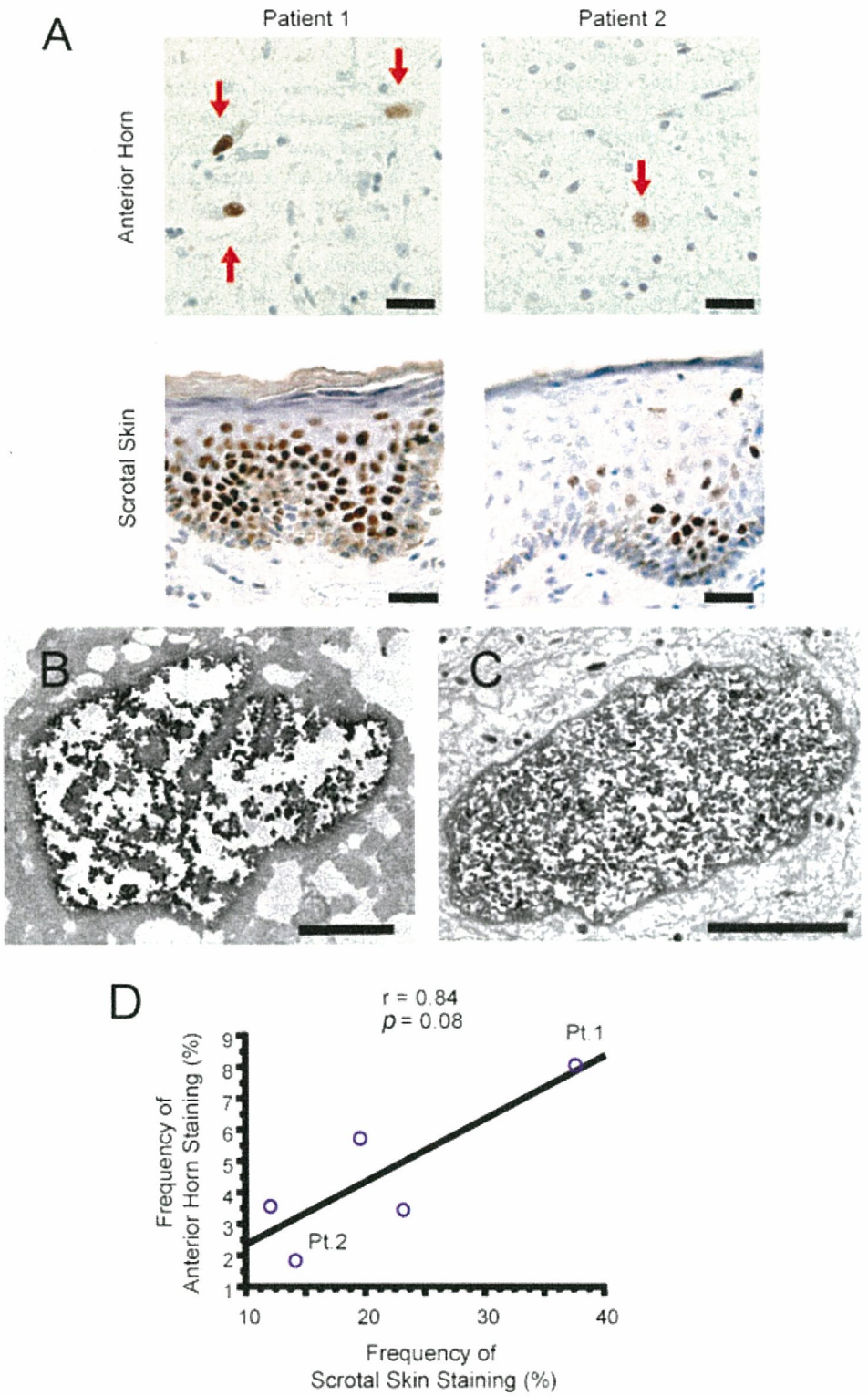

Fig 1. Mutant androgen receptor (AR) nuclear accumulation in scrotal skin and spinal motor neurons. (A) Mutant AR accumulation was remarkable in both spinal motor neurons (arrows) and scrotal skin of Patient 1, but was less remarkable in both motor neurons (arrows) and skin in Patient 2. Bar = 30μm. (B, C) Electron microscopic immunohistochemistry for 1C2 demonstrated granular dense and amorphous aggregates corresponding to diffuse nuclear staining in both spinal motor neurons and epithelial cells of scrotal skin. Bar =3μm. (D) The extent of mutant AR accumulation in scrotal skin epithelial cells showed a tendency to correlate with that in anterior horn cells. Circles (Pt. 1, Pt. 2) correspond to Patient 1 and 2 in Fig 1A.

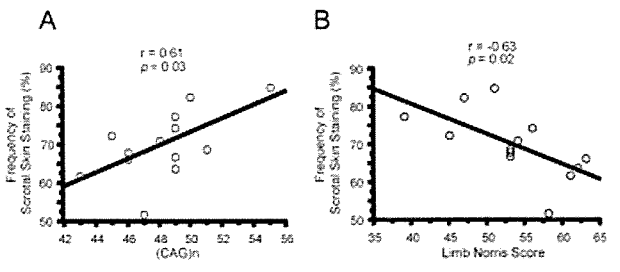

Fig 2. Correlation of the frequency of scrotal skin staining to CAG repeats and limb Norris score. The frequency of 1C2-positive cells in the scrotal skin biopsies correlated significantly with (A) CAG repeat length and (B) inversely correlated with the amyotrophic lateral sclerosis functional scale assessed by the Norris score on limbs. (CAG)n = number of expanded CAG repeats in the spinal and bulbar muscular atrophy allele.

fuse nuclear mutant AR accumulation, but not that of NIs, in the spinal cord.[16] Accordingly, neuronal dysfunction is likely to be caused by diffuse nuclear accumulation of mutant AR in the affected tissues. In this study, the extent of mutant AR nuclear accumulation in scrotal skin cells paralleled that in the anterior horn cells in autopsied cases. Electron microscopic immunohistochemistry for 1C2 anti-expanded PolyQ demonstrated granular dense and amorphous aggregates corresponding to diffuse nuclear staining in both spinal motor neurons and epithelial cells of scrotal skin. Furthermore, the fine structure of the aggregates in spinal motor neurons and epithelial cells was quite similar. Biopsy analyses in this study also suggested that scrotal skin findings were correlated with the motor functional scores of SBMA patients.

Our findings suggest that nuclear mutant AR assessed by 1C2 immunostaining in the scrotal skin is a practical procedure to estimate the severity of SBMA pathogenesis in the nervous system. In support of this view, decreases in mutant AR accumulation in the motor neurons paralleled that in nonneuronal cells in the androgen deprivation therapy tested in the mouse model of SBMA. In addition, leuprorelin treatment markedly reduced serum testosterone levels, as well as nuclear accumulation of mutant AR in the scrotal skin, suggesting that medical castration with leuprorelin intervenes in the pathogenic process of human SBMA, as demonstrated in the animal study. Moreover, serum CK levels were significantly decreased in this leuprorelin study. Because high CK values are common in SBMA patients and histopathological examinations have shown myogenic changes together with neurogenic findings in this disease,[1,3] presumably, a decrease in CK values with leuprorelin treatment implies muscular protection. Serum CK levels, however, did not significantly correlate with the Norris score on limbs or with scrotal skin biopsy findings in our cross-sectional study.

As defined by the Biomarkers Definitions Working Group, a disease biomarker should be objectively measurable and evaluated as an indicator of pathogenic processes or pharmacological responses to a therapeutic intervention.[28] Based on the observations described earlier, 1C2-stained mutant AR accumulation in the biopsied scrotal skin is likely to be a potent biomarker reflecting pathogenic processes of SBMA. Particularly, the correlation of the extent of mutant AR nuclear accumulation in the spinal motor neurons with that in scrotal skin biopsies in the autopsied cases suggests that findings in the scrotal skin can predict pathogenic processes in the motor neurons.

Although its precise natural history has not been evaluated, SBMA is a slowly progressive disease.[1,5] Thus, extremely long-term clinical trials are necessary to assess whether certain drugs can alter the natural disease progression by targeting clinical end points such as occurrence of aspiration pneumonia or becoming wheelchair bound. Suitable surrogate end points, which reflect the pathogenesis and severity of SBMA, are substantial to assess the therapeutic efficacy in drug trials. Although it is not practical to obtain biopsy specimens from the central nervous system (CNS), a punch biopsy of the scrotal skin enables a safe and accessible examination for patients.

It has also been suggested that reliance on surrogate end points can be misleading because they may not accurately predict the actual effects that treatments have on the health of a patient, as was seen with the CD4 counts in human immunodeficiency virus trials, the bone mineral density in osteoporosis trials, and others.[29] However, several factors have been suggested to consider the decision to rely on a surrogate.[30] In SBMA, mutant AR accumulation assessed by scrotal skin biopsy can be a candidate for a surrogate end point in light of several pieces of evidence. First, a credible SBMA animal model demonstrated dramatic functional motor recovery in response to testosterone deprivation therapy that depleted mutant AR accumulation in the central nervous system, as well as in nonneuronal tissues.[14,15] Second, the degree of diffuse nuclear accumulation of mutant AR in both the CNS and scrotal skin correlates well with CAG repeat length and disease severity, indicating that it is a natural phenomenon of and reflects the underlying pathology of the disease. Third, autopsy studies show that levels of nuclear AR accumulation in the scrotal skin are correlated with those in the CNS. Moreover, levels of nuclear translocated mutant AR in the scrotal skin decreased significantly in response to drug therapy that has been shown to deplete such accumulations in the CNS of SBMA mice, to significantly rescue motor dysfunction in these mice, and to partially stabilize neurological symptoms in one reported case of human SBMA.[21]

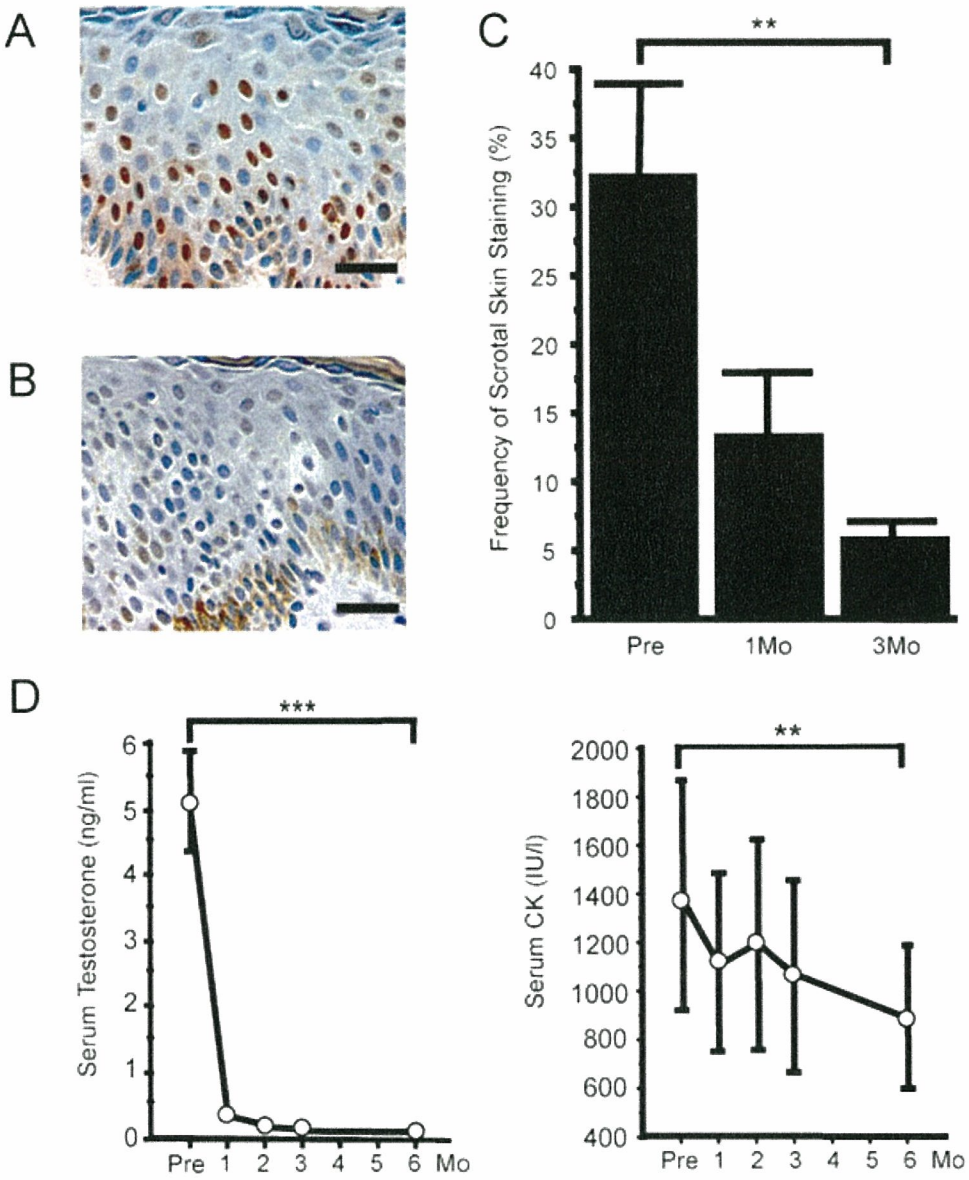

Fig 3. Effects of leuprorelin on mutant androgen receptor (AR) accumulation in scrotal skin, serum testosterone, and creatine kinase (CK). (A) Scrotal skin shows intense and frequent staining for anti-polyglutamine antibody in the nucleus before therapy. (B) Twelve weeks after therapy, both intensity and frequency of nuclear staining markedly decreased. Bar = 30μm. (C) Quantitative analysis of immunohistochemistry demonstrated a significant decrease in the number of positively stained nuclei. (D) Serum testosterone and CK decreased significantly in 6 months. Frequency of staining was calculated from counts of more than 500 nuclei in randomly selected areas and was expressed as mean ± standard deviation for 5 patients. $^{**}p < 0.01$; $^{***}p < 0.0001$.

Although our results were obtained from a small sample, nuclear accumulation of mutant AR in the scrotal skin appears to be a potent pathogenic biomarker of SBMA. A correlation between decline in validated clinical scales and nuclear mutant AR accumulations must be demonstrated in a longitudinal study to verify this histopathological feature as a biomarker for clinical severity. Similarly, validation of the scrotal skin biopsy findings as a surrogate end point in clinical trials will require a longitudinal study verifying that suppression of nuclear staining correlates with improvement on a validated clinical scale and the true clinical outcome events such as the need for a wheelchair, the presence of aspiration pneumonia, or death.

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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. Sperfeld AD, Karitzky J, Brummer D, et al. X-linked bulbospinal neuropathy: Kennedy disease. Arch Neurol 2002;59: 1921–1926.
3. Sobue G, Hashizume Y, Mukai E, et al. X-linked recessive bulbospinal neuronopathy: a clinicopathological study. Brain 1989; 112:209–232.
4. Katsuno M, Adachi H, Tanaka F, et al. Spinal and bulbar muscular atrophy (SBMA): ligand-dependent pathogenesis and therapeutic perspective. J Mol Med 2004;82:298–307.
5. Sobue G, Adachi H, Katsuno M. Spinal and bulbar muscular atrophy (SBMA). In: Dickinson D, ed. Neurodegeneration: the molecular pathology of dementia and movement disorders. Basel: INS Neuropathology, 2003:275–279.
6. La Spada AR, Wilson EM, Lubahn DB, et al. Androgen receptor gene mutations in X-linked spinal and bulbar muscular atrophy. Nature 1991;352:77–79.
7. Tanaka F, Doyu M, Ito Y, et al. Founder effect in spinal and bulbar muscular atrophy (SBMA). Hum Mol Genet 1996;5: 1253–1257.
8. Doyu M, Sobue G, Mukai E, et al. Severity of X-linked recessive bulbospinal neuronopathy correlates with size of the tandem CAG repeat in androgen receptor gene. Ann Neurol 1992; 32:707–710.
9. Shimada N, Sobue G, Doyu M, et al. X-linked recessive bulbospinal neuronopathy: clinical phenotypes and CAG repeat size in androgen receptor gene. Muscle Nerve 1995;18: 1378–1384.
10. Zoghbi HY, Orr HT. Glutamine repeats and neurodegeneration. Annu Rev Neurosci 2000;23:217–247.
11. Li M, Miwa S, Kobayashi Y, et al. Nuclear inclusions of the androgen receptor protein in spinal and bulbar muscular atrophy. Ann Neurol 1998;44:249–254.
12. Li M, Nakagomi Y, Kobayashi Y, et al. Nonneural nuclear inclusions of androgen receptor protein in spinal and bulbar muscular atrophy. Am J Pathol 1998;153:695–701.
13. Arrasate M, Mitra S, Schweitzer ES, et al. Inclusion body formation reduces levels of mutant huntingtin and the risk of neuronal death. Nature 2004;431:805–810.
14. Katsuno M, Adachi H, Kume A, et al. Testosterone reduction prevents phenotypic expression in a transgenic mouse model of spinal and bulbar muscular atrophy. Neuron 2002;35: 843–854.
15. Katsuno M, Adachi H, Doyu M, et al. Leuprorelin rescues polyglutamine-dependent phenotypes in a transgenic mouse model of spinal and bulbar muscular atrophy. Nat Med 2003; 9:768–773.
16. Adachi H, Katsuno M, Minamiyama M, et al. Widespread nuclear and cytoplasmic accumulation of mutant androgen receptor in SBMA patients. Brain 2005;128:659–670.
17. Sobue G, Doyu M, Kachi T, et al. Subclinical phenotypic expressions in heterozygous females of X-linked recessive bulbospinal neuronopathy. J Neurol Sci 1993;117:74–78.
18. Schmidt BJ, Greenberg CR, Allingham-Hawkins DJ, Spriggs EL. Expression of X-linked bulbospinal muscular atrophy (Kennedy disease) in two homozygous women. Neurology 2002;59:770–772.
19. Takeyama K, Ito S, Yamamoto A, et al. Androgen-dependent neurodegeneration by polyglutamine-expanded human androgen receptor in Drosophila. Neuron 2002;35:855–864.
20. Chevalier-Larsen ES, O'Brien CJ, Wang H, et al. Castration restores function and neurofilament alterations of aged symptomatic males in a transgenic mouse model of spinal and bulbar muscular atrophy. J Neurosci 2004;24:4778–4786.
21. Shimohata T, Kimura T, Nishizawa M, et al. Five year follow up of a patient with spinal and bulbar muscular atrophy treated with leuprorelin. J Neurol Neurosurg Psychiatry 2004;75: 1206–1207.
22. Norris FH Jr, Calanchini PR, Fallat RJ, et al. The administration of guanidine in amyotrophic lateral sclerosis. Neurology 1974;24:721–728.
23. Trottier Y, Lutz Y, Stevanin G, et al. Polyglutamine expansion as a pathological epitope in Huntington's disease and four dominant cerebellar ataxias. Nature 1995;378:403–406.
24. Terao S, Sobue G, Hashizume Y, et al. Age-related changes in human spinal ventral horn cells with special reference to the loss of small neurons in the intermediate zone: a quantitative analysis. Acta Neuropathol (Berl) 1996;92:109–114.
25. DiFiglia M, Sapp E, Chase KO, et al. Aggregation of huntingtin in neuronal intranuclear inclusions and dystrophic neurites in brain. Science 1997;277:1990–1993.
26. Hayashi Y, Kakita A, Yamada M, et al. Hereditary dentatorubral-pallidoluysian atrophy: ubiquitinated filamentous inclusions in the cerebellar dentate nucleus neurons. Acta Neuropathol (Berl) 1998;95:479–482.
27. Paulson HL, Perez MK, Trottier Y, et al. Intranuclear inclusions of expanded polyglutamine protein in spinocerebellar ataxia type 3. Neuron 1997;19:333–344.
28. Biomarkers Definitions Working Group. Biomarkers and surrogate endpoints: preferred definitions and conceptual framework. Clin Pharmacol Ther 2001;69:89–95.
29. Fleming TR, DeMets DL. Surrogate end points in clinical trials: are we being misled? Ann Intern Med 1996;125: 605–613.
30. Temple R. Are surrogate markers adequate to assess cardiovascular disease drugs? JAMA 1999;282:790–795.

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Natural history of spinal and bulbar muscular atrophy (SBMA): a study of 223 Japanese patients

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Spinal and bulbar muscular atrophy (SBMA) is an adult-onset motoneuron disease caused by a CAG-repeat expansion in the androgen receptor (*AR*) gene and for which no curative therapy exists. However, since recent research may provide opportunities for medical treatment, information concerning the natural history of SBMA would be beneficial in planning future clinical trials. We investigated the natural course of SBMA as assessed by nine activities of daily living (ADL) milestones in 223 Japanese SBMA patients (mean age at data collection = 55.2 years; range = 30–87 years) followed from 1 to 20 years. All the patients were diagnosed by genetic analysis. Hand tremor was an early event that was noticed at a median age of 33 years. Muscular weakness occurred predominantly in the lower limbs, and was noticed at a median age of 44 years, followed by the requirement of a handrail to ascend stairs at 49, dysarthria at 50, dysphagia at 54, use of a cane at 59 and a wheelchair at 61 years. Twenty-one of the patients developed pneumonia at a median age of 62 and 15 of them died at a median age of 65 years. The most common cause of death in these cases was pneumonia and respiratory failure. The ages at onset of each ADL milestone were strongly correlated with the length of CAG repeats in the *AR* gene. However CAG-repeat length did not correlate with the time intervals between each ADL milestone, suggesting that although the onset age of each ADL milestone depends on the CAG-repeat length in the *AR* gene, the rate of disease progression does not. The levels of serum testosterone, an important triggering factor for polyglutamine-mediated motoneuron degeneration, were maintained at relatively high levels even at advanced ages. These results provide beneficial information for future clinical therapeutic trials, although further detailed prospective studies are also needed.

Keywords: natural history; motoneuron disease; SBMA; Kennedy disease; ADL milestone

Abbreviations: ADL = activities of daily living; ALT = alanine aminotransferase; AR = androgen receptor; AST = aspartate aminotransferase; CK = creatine kinase; HbA1c = haemoglobin A1c; SBMA = spinal and bulbar muscular atrophy

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Introduction

Spinal and bulbar muscular atrophy (SBMA) is a neurodegenerative disorder of motoneurons characterized by proximal limb muscular atrophy, bulbar involvement, marked contraction fasciculation, hand tremor and gynaecomastia (Kennedy *et al.*, 1968; Sobue *et al.*, 1989). SBMA is caused by a CAG-repeat expansion in the first exon of the androgen receptor (*AR*) gene on the X-chromosome (La Spada *et al.*, 1991). Similar to other triplet repeat diseases, the age at onset of disease has been inversely linked to the size of the CAG-repeat expansions (Andrew *et al.*, 1993; Sasaki *et al.*, 1996; Rosenblatt *et al.*, 2003). For example, an association between the age at onset of limb muscle weakness and the CAG-repeat length has been demonstrated (Doyu *et al.*, 1992; Igarashi *et al.*, 1992; La Spada *et al.*, 1992; Shimada *et al.*, 1995; Sinnreich *et al.*, 2004). Nuclear accumulation of mutant *AR* with expanded polyglutamines in motoneurons, as well as in other cells, has been shown to be a major pathogenic process (Li *et al.*, 1998*a*, *b*; Adachi *et al.*, 2005). However, the progression and prognosis of SBMA has not been assessed in detail, particularly concerning the influence of CAG-repeat size, the decline of the activities of daily living (ADL) with disease progression and the determination of functional

prognosis. Some SBMA studies reported no correlation between the progression of the clinical course and the number of CAG repeats (Lund *et al.*, 2001; Sperfeld *et al.*, 2002), while other studies revealed an age-assessed severity of limb-muscle weakness (Doyu *et al.*, 1992) or only a weak correlation between the decline of ADL and CAG-repeat expansion (La Spada *et al.*, 1992). Since most of the studies performed thus far contained small sample sizes, the influence of CAG-repeat length on the clinical course of SBMA patients remains obscure. In other CAG-repeat diseases such as Huntington's disease, spinocerebellar ataxia type 3 (SCA3) and dentatorubral-pallidoluysian atrophy (DRPLA), an age-assessed residual cell population, a variety of clinical manifestations and MRI-assessed cerebellar volume have been reported to correlate with CAG-repeat length (Koide *et al.*, 1994; Furtado *et al.*, 1996; Penney *et al.*, 1997; Abe *et al.*, 1998). However, it is still not known how CAG-repeat length influences the progression and prognosis of CAG-repeat diseases.

Recent research has suggested therapeutic approaches to SBMA. In a transgenic mouse model expressing the human *AR* gene with expanded CAG repeats, progressive muscular atrophy and weakness associated with the nuclear accumulation of mutant AR protein was observed. These phenotypes were significantly ameliorated by anti-testosterone therapy (Katsuno *et al.*, 2002, 2003), and the clinical and pathological phenotypes of these mice were markedly improved by the overexpression of heat shock proteins (Adachi *et al.*, 2003; Katsuno *et al.*, 2005). Furthermore, 17-allylamino-17-demethoxygeldanamycin (17-AAG), a potent HSP90 inhibitor, was recently shown to ameliorate motor function deficits and pathological changes in SBMA transgenic mice (Waza *et al.*, 2005). These remarkable therapeutic effects in the transgenic mouse model strongly suggest the possibility of using these approaches in human clinical trials. In order to prepare for such a therapeutic approach, it is important to establish the natural history of clinical symptoms of SBMA based on a large number of patients.

In the present study, we investigated the natural course of SBMA as assessed by 9 ADL milestones in 223 Japanese SBMA patients, and correlated the age of onset of specific milestones during the course of the disease with the CAG-repeat length in the *AR* gene.

Patients and methods

Patients and clinical evaluations

Our laboratory diagnosed 303 patients as SBMA by genetic analysis between 1992 and 2004. Two-thirds of the patients were followed in Nagoya University Hospital or affiliated hospitals, while the other patients were from other hospitals nationwide. These patients were followed by neurologists from 1 year to >20 years. We reviewed the clinical course of the disease in 223 out of 303 patients. The initial symptoms and onset of nine ADL milestones were assessed to evaluate the clinical course of the disease. The ADL milestones were defined as follows: hand tremor (patient awareness of hand tremor), muscular weakness (initial patient awareness of muscular weakness in any part of the body), requirement of a handrail (patient was unable to ascend stairs without the use of a handrail), dysarthria (patient was unable to articulate properly and had intelligible speech only with repetition), dysphagia (patient choked occasionally at meals), use of a cane (patient used a cane constantly when away from home), use of a wheelchair (patient used a wheelchair when away from home) and development of pneumonia (patient developed pneumonia that required in-hospital care). The age at death and cause of death were also investigated. We assessed the age at which the ADL milestones first occurred and the age at death by direct interview, examination of the patients, family interviews and by reviewing the patient's clinical record. The milestones that could be recognized by family members, such as the use of a cane, the use of a wheelchair or the development of pneumonia, were confirmed by them wherever possible.

All evaluators used similar criteria to assess each milestone. To verify these nine ADL milestones as characteristic landmarks in the progression of SBMA symptoms, two neurologists independently assessed their onset in SBMA patients. The accordance between the evaluators of the age of onset of each ADL milestone was verified in 20 SBMA patients with Pearson's correlation coefficients ranging from 0.95 to 0.99.

The clinical landmarks adopted in the previous studies that showed clinical courses of SBMA, based on the characteristic symptoms, were onset of weakness, difficulty climbing stairs, being wheelchair-bound, tremor, gynaecomastia, fasciculations, premature exhaustion of muscles and chewing, muscle cramps, muscle pain, dysarthria and dysphagia (Doyu *et al.*, 1992; La Spada *et al.*, 1992; Shimada *et al.*, 1995; Sperfeld *et al.*, 2002; Sinnreich *et al.*, 2004). We excluded development of gynaecomastia and fasciculation from the ADL milestones, since more than one-third of the patients were not aware of these symptoms, despite their presence. The appearance of muscle cramp and exhaustion of muscles and chewing were also excluded as their recognition was extremely variable among the patients. Some patients recognized them at a very early phase, while others did so only at later stages or not at all.

We used the modified Rankin scale (van Swieten *et al.*, 1988) for the assessment of clinical disability in daily life and examined the serum levels of creatine kinase (CK), aspartate aminotransferase (AST), alanine aminotransferase (ALT), total cholesterol, total testosterone and haemoglobin A1c (HbA1c) as laboratory markers for disease status. As controls, we used the serum levels of CK, AST, ALT, total cholesterol and HbA1c from health screening data of 62–70 males aged 24–79 years, free from neuromuscular diseases. For serum testosterone levels, we adopted published control data from 1143 Japanese males determined by the same assay method that we used in this study (Iwamoto *et al.*, 2004).

We implemented the ethics guideline for human genome/gene analysis research and the ethics guideline for epidemiological studies endorsed by the Japanese government. Before we interviewed the patients, we obtained written informed consent. In cases where this was not possible, such as deceased patients, we used only existing material without informed consent and strictly protected anonymity. All of the study plans were approved by the ethics committee of Nagoya University Graduate School of Medicine.

Genetic analysis

Genomic DNA was extracted from peripheral blood of the SBMA patients using conventional techniques. PCR amplification of the

CAG repeat in the *AR* gene was performed using a fluorescein-labelled forward primer (5′-TCCAGAATCTGTTCCAGAGCGT-GC-3′) and a non-labelled reverse primer (5′-TGGCCTCGCTCAG-GATGTCTTTAAG-3′). Detailed PCR conditions were described previously (Tanaka *et al.*, 1999). Aliquots of PCR products were combined with loading dye and separated by electrophoresis with an autoread sequencer SQ-5500 (Hitachi Electronics Engineering, Tokyo, Japan). The size of the CAG repeat was analysed on Fraglys software version 2.2 (Hitachi) by comparison with co-electrophoresed PCR standards with known repeat sizes. The CAG-repeat size of the PCR standard was determined by direct sequence as described previously (Doyu *et al.*, 1992).

Data analysis

All variables were summarized using descriptive statistics, including median, mean, SD, percentile and percentages. Age at ADL milestone data from a sufficient number of the patients was evaluated by Kaplan–Meier analyses, and log rank test statistics were used to determine whether Kaplan–Meier transition curves differed among subgroups. Relationships between the age at each ADL milestone and the length of CAG repeat of *AR* gene were analysed using Pearson's correlation coefficient. Correlations between laboratory test value and the age at examination were also analysed using Pearson's correlation coefficient. *P*-values of <0.05 were considered to be statistically significant. Calculations were performed using the statistical software package Dr SPSS II for Windows (SPSS Japan Inc., Tokyo, Japan).

Results

Clinical and genetic backgrounds of SBMA patients

A total of 223 SBMA patients were included in this study (Table 1). All of the patients were of Japanese nationality. The mean age at the time of data collection was 55.2 ± 10.5 years (range = 30–87 years). The mean duration from onset assessed by the patient's initial awareness of muscle weakness was 9.8 ± 7.2 years (range = 0–37).

The mean number of CAG repeats in the *AR* gene was 46.6 ± 3.5 (range = 40–57). The location of the initial noticeable muscular weakness was lower extremities in 70.5%, upper extremities in 31.0%, bulbar symptoms in 11.4% and facial weakness in 2.4%. Some patients noticed muscle weakness initially in two locations simultaneously; thus overlap between the groups existed. Weakness in the lower extremities was noticed most often as difficulty in climbing stairs, followed by difficulty in walking for long distances and difficulty in standing from a sitting position. Bulbar symptoms were first noticed as a difficulty in articulating properly. ADL assessed by a modified Rankin scale at examination was 0–1 in 17.2%, 2–3 in 66.1% and 4–6 in 16.7% of the patients. Serum CK levels were 863.5 ± 762.5 IU/l (range = 31–4955; normal value = 45–245 IU/l), HbA1c levels were 5.7 ± 1.1% (range = 4.3–9.6; normal value = 4.3–5.8%), serum testosterone levels were 6.48 ± 1.83 ng/ml (range = 2.85–10.20; normal value = 2.7–10.7 ng/ml), serum AST levels were 44.3 ± 29.4 IU/l (range = 17–238; normal value = 0–41 IU/l), serum ALT levels were 52.6 ± 37.1 IU/l (range = 12–248; normal value = 0–45 IU/l) and serum total cholesterol levels were 219.3 ± 42.3 mg/dl (range = 119–413; normal value = 120–220 mg/dl).

Table 1 Clinical and genetic backgrounds of SBMA patients

Clinical and genetic features	Mean ± SD (range)
Age at examination (years)	55.2 ± 10.5 (30–87)
Duration from onset (years)[a]	9.8 ± 7.2 (0–37)
CAG-repeat length in *AR* gene (number)	46.6 ± 3.5 (40–57)
Location of initial muscular weakness the patients perceived (%)[b]	
Facial	2.4
Bulbar	11.4
Upper extremities	31.0
Lower extremities	70.5
Modified Rankin scale at examination (%)	
0–1	17.2
2–3	66.1
4–6	16.7
Serum markers at examination	
Serum CK (*n* = 182) (IU/l)	863.5 ± 762.5 (31–4955)
HbA1c level (*n* = 76) (%)	5.7 ± 1.1 (4.3–9.6)
Serum testosterone level (*n* = 61) (ng/ml)	6.48 ± 1.83 (2.85–10.20)
Serum AST (*n* = 130) (IU/l)	44.3 ± 29.4 (17–238)
Serum ALT (*n* = 133) (IU/l)	52.6 ± 37.1 (12–248)
Total cholesterol level (*n* = 82) (mg/dl)	219.3 ± 42.3 (119–413)

Normal values for serum CK range = 45–245 IU/l; HbA1c range = 4.3–5.8%; serum testosterone range = 2.7–10.7 ng/ml; serum AST range = 0–41 IU/l; serum ALT range = 0–45 IU/l; and total cholesterol range = 120–220 mg/dl. [a]Onset was assessed by patients' initial awareness of muscle weakness; [b]some patients noticed muscle weakness in two locations simultaneously.

Age at which ADL milestones appear

Age distributions at which the ADL milestones initially appeared are summarized in Fig. 1. Hand tremor was the earliest of the ADL milestones that the patients noticed, and it occurred at a median age of 33 years. Hand tremor was particularly noticed when patients used their hands such as in holding a drinking glass. Muscular weakness, predominantly in the lower extremities, was noticed at a median age of 44 years, followed by the need of a handrail when going up stairs at a median age of 49 years. Dysarthria, dysphagia and the use of a cane appeared at median ages of 50, 54 and 59 years, respectively. The use of a wheelchair started at a median age of 61 years. Patients developed pneumonia owing to aspiration and required in-hospital care at a median age of 62 years. The median age of those 15 patients who died before this report was 65 years. The predominant cause of death in eight of these cases was aspiration pneumonia. One patient died of lung cancer, and another patient died from ischaemic heart disease. One patient committed suicide. The causes of death of the other four patients were unknown. The ages of

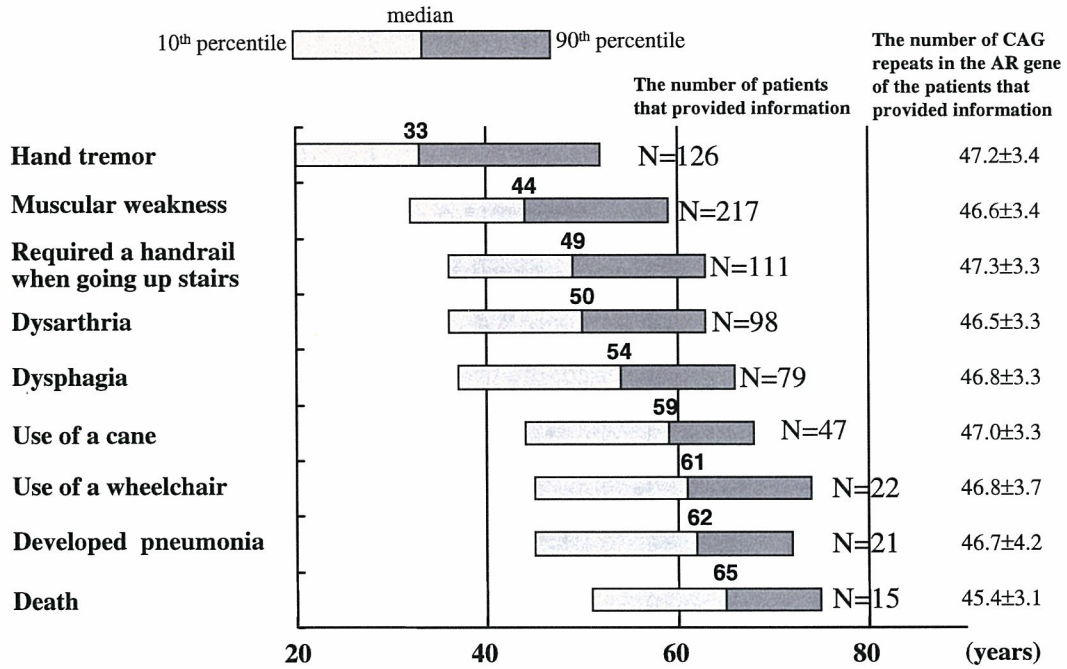

Fig. 1 Age distribution of ADL milestones for 223 SBMA patients. The mean number of CAG repeats in the *AR* gene of the patients does not differ significantly as shown at the right.

onset of each ADL milestone showed a considerable wide-ranged distribution from 25 to 30 years when assessed from the 10th to 90th percentile range. Although there were no significant differences in the mean number of CAG repeats in the *AR* gene of the patients in which we assessed the age at onset of each ADL milestone (Fig. 1), suggesting that age distributions at each milestone were derived from genetically uniform patients, we tested the hypothesis that the wide range in ages of onset at each milestone may be due to individual differences in CAG-repeat lengths.

Age at onset of each ADL milestone correlates well with CAG-repeat length

As shown in Fig. 2, the onset age of the individual ADL milestones examined showed significant correlations with the CAG-repeat length of the patients reporting on these symptoms ($r = -0.853$ to -0.447, $P < 0.016$–0.001). Of these, age at onset of hand tremor, requirement of a handrail, use of a wheelchair, developing pneumonia requiring in-hospital care and death were strongly correlated with the CAG repeats with $r < -0.5$. Furthermore, the onset ages of pneumonia and death were highly correlated with the CAG repeats with $r = -0.78$ and -0.85, respectively, indicating that these specific events, the onset ages of which the patients or their families were able to indicate more definitely, showed a more significant correlation with the CAG-repeat length than other ADL milestones.

Since 47 repeats was the median CAG-repeat length of the entire patient group, we further compared the Kaplan–Meier curves for age at onset of hand tremor, muscular weakness and requirement of a handrail between the patient group with 47 CAG repeats or more and those with <47 CAG repeats (Fig. 3). We assessed only these three ADL milestones, since the number of patients in these groups was sufficient to perform a log rank test analysis. The patients with <47 CAG repeats showed regression curves shifted by ~10 years compared with those with ≥47 CAG repeats (Fig. 3, $P < 0.001$ in log rank test). Together, these observations strongly suggest that the onset age of each ADL milestone is highly dependent on CAG-repeat length in the *AR* gene.

CAG-repeat length does not correlate with the rate of disease progression assessed by ADL milestones

In order to assess whether CAG-repeat length influences the disease progression rate, we examined the relationship between the time intervals from onset age of muscular weakness to that of requirement of a handrail when going up stairs, use of a cane, use of a wheelchair, development of pneumonia and death and the CAG-repeat lengths in these groups (Fig. 4). We did not find any significant correlations of the intervals among the onset age of the various milestones with the CAG-repeat length, suggesting that the progression rate of the disease is not significantly influenced by the CAG-repeat size.

In addition, we examined the declining regression assessed by those ADL milestones in individual patients with ≥47 CAG repeats compared with those with <47 (Fig. 5). These regression lines were divergent from each other, possibly because of divergent CAG-repeat size, while the mean slopes

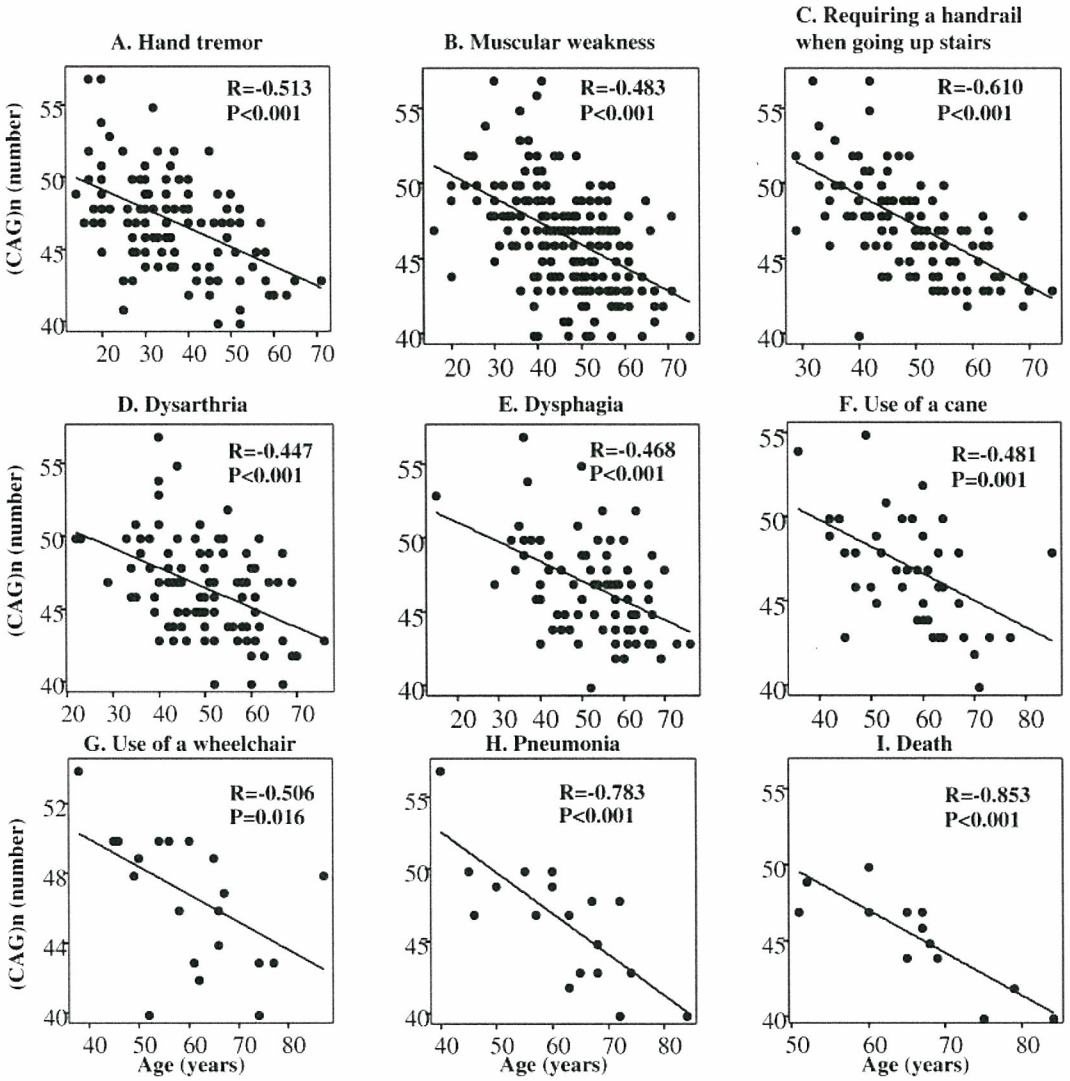

Fig. 2 (**A–I**) Correlation of the CAG-repeat number of and the age at each ADL milestone. There were significant correlations between CAG number and age at all milestones analysed using Pearson's correlation coefficient.

of the regression lines of the two groups were likely to be parallel. There were no significant differences between the interval times of the two groups as assessed by unpaired *t*-test (Fig. 5), suggesting, again, that the rate of disease progression was not markedly dependent upon the size of the CAG repeats.

Age-related changes of laboratory data and their relation to CAG repeats

Glucose intolerance, serum CK and ALT elevation and androgen insensitivity of SBMA patients have been reported (Sobue *et al.*, 1989; Shimada *et al.*, 1995; Dejager *et al.*, 2002; Sinnreich *et al.*, 2004). We examined the relationship between serum CK, HbA1c, testosterone, total cholesterol, AST and ALT levels and the age and CAG-repeat length of the patients. The serum levels of CK, AST and ALT were elevated in subpopulations of patients, particularly in the early phase of the disease, while these levels gradually declined with age (Fig. 6A, E and F). In advanced ages, the levels of these serum markers had declined to nearly normal levels. Serum testosterone levels were slightly elevated from control values in one-third of patients; in general, they declined slightly with age (Fig. 6C). However, even at these advanced ages testosterone levels were within or above the normal range. In contrast, HbA1c levels were within the normal range in the patients with short disease durations, but they gradually increased to above the normal range as the age of patients increased (Fig. 6B). Cholesterol levels were mildly elevated in some patients, but there was no particular age-dependent change observed (Fig. 6D). Elevated levels of these serum markers were not correlated with the CAG-repeat sizes (data not shown). Therefore, the levels of these markers appear to reflect the active pathological process of the disease, especially in the early or late phases, but their significance should be examined further.