A

RhoA   Rac1

Control RNA   +   +

TDP-43 siRNA   +   +

Input

$[^{14}C]$

B

$p = 0.04$ $p = 0.05$

C

GGTI   −   +   −   +

Rac1

Input $[^{14}C]$

FIGURE 8. Protein prenylation of RhoA and Rac1 in Neuro-2a cells. *A*, Neuro-2a cells were metabolically labeled with $[^{14}C]$MVA: *left*, Western blot with anti-RhoA or Rac1 antibody of cell lysates; *right*, incorporation of $[^{14}C]$MVA into RhoA or Rac1. *B*, densitometric quantitation of four independent experiments. *Error bars* indicate S.D. *C*, incorporation of $[^{14}C]$MVA into Rac1 in Neuro-2a cells incubated with or without GGTI-298 (*GGTI*).

family member has a distinct effect on cell morphology and plays an important role in the regulation of neuronal survival, we demonstrated that pan-inhibition of Rho family proteins by *C. difficile* toxin B suppressed neurite outgrowth and reduced cell viability as reported previously (19–21, 32, 33). We demonstrated here that the activities of RhoA, Rac1, and Cdc42 were all decreased in TDP-43-depleted cells, suggesting that knockdown of TDP-43 induces inhibition of neurite outgrowth and cell death through the inactivation of Rho family GTPases. There was no evidence of apoptosis mediated by TDP-43 knockdown in the model we used perhaps because of the biological characteristics of Neuro-2a cells in which the induction of apoptosis is known to be difficult (34, 35). In support of this view, we confirmed that GGTI-298 had no effect on apoptosis in Neuro-2a cells under the present conditions (Fig. 6*C*), although this reagent has been shown to efficiently induce apoptosis in non-neuronal cells (36, 37).

Inhibited Protein Geranylgeranylation of Rho Family Members in TDP-43-depleted Cells—How does TDP-43 regulate the activities of the Rho family? Various molecules have been shown to regulate the activity of Rho, Rac, and Cdc42, but few are capable of regulating all three GTPases concomitantly. To elucidate the molecular mechanism by which TDP-43 regulates Rho, Rac, and Cdc42, we directed our attention to the fact that membrane localization is the key regulatory factor common to these molecules. Small G proteins, including members of the Rho and Ras families, act as molecular switches cycling between an active, GTP-bound state and an inactive, GDP-bound state (18, 38, 39). Post-translational modification with a C-terminal prenyl moiety allows small G proteins to associate with the membrane where they can interact with and activate their effectors (29). Proteins that require prenylation include the farnesyl group, such as the Ras family, and the geranylgeranyl group, such as the Rho or the Rab family (29, 40). In the present study, we showed that knockdown of TDP-43 decreased membrane-bound RhoA, Rac1, and Cdc42 but did not affect the intracellular distribution of H-Ras or Rab5. TDP-43 knockdown also inhibited the incorporation of $[^{14}C]$MVA, a tracer of the mevalonate pathway, into RhoA and Rac1 in differentiated Neuro-2a cells. In contrast, the expression level of Rho GDP dissociation inhibitor, which functions by extracting Rho family GTPases from membranes and solubilizing them in the cytosol (41, 42), was not significantly altered by TDP-43 knockdown. In addition, inhibition of geranylgeranylation by GGTI-298 reproduced the cytotoxic effects of TDP-43 depletion, whereas GGPP, the substrate of the geranylgeranylation pathway, restored cell viability and neurite outgrowth in TDP-43-depleted Neuro-2a cells. Furthermore in GGTI-298 treated cells, overexpression of TDP-43 restored the membrane localization of Rho GTPases as well as cell viability and neurite outgrowth. These findings suggest that TDP-43 depletion inactivates Rho family GTPases through inhibition of protein geranylgeranylation.

Protein geranylgeranylation of Rho family members is catalyzed by geranylgeranyltransferase-I using GGPP produced by geranylgeranyl pyrophosphate synthase-1 as the substrate (43). Geranylgeranyltransferase-I consists of two subunits, α and β, and the α subunit is also a component of protein farnesyltransferase (44). We thus assessed the mRNA and protein expression of geranylgeranyltransferase-Iβ and geranylgeranyl pyrophosphate synthase-1 in Neuro-2a cells. However, the expression levels of these enzymes were not altered by TDP-43 knockdown. These results imply that TDP-43 depletion decreases the activities of these enzymes.

Loss of TDP-43 Function in the Pathophysiology of TDP-43 Proteinopathies—Ubiquitinated cytoplasmic inclusions are a histopathological hallmark of ALS and frontotemporal lobar degeneration with ubiquitin-positive inclusions. Although the nature of these aggregates has not been fully elucidated, recent studies have identified TDP-43 as the major component of the ubiquitin-immunoreactive neuronal inclusions seen in ALS and frontotemporal lobar degeneration with ubiquitin-positive inclusions (1, 2). There has been a great deal of debate about whether loss or gain of function of TDP-43 causes neuronal dysfunction and eventual cell death. Although TDP-43 is a

- 135 -

Dysregulation of Rho Families by TDP-43 Depletion

ubiquitously expressed, highly conserved nuclear protein, under the pathological conditions in ALS and frontotemporal lobar degeneration with ubiquitin-positive inclusions, TDP-43 completely disappears from the nuclei of the affected neurons (1, 2). These histopathological findings indicate that loss of nuclear TDP-43 may underlie neuronal degeneration, although it is also possible that neuronal inclusions possess cytotoxic properties. It has been reported that TDP-43 depletion leads to up-regulation of cyclin-dependent kinase 6 protein and transcript levels followed by misregulation of the cell cycle and apoptosis in cultured human epithelial cancer cells (14). In the present study, knockdown of TDP-43 inactivated Rho family GTPases and thereby induced cell death in differentiated Neuro-2a cells. Although this is not the model imitating the dislocation of TDP-43 from the nucleus to the cytoplasm, our findings suggest that loss of function of TDP-43 may induce neuronal degeneration probably through dysregulation of Rho family GTPases.

In summary, we have demonstrated that TDP-43 depletion inhibits neurite outgrowth and induces neuronal cell death. This phenomenon possibly results from a reduced membrane localization of Rho family GTPases due to the inhibition of protein geranylgeranylation.

REFERENCES

1. Neumann, M., Sampathu, D. M., Kwong, L. K., Truax, A. C., Micsenyi, M. C., Chou, T. T., Bruce, J., Schuck, T., Grossman, M., Clark, C. M., McCluskey, L. F., Miller, B. L., Masliah, E., Mackenzie, I. R., Feldman, H., Feiden, W., Kretzschmar, H. A., Trojanowski, J. Q., and Lee, V. M. (2006) *Science* **314**, 130–133
2. Arai, T., Hasegawa, M., Akiyama, H., Ikeda, K., Nonaka, T., Mori, H., Mann, D., Tsuchiya, K., Yoshida, M., Hashizume, Y., and Oda, T. (2006) *Biochem. Biophys. Res. Commun.* **351**, 602–611
3. Gitcho, M. A., Baloh, R. H., Chakraverty, S., Mayo, K., Norton, J. B., Levitch, D., Hatanpaa, K. J., White, C. L., 3rd, Bigio, E. H., Caselli, R., Baker, M., Al-Lozi, M. T., Morris, J. C., Pestronk, A., Rademakers, R., Goate, A. M., and Cairns, N. J. (2008) *Ann. Neurol.* **63**, 535–538
4. Yokoseki, A., Shiga, A., Tan, C. F., Tagawa, A., Kaneko, H., Koyama, A., Eguchi, H., Tsujino, A., Ikeuchi, T., Kakita, A., Okamoto, K., Nishizawa, M., Takahashi, H., and Onodera, O. (2008) *Ann. Neurol.* **63**, 538–542
5. Kabashi, E., Valdmanis, P. N., Dion, P., Spiegelman, D., McConkey, B. J., Vande Velde, C., Bouchard, J. P., Lacomblez, L., Pochigaeva, K., Salachas, F., Pradat, P. F., Camu, W., Meininger, V., Dupre, N., and Rouleau, G. A. (2008) *Nat. Genet.* **40**, 572–574
6. Sreedharan, J., Blair, I. P., Tripathi, V. B., Hu, X., Vance, C., Rogelj, B., Ackerley, S., Durnall, J. C., Williams, K. L., Buratti, E., Baralle, F., de Belleroche, J., Mitchell, J. D., Leigh, P. N., Al-Chalabi, A., Miller, C. C., Nicholson, G., and Shaw, C. E. (2008) *Science* **319**, 1668–1672
7. Van Deerlin, V. M., Leverenz, J. B., Bekris, L. M., Bird, T. D., Yuan, W., Elman, L. B., Clay, D., Wood, E. M., Chen-Plotkin, A. S., Martinez-Lage, M., Steinbart, E., McCluskey, L., Grossman, M., Neumann, M., Wu, I. L., Yang, W. S., Kalb, R., Galasko, D. R., Montine, T. J., Trojanowski, J. Q., Lee, V. M., Schellenberg, G. D., and Yu, C. E. (2008) *Lancet Neurol.* **7**, 409–416
8. Ayala, Y. M., Pantano, S., D'Ambrogio, A., Buratti, E., Brindisi, A., Marchetti, C., Romano, M., and Baralle, F. E. (2005) *J. Mol. Biol.* **348**, 575–588
9. Buratti, E., Brindisi, A., Giombi, M., Tisminetzky, S., Ayala, Y. M., and Baralle, F. E. (2005) *J. Biol. Chem.* **280**, 37572–37584
10. Wang, I. F., Reddy, N. M., and Shen, C. K. (2002) *Proc. Natl. Acad. Sci. U.S.A.* **99**, 13583–13588
11. Wang, H. Y., Wang, I. F., Bose, J., and Shen, C. K. (2004) *Genomics* **83**, 130–139
12. Bose, J. K., Wang, I. F., Hung, L., Tarn, W. Y., and Shen, C. K. (2008) *J. Biol. Chem.* **283**, 28852–28859
13. Strong, M. J., Volkening, K., Hammond, R., Yang, W., Strong, W., Leystra-Lantz, C., and Shoesmith, C. (2007) *Mol. Cell. Neurosci.* **35**, 320–327
14. Ayala, Y. M., Misteli, T., and Baralle, F. E. (2008) *Proc. Natl. Acad. Sci. U.S.A.* **105**, 3785–3789
15. Kaibuchi, K., Kuroda, S., and Amano, M. (1999) *Annu. Rev. Biochem.* **68**, 459–486
16. Burridge, K., and Wennerberg, K. (2004) *Cell* **116**, 167–179
17. Jaffe, A. B., and Hall, A. (2005) *Annu. Rev. Cell Dev. Biol.* **21**, 247–269
18. Hall, A. (1998) *Science* **279**, 509–514
19. Just, I., Selzer, J., Wilm, M., von Eichel-Streiber, C., Mann, M., and Aktories, K. (1995) *Nature* **375**, 500–503
20. Bradke, F., and Dotti, C. G. (1999) *Science* **283**, 1931–1934
21. Ahmed, I., Calle, Y., Iwashita, S., and Nur-E-Kamal, A. (2006) *Mol. Cell. Biochem.* **281**, 17–25
22. Cairns, N. J., Neumann, M., Bigio, E. H., Holm, I. E., Troost, D., Hatanpaa, K. J., Foong, C., White, C. L., 3rd, Schneider, J. A., Kretzschmar, H. A., Carter, D., Taylor-Reinwald, L., Paulsmeyer, K., Strider, J., Gitcho, M., Goate, A. M., Morris, J. C., Mishra, M., Kwong, L. K., Stieber, A., Xu, Y., Forman, M. S., Trojanowski, J. Q., Lee, V. M., and Mackenzie, I. R. (2007) *Am. J. Pathol.* **171**, 227–240
23. Nakagawa, M., Fukata, M., Yamaga, M., Itoh, N., and Kaibuchi, K. (2001) *J. Cell Sci.* **114**, 1829–1838
24. Watanabe, T., Wang, S., Noritake, J., Sato, K., Fukata, M., Takefuji, M., Nakagawa, M., Izumi, N., Akiyama, T., and Kaibuchi, K. (2004) *Dev. Cell* **7**, 871–883
25. Niwa, J., Yamada, S., Ishigaki, S., Sone, J., Takahashi, M., Katsuno, M., Tanaka, F., Doyu, M., and Sobue, G. (2007) *J. Biol. Chem.* **282**, 28087–28095
26. Katsuno, M., Adachi, H., Minamiyama, M., Waza, M., Tokui, K., Banno, H., Suzuki, K., Onoda, Y., Tanaka, F., Doyu, M., and Sobue, G. (2006) *J. Neurosci.* **26**, 12106–12117
27. Katsuno, M., Adachi, H., Doyu, M., Minamiyama, M., Sang, C., Kobayashi, Y., Inukai, A., and Sobue, G. (2003) *Nat. Med.* **9**, 768–773
28. Feng, J., Ito, M., Ichikawa, K., Isaka, N., Nishikawa, M., Hartshorne, D. J., and Nakano, T. (1999) *J. Biol. Chem.* **274**, 37385–37390
29. Adamson, P., Marshall, C. J., Hall, A., and Tilbrook, P. A. (1992) *J. Biol. Chem.* **267**, 20033–20038
30. Tanaka, T., Tatsuno, I., Uchida, D., Moroo, I., Morio, H., Nakamura, S., Noguchi, Y., Yasuda, T., Kitagawa, M., Saito, Y., and Hirai, A. (2000) *J. Neurosci.* **20**, 2852–2859
31. Meske, V., Albert, F., Richter, D., Schwarze, J., and Ohm, T. G. (2003) *Eur. J. Neurosci.* **17**, 93–102
32. Linseman, D. A., Laessig, T., Meintzer, M. K., McClure, M., Barth, H., Aktories, K., and Heidenreich, K. A. (2001) *J. Biol. Chem.* **276**, 39123–39131
33. Loucks, F. A., Le, S. S., Zimmermann, A. K., Ryan, K. R., Barth, H., Aktories, K., and Linseman, D. A. (2006) *J. Neurochem.* **97**, 957–967
34. Sueyoshi, N., Maehara, T., and Ito, M. (2001) *J. Lipid Res.* **42**, 1197–1202
35. Saito, M., Saito, M., Cooper, T. B., and Vadasz, C. (2005) *Alcohol Clin. Exp. Res.* **29**, 1374–1383
36. Xia, Z., Tan, M. M., Wong, W. W., Dimitroulakos, J., Minden, M. D., and Penn, L. Z. (2001) *Leukemia* **15**, 1398–1407
37. Li, X., Liu, L., Tupper, J. C., Bannerman, D. D., Winn, R. K., Sebti, S. M., Hamilton, A. D., and Harlan, J. M. (2002) *J. Biol. Chem.* **277**, 15309–15316
38. Lamarche, N., and Hall, A. (1994) *Trends Genet* **10**, 436–440
39. Zhou, K., Wang, Y., Gorski, J. L., Nomura, N., Collard, J., and Bokoch, G. M. (1998) *J. Biol. Chem.* **273**, 16782–16786
40. Cox, A. D., and Der, C. J. (1992) *Curr. Opin. Cell Biol.* **4**, 1008–1016
41. Fukumoto, Y., Kaibuchi, K., Hori, Y., Fujioka, H., Araki, S., Ueda, T., Kikuchi, A., and Takai, Y. (1990) *Oncogene* **5**, 1321–1328
42. Dovas, A., and Couchman, J. R. (2005) *Biochem. J.* **390**, 1–9
43. Ericsson, J., Greene, J. M., Carter, K. C., Shell, B. K., Duan, D. R., Florence, C., and Edwards, P. A. (1998) *J. Lipid Res.* **39**, 1731–1739
44. Moomaw, J. F., and Casey, P. J. (1992) *J. Biol. Chem.* **267**, 17438–17443

- 136 -

Phase 2 Trial of Leuprorelin in Patients with Spinal and Bulbar Muscular Atrophy

Haruhiko Banno, MD, PhD,[1] Masahisa Katsuno, MD, PhD,[1,2] Keisuke Suzuki, MD, PhD,[1]
Yu Takeuchi, MD,[1] Motoshi Kawashima, MD,[1] Noriaki Suga, MD,[1] Motoko Takamori, MD, PhD,[3]
Mizuki Ito, MD, PhD,[1] Tomohiko Nakamura, MD, PhD,[1] Koji Matsuo, MD, PhD,[1]
Shinichi Yamada, MD, PhD,[1] Yumiko Oki, MD, PhD,[4] Hiroaki Adachi, MD, PhD,[1]
Makoto Minamiyama, MD, PhD,[1] Masahiro Waza, MD, PhD,[1] Naoki Atsuta, MD, PhD,[1]
Hirohisa Watanabe, MD, PhD,[1] Yasushi Fujimoto, MD, PhD,[5] Tsutomu Nakashima, MD, PhD,[5]
Fumiaki Tanaka, MD, PhD,[1] Manabu Doyu, MD, PhD,[6] and Gen Sobue, MD, PhD[1]

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). Animal studies have shown that the pathogenesis of SBMA is dependent on serum testosterone level. This study is aimed at evaluating the efficacy and safety of androgen deprivation by leuprorelin acetate in patients with SBMA.

Methods: Fifty SBMA patients underwent subcutaneous injections of leuprorelin acetate or placebo in a randomized, placebo-controlled trial for 48 weeks, followed by an open-label trial for an additional 96 weeks, in which 19 patients of the leuprorelin group and 15 of the placebo group received leuprorelin acetate. The patients who did not participate in the open-label trial were also followed up for the 96-week period (UMIN000000474).

Results: Leuprorelin acetate significantly extended the duration of cricopharyngeal opening in videofluorography and decreased mutant AR accumulation in scrotal skin biopsy. The patients treated with leuprorelin acetate for 144 weeks exhibited significantly greater functional scores and better swallowing parameters than those who received placebo. Autopsy of one patient who received leuprorelin acetate for 118 weeks suggested that androgen deprivation inhibits the nuclear accumulation or stabilization, or both, of mutant AR in the motor neurons of the spinal cord and brainstem.

Interpretation: These observations suggest that administration of leuprorelin acetate suppresses the deterioration of neuromuscular impairment in SBMA by inhibiting the toxic accumulation of mutant AR. The results of this phase 2 trial support the start of large-scale clinical trials of androgen deprivation for SBMA.

Spinal and bulbar muscular atrophy (SBMA), also known as Kennedy's disease, is the first of the neurodegenerative diseases for which the molecular basis was discovered to be the expansion of a trinucleotide CAG repeat in the gene of the causative protein. SBMA is an adult-onset, motor neuron disease characterized by muscle atrophy, weakness, contraction fasciculations, and bulbar involvement.[1–4] Its prevalence has been estimated to be 1 to 2 per 100,000, although a considerable number of patients may be misdiagnosed with other neuromuscular diseases such as amyotrophic lateral sclerosis (ALS).[5,6] The progression of SBMA is usually slow, but life-threatening respiratory tract infections often occur in the advanced stage of the disease, resulting in death.[7] Laboratory tests show increased serum levels of creatine kinase and liver enzymes in most cases. The expanded CAG triplet repeat sequence, which encodes a polyglutamine tract, is found in the androgen receptor gene *(AR)*.[8] The CAG repeat numbers range from 38 to 62 in SBMA patients, whereas healthy individuals have 9 to 36 CAGs.[5,8–10] The number of CAGs is correlated with disease severity and inversely correlated with the age of onset, as observed in other polyglutamine-related neurodegenerative dis-

eases, including Huntington's disease and several forms of spinocerebellar ataxia.[11,12] In polyglutamine diseases, nuclear localization of the respective mutant protein is considered important for inducing neuronal cell dysfunction and degeneration.[13-15] The extent of diffuse nuclear accumulation of mutant AR in spinal motor neurons is closely related to the CAG repeat length in autopsied SBMA cases.[16] In addition, nuclear accumulation of mutant AR in scrotal skin correlates with both disease severity and CAG repeat length, suggesting that the number of scrotal skin epithelial cells positive for 1C2, an anti-polyglutamine antibody, is a potent pathogenic marker of SBMA and can serve as a useful biomarker in therapeutic trials.[17]

A characteristic clinical feature of SBMA is that full disease manifestations occur in male but not in female individuals even when they are homozygous for the mutation.[18,19] The sex dependency of disease manifestation in SBMA may arise from a testosterone-dependent nuclear accumulation of mutant AR.[20-23] In mouse models of SBMA, surgical castration delays disease onset and progression, as well as reverses neuromuscular phenotypes.[20,23] Leuprorelin acetate, a luteinizing hormone–releasing hormone agonist that reduces testosterone release from the testis and inhibits nuclear accumulation of mutant AR, ameliorates motor dysfunction in male transgenic mice carrying full-length mutant human *AR* with an expanded polyglutamine tract.[21]

Although data from animal studies indicated that androgen deprivation via leuprorelin acetate is a promising therapeutic agent for SBMA,[20,21] clinical experience using this drug in SBMA patients is limited.[24] The safety and efficacy of leuprorelin acetate were demonstrated for treating prostate cancer, endometriosis, uterine fibroids, and central precocious puberty in children.[25] To determine whether androgen deprivation therapy prevents the progression of SBMA in humans, we conducted a two-arm, randomized, placebo-controlled, phase 2 clinical trial of leuprorelin acetate in patients with SBMA for 48 weeks, followed by an open-label trial for an additional 96 weeks.

Patients and Methods

Patients
Inclusion criteria of this trial included: (1) genetically confirmed SBMA male Japanese patients with more than one of the following symptoms: muscle weakness, muscle atrophy, bulbar palsy, or hand tremor; (2) patients whose *AR* CAG repeat length was more than 38; (3) patients who were 30 to 70 years old at the time of informed consent; (4) patients who had no desire to father a child; and (5) patients who gave written informed consent. Patients were excluded if they met any of the following criteria: (1) medical history of allergy to leuprorelin acetate; (2) had taken testosterone within 8 weeks before the informed consent; (3) had severe

complications; or (4) were not eligible for other reasons (eg, previous use of luteinizing hormone–releasing hormone agonists or medical history of allergy to barium sulfate).

Study Design
We conducted a 48-week, prospective, randomized, placebo-controlled, single-site trial and a 96-week open-label follow-up trial at Nagoya University Hospital (Fig 1). Fifty patients were included between September 2003 and March 2004. The last patient terminated the randomized controlled trial (RCT) in February 2005. The protocol for the trial was filed with the open clinical trial registry (www.umin.ac.jp/ctr/index.htm) under the Identifier Number UMIN000000474. In the 48-week RCT, patients were randomized in a 1:1 ratio of leuprorelin acetate or identically appearing placebo using the minimization method by an independent investigator. Dynamic allocation was performed based on patient age and severity to reduce bias.[26] Patients were blinded throughout the RCT, and at week 48, they decided whether to participate in the follow-up trial without knowing to which drug group they had been allocated. As a result, 19 patients in the leuprorelin group and 15 in the placebo group entered the open-label follow-up trial between August 2004 and March 2005. The remaining 15 patients who declined to participate in the open-label trial were followed up for these 96 weeks; 1 patient who discontinued early in the 48-week RCT was not followed up. The last patient terminated the follow-up trial in February 2007.

All the examinations and treatments were performed at the Nagoya University Hospital throughout the trials. The patients were hospitalized for 7 days at weeks 0 and 48, and were evaluated every 4 weeks in the 48-week RCT. During the 96-week follow-up trial, they were examined every 12 weeks. Blinding was ensured by the use of identical opaque injection syringes. Clinical scores and muscle strength were assessed by blinded neurologists throughout the RCT period.

Treatment
Leuprorelin acetate was subcutaneously injected at a dose of 3.75mg every 4 weeks in the 48-week RCT, and 11.25mg was administrated every 12 weeks in the 96-week follow-up trial. Leuprorelin acetate suppresses testosterone release by downregulating luteinizing hormone–releasing hormone receptors in the pituitary. We did not conduct a dose–response study in this trial, because previous studies suggested that leuprorelin-mediated androgen deprivation is incomplete at dosages less than 3.75mg/4 weeks in men.[27,28]

Outcome Measures
The primary end point of this trial was motor function measured by the widely used and validated Revised ALS Functional Rating Scale (ALSFRS-R; Japanese edition). Although there are no validated scales for SBMA, all the items in the ALSFRS-R are applicable to this disease.[17,29,30] Secondary outcome measures included cricopharyngeal opening duration visualized by videofluorography (VF), the frequency of 1C2-positive cells in scrotal skin biopsies, lung function values [forced expiratory volume in 1 second/forced vital capacity (FEV_1/FVC) and vital capacity as the percentage of pre-
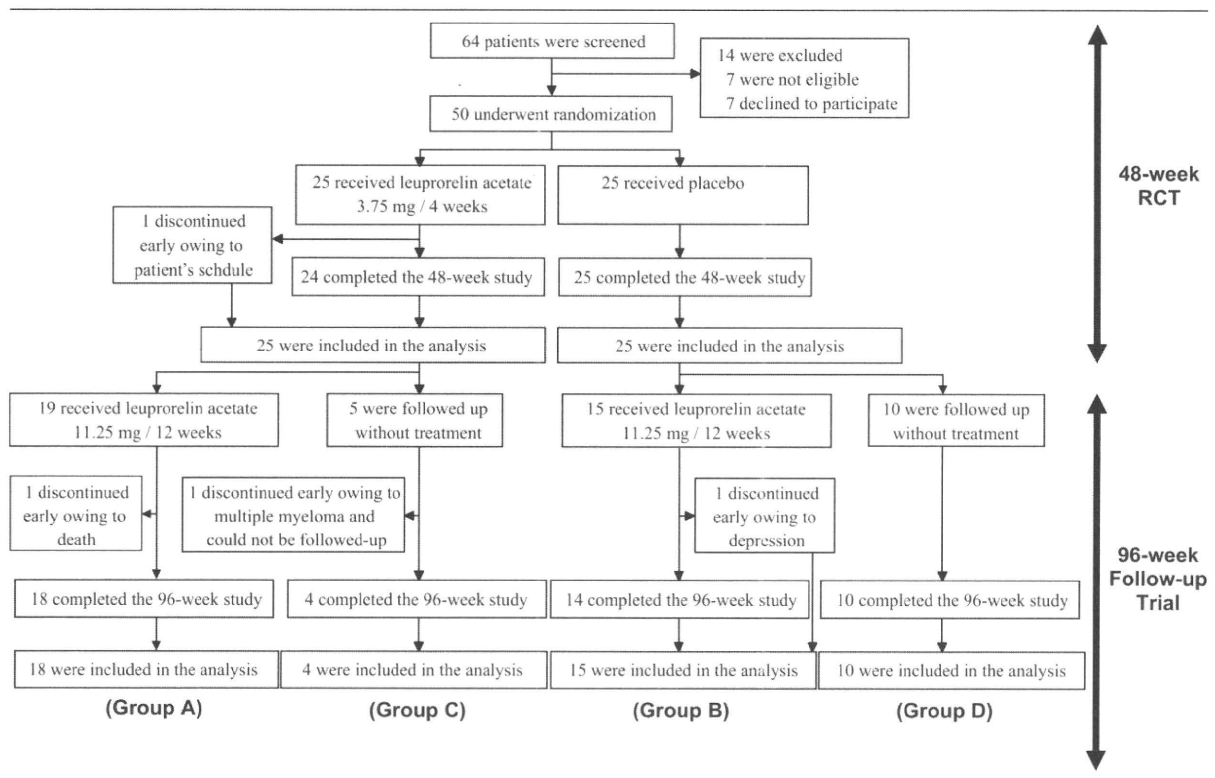
Fig 1. Patient selection flow diagram. RCT = randomized controlled trial.

dicted (%VC)], and serum levels of the following enzymes: L-aspartate aminotransferase, L-alanine aminotransferase, and creatine kinase. As other outcome measures, we analyzed the sum of the following three ALSFRS-R subscores: speech, salivation, and swallowing. We also measured muscle strength using maximum voluntary isometric contraction and conducted a nerve conduction study. The Beck depression inventory and standard laboratory parameters were checked for evaluating safety. Scrotal skin biopsies were performed at weeks 0 and 48 of the 48-week RCT. The ALSFRS-R, lung function test, maximum voluntary isometric contraction, and Beck depression inventory were measured every 24 weeks throughout the trials, whereas VF was examined at weeks 0 and 48 in the 48-week RCT, and at the end of the 96-week follow-up trial. The nerve conduction study was conducted at weeks 0 and 48 in the 48-week RCT. All laboratory tests were performed at weeks 0, 4, 8, 12, 24, 36, and 48 in the 48-week RCT, and every 12 weeks in the 96-week follow-up trial. Outcome for the efficacy analysis was assessed at the final visit at the end of 48-week RCT and at the end of the 96-week follow-up trial.

In VF examinations, patients were instructed to swallow 3ml of 40% wt/vol barium sulfate twice in a standing position. VF data were recorded on Mini DV videotape (Sony, Tokyo, Japan) at 30 frames/sec. For scrotal skin biopsies at 0 and 48 weeks, three specimens were taken from each patient at each time by punch biopsy using a 3mm diameter Dermapunch (Nipro, Tokyo, Japan) under local anesthesia (lidocaine acetate, 10ml) and processed for immunohistochem-

ical analysis using an anti-polyglutamine (1C2) antibody, as described later. All 50 patients underwent biopsies at week 0, but 2 patients' specimens did not attach to the slides and were not included in the analyses. All patients who underwent biopsy sterilized the wound for several days themselves and underwent antibiotic therapy after the procedure, as needed. Serum creatine kinase levels were determined in blood samples obtained on the second day of admission by ultraviolet measurement using hexokinase and glucose-6-phosphate.[31] Serum testosterone levels were measured by radioimmunoassay using the DPC total testosterone kit (Diagnostic Products Corporation, Los Angeles, CA).

Quantitative Measurement in Videofluorography

To identify the appropriate parameters for the outcome measure in this trial, we performed a preliminary analysis of quantitative swallowing evaluation using VF in 18 additional SBMA patients (see Supplemental Tables 1 and 2). In this preliminary study, we assessed the reliability and validity of pharyngeal residue and those of the various temporal measures: pharyngeal delay time, cricopharyngeal opening duration, and total duration of maximal laryngeal elevation. As a result, we found that cricopharyngeal opening duration was the most reliable measurement of swallowing, and that this duration correlated well with functional scores such as the Norris Scores and the ALSFRS-R. None of the other parameters exhibited both high reliability and correlation with functional scores. Therefore, we adopted cricopharyngeal

opening duration as the secondary end point of this trial and measured other parameters as references. All the parameters were measured blindly by two independent evaluators according to standard procedures.[32,33] In brief, duration of cricopharyngeal opening was defined as the length of time during which the cricopharyngeal sphincter was open. Pharyngeal delay time was defined as the interval from the bolus passing the base of the tongue to the onset of laryngeal elevation, whereas duration of maximum laryngeal elevation was the length of time during which the larynx was maximally elevated from its rest position. Pharyngeal residue was measured using semiquantitative scales: 0, 2, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100%.

Immunohistochemical Detection of Mutant Androgen Receptor

Immunohistochemistry of scrotal skin (from biopsies), spinal cord, and pontine base (from autopsies) specimens were conducted as described previously.[16,17] In brief, 6μm-thick, formalin-fixed, paraffin-embedded sections were prepared, deparaffinized, rehydrated, and pretreated by immersing in 98% formic acid for 5 minutes and then microwaving for 15 minutes in 10mM citrate buffer at pH 6.0. Sections were incubated with a mouse antiexpanded polyglutamine antibody (1C2; 1:20,000; Chemicon, Temecula, CA)[34] to evaluate the nuclear accumulation of mutant AR.[16,20,21] Immune complexes were visualized using the Envision-plus kit (Dako, Glostrup, Denmark). Sections were counterstained with Mayer's hematoxylin. Quantitative assessment of 1C2-positive cells in scrotal skin was performed as described previously.[17] In brief, the frequency of diffuse nuclear staining was calculated from counts of more than 500 nuclei in 5 randomly selected fields of each section (BX51TF; Olympus, Tokyo, Japan). To assess the nuclear accumulation of mutant AR in spinal cord motor neurons, we prepared at least 100 serial transverse sections from the cervical spinal cord and immunostained every 10th section with the anti-polyglutamine 1C2 antibody. For the purposes of counting, a neuron was defined by the presence of its obvious nucleolus in a given 6μm-thick section. The numbers of 1C2-positive and -negative cells within the ventral horn on both the right and left sides were counted under the light microscope with a computer-assisted image analyzer (BX51TF; Olympus), as described previously.[16,35,36] For quantification of 1C2-positive neurons within the pontine base, the frequency of diffuse nuclear staining was calculated from counts of more than 500 neurons in a total of 50 or more fields from each section (BX51N-34; Olympus), as described previously.[37] Populations of 1C2-positive cells were expressed as percentages of the total cell counts.

Autopsy Study

Autopsy specimens of cervical spinal cord (seven patients) and pons (five patients) were obtained from nine control, genetically confirmed SBMA patients who had not participated in any therapeutic trials (52–83 years old; men; 41–52 CAG repeats) and one subject (70 years old) who died at week 67 of the 96-week follow-up study (Patient 16), who had been allocated to the leuprorelin group in the 48-week RCT and had continued leuprorelin administration in the

96-week follow-up trial. The last administration of leuprorelin acetate was at week 60 of the follow-up trial. The causes of death of the control patients were pneumonia in three, respiratory failure in three, unknown in two, and lung cancer in one. Immunohistochemistry of the specimens was performed as described earlier. The collection of tissues and their use for this study were approved by the Ethics Committee of Nagoya University Graduate School of Medicine.

Genetic Analysis

Genomic DNA was extracted from peripheral blood of the patients using conventional techniques, and the CAG repeat size was determined as described previously.[9,11,38] In brief, polymerase chain reaction amplification of the CAG repeat in exon 1 of the *AR* gene was performed using a fluorescein-labeled forward primer (5'-TCCAGAATCTGTTCCAGA-GCGTGC-3') and a nonlabeled reverse primer (5'-TGG-CCTCGCTCAGGATGTCTTTAAG-3'). Size of the CAG repeat was analyzed using Fraglys software version 2.2 (Hitachi Electronics Engineering, Tokyo, Japan) by comparison with coelectrophoresed polymerase chain reaction standards with known repeat sizes. Patients with 38 or more CAGs were diagnosed with SBMA.[10] All patients gave their written informed consent to genetic analyses.

Statistical Analyses

The effectiveness analysis and safety evaluation were conducted on data from the intention-to-treat population in the 48-week RCT. We analyzed the data by Pearson's coefficient, Spearman's rank correlation, and Student's t test. The Mann–Whitney U test was used to analyze serum testosterone levels. p values less than 0.05 were considered indicative of significance. For multiple comparisons, p values were corrected using the Dunnett test. Computations were performed with SPSS software (version 14.0J for Windows; SPSS Japan, Tokyo, Japan).

Ethics

This study was conducted according to the Declaration of Helsinki (Hong Kong Amendment). Written informed consent was obtained from each patient. Patients were free to withdraw from the study at any time for any reason. The protocol was approved by the Nagoya University Hospital Institutional Review Board. Confidentiality was ensured by assigning a study code to each patient. All studies conformed to the ethics guidelines for human genome/gene analysis research and the ethics guidelines for epidemiological studies endorsed by the Japanese government.

Results

Demographics

Fifty participants met the eligibility criteria, gave informed consent, and were assigned to either the leuprorelin or placebo group. There were no significant differences in the characteristics of the two groups (Table 1). There were no protocol deviations, although one patient in the leuprorelin group discontinued the drug after 16 weeks because of the patient's schedule, but this patient was included in the end-point analyses.

Table 1. Characteristics of Patients in the 48-Week Randomized Controlled Trial (RCT)

Characteristics	Leuprorelin (n = 25)	Placebo (n = 25)	p
Mean age ± SD, yr	52.8 ± 7.4	52.0 ± 8.9	NS
Mean height ± SD, cm	167.5 ± 6.2	168.1 ± 6.1	NS
Mean weight ± SD, kg	58.4 ± 5.7	60.2 ± 6.2	NS
Mean duration of weakness ± SD, yr	10.8 ± 6.3	12.9 ± 8.2	NS
Mean (CAG)n ± SD	48.5 ± 3.2	48.1 ± 2.5	NS
Mean ALSFRS-R score ± SD (Japanese edition)	41.1 ± 3.7	42.0 ± 3.4	NS
ADL (cane-assisted/independent)	6/19	7/18	NS

SD = standard deviation; NS = not significant; (CAG)n = number of expanded CAG repeats in the *androgen receptor* gene; ALSFRS-R = revised amyotrophic lateral sclerosis functional rating scale; ADL = activities of daily living.

No patients discontinued treatment prematurely because of adverse events during the 48-week RCT. At the end of the 48-week RCT, 34 of the 50 patients elected to receive leuprorelin administration in the follow-up trial before the key was broken. During this 96-week follow-up trial, one patient discontinued treatment mainly because of depression but was followed up without leuprorelin administration. One patient (Patient 16) died of acute cardiac failure at week 67 and was not included in the end-point analyses (see Fig 1).

Forty-eight-Week Randomized Controlled Trial
The outcome measures of the 48-week RCT are shown in Figure 2. In patients who received leuprorelin acetate, serum testosterone levels decreased to near zero within 4 weeks of the treatment (see Fig 2A). In the placebo group, ALSFRS-R scores had declined by 0.9 point at week 48, suggesting that the change in motor function of patients in this trial was similar to that in a previous study on the natural history of SBMA.[39] Although there was no significant difference in the changes in ALSFRS-R *total* scores at week 48 in the leuprorelin and placebo groups (see Fig 2B), there was a tendency for the swallowing subscores to be improved in the leuprorelin group (see Fig 2C). This view was supported by the fact that the cricopharyngeal opening duration was significantly extended in the leuprorelin group compared with the placebo group, suggesting that androgen deprivation suppressed deterioration of swallowing function in SBMA ($p < 0.05$; see Fig 2D). The serum level of creatine kinase, a marker of muscular involvement in SBMA, and those of liver enzymes also tended to be decreased in the leuprorelin group (see Fig 2E; see Supplemental Table 3). Diffuse nuclear staining was predominantly observed in the scrotal skin biopsy. The frequency of 1C2-positive cells in the scrotal epithelium was significantly decreased at week 48 in the leuprorelin group ($p < 0.001$; see Fig 2F). There were no significant effects of leuprorelin ac-

etate on all other secondary end points (see Supplemental Table 3). Although we performed stratified analyses, neither CAG repeat size nor age had any influence on the outcome measures (data not shown).

Ninety-six-Week Follow-up Trial
All but one patient, who discontinued treatment early in the 48-week RCT, underwent an additional 96-week follow-up. Fifteen patients declined to continue leuprorelin administration mostly because of economic reasons. As shown in Table 2, at the time of enrollment in the follow-up trial, there were no differences in the characteristics of patients who participated and those who were not enrolled, indicating no selection bias for the enrollment.

In the follow-up trial, we compared ALSFRS-R scores and VF findings of the following groups: Group A—patients who were allocated to the leuprorelin group for 48 weeks and received leuprorelin for an additional 96 weeks; Group B—patients who were allocated to the placebo group and received leuprorelin for an additional 96 weeks; Group C—patients who were allocated to the leuprorelin group for 48 weeks but did not receive treatment during the 96-week follow-up; and Group D—patients who were allocated to the placebo group and were followed up without leuprorelin treatment for 96 weeks. Multiple comparisons were performed with Group D as the control. We did not include the following two subjects in these analyses: one patient in Group A who died during the follow-up period and one in Group C who was diagnosed with multiple myelomas during the follow-up period. At week 96 of the follow-up trial, ALSFRS-R scores were significantly greater in Groups A and B than in Group D (Figs 3A, B). Similarly, the swallowing subscores of the ALSFRS-R were significantly greater in Group A than in Group D (see Fig 3C). Cricopharyngeal opening duration in VF was also significantly longer in Groups A and B than in Group D (see Fig 3D).
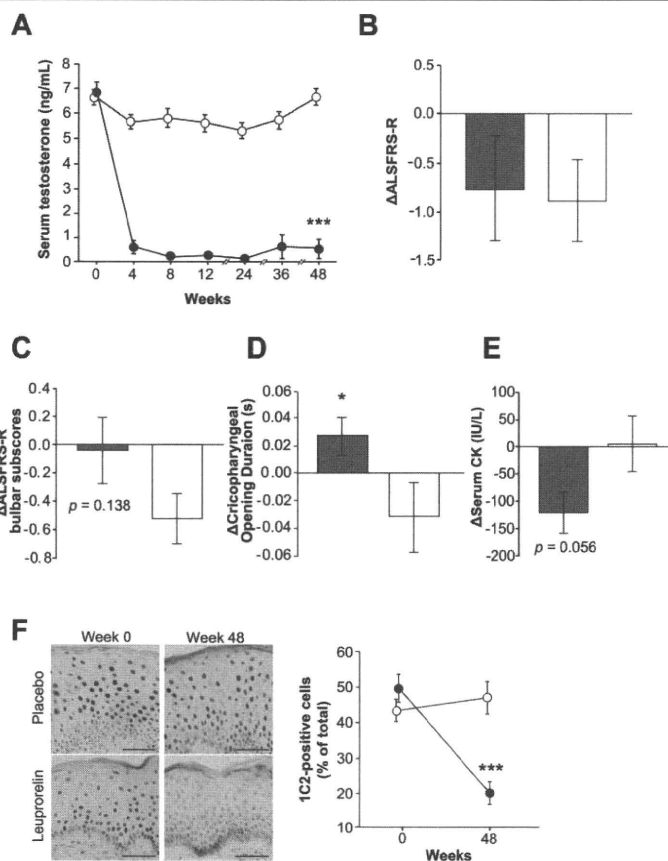
Fig 2. *Efficacy results of the 48-week randomized controlled trial (RCT). (A) Treatment with leuprorelin acetate* (black circles) *rapidly depleted serum testosterone levels.* White circles *represent placebo group. (B) There was no significant difference in the change in Revised Amyotrophic Lateral Sclerosis Functional Rating Scale (ALSFRS-R) score between the groups. (C) There was a favorable tendency in the swallowing subscores of the ALSFRS-R in the leuprorelin group. (D) Cricopharyngeal opening duration was significantly extended by the 48-week leuprorelin treatment. (E) Serum creatine kinase (CK) levels also tended to be decreased in the leuprorelin group. (F) The frequency of diffuse nuclear 1C2 staining (indicative of mutant androgen receptor [AR]) in the scrotal epithelial cells was significantly decreased after the 48-week administration of leuprorelin acetate.* White bars *represent placebo group;* black bars *represent leuprorelin acetate group. Scale bars = 50μm. Data are expressed as means ± standard error of the mean.* *p < 0.05; ***p < 0.001.*

Safety and Tolerability

There were a total of 58 adverse events recorded during the 48-week RCT; none was so serious as to require hospitalization (Table 3). The most frequent adverse event in the leuprorelin group was a loss of sexual function, recorded as erectile dysfunction, but this symptom was also often seen in the placebo group, suggesting androgen insensitivity in SBMA patients. Although increases in total cholesterol, triglyceride, fasting blood sugar, or glycosylated hemoglobin (HbA1c) were seen in the leuprorelin group, no marked exacerbations were observed. The details of adverse events during the 96-week follow-up trial were obtained from Groups A, B, and D. As shown in Table 4, there were no treatment duration–dependent adverse effects of leuprorelin acetate as reported previously.[40]

Autopsy Study

One participant (Patient 16) who received leuprorelin acetate in the 48-week RCT and continued to receive leuprorelin acetate in the 96-week follow-up trial died 118 weeks after initiation of the treatment. Autopsy of the patient indicated acute cardiac failure caused by cardiac arrhythmia as a possible cause of death. Otherwise, no specific causes of death were reported. Autopsied specimens were assessed by anti-polyglutamine (1C2) immunohistochemistry as in the scrotal skin biopsy and were compared with the findings of previously autopsied SBMA cases who had not been treated with leuprorelin acetate or with relevant drugs. In the spinal motor neurons, diffuse nuclear staining of 1C2 was predominantly observed, and nuclear inclusions were less frequent. The frequencies of 1C2-positive

Table 2. Characteristics of Patients in the 96-week Follow-up Trial

Treatment in 96-week Follow up	Leuprorelin in 48-week RCT (n = 22)				Placebo in 48-week RCT (n = 25)			
	Leuprorelin (Group A, n = 18)	No Treatment (Group C, n = 4)	p		Leuprorelin (Group B, n = 15)	No Treatment (Group D, n = 10)	p	
Age (yr)	52.0 ± 6.5	56.3 ± 8.1	NS		52.5 ± 8.2	51.3 ± 10.2	NS	
Height (cm)	168.6 ± 5.8	164.3 ± 9.4	NS		168.5 ± 5.0	167.6 ± 7.7	NS	
Weight (kg)	58.6 ± 5.9	58.8 ± 6.2	NS		59.6 ± 5.6	61.2 ± 7.3	NS	
Duration of Weakness (yr)	11.7 ± 6.4	8.3 ± 7.4	NS		12.8 ± 5.5	13.0 ± 11.5	NS	
(CAG)n	49.1 ± 3.3	45.8 ± 2.2	NS		48.0 ± 2.5	48.2 ± 2.6	NS	
ALSFRS-R (Japanese Edition) [a]	41.3 ± 2.8	40.5 ± 7.7	NS		42.1 ± 2.6	42.0 ± 4.5	NS	
	41.2 ± 3.7	39.8 ± 6.7	NS		40.7 ± 3.6	41.9 ± 5.0	NS	
ADL (cane-assisted/independent) [a]	3/15	1/3	NS		4/11	3/7	NS	
	7/11	2/2	NS		6/9	3/7	NS	

[a]Upper values indicate data at inclusion in the 48-week RCT, and lower values those at inclusion in the 96-week follow-up trial. Other values are data at inclusion of 48-week RCT. (CAG)n = number of expanded CAG repeats in the *androgen receptor* gene; ALSFRS-R = revised amyotrophic lateral sclerosis functional rating scale. Data represent means ± SD except for ADL.
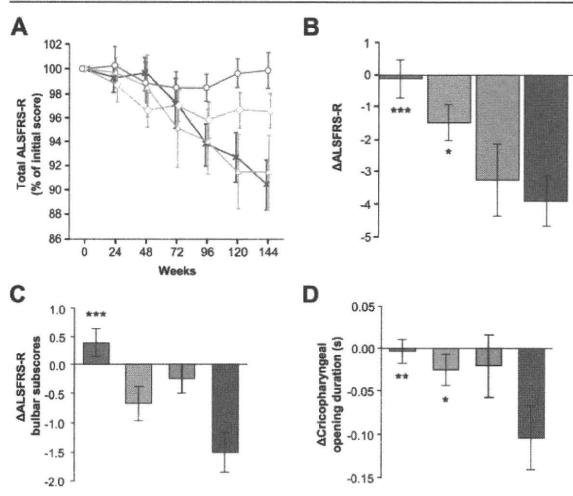

Fig 3. Efficacy results of the 96-week follow-up trial. (A, B) Changes in the Revised Amyotrophic Lateral Sclerosis Functional Rating Scale (ALSFRS-R) scores showed treatment duration–dependent improvements in the leuprorelin-treated groups. (C, D) The ALSFRS-R bulbar subscores (C) and videofluorography (VF) findings (D) were also significantly improved in the leuprorelin-treated patients. Data are expressed as means ± standard error of the mean. *p < 0.05; **p < 0.005; ***p < 0.001 with respect to Group D. Red represents Group A: 48-week leuprorelin/96-week leuprorelin (n = 18); orange represents Group B: 48-week placebo/96-week leuprorelin (n = 15); light blue represents Group C: 48-week leuprorelin/96-week no treatment (n = 4); blue represents Group D: 48-week placebo/96-week no treatment (n = 10).
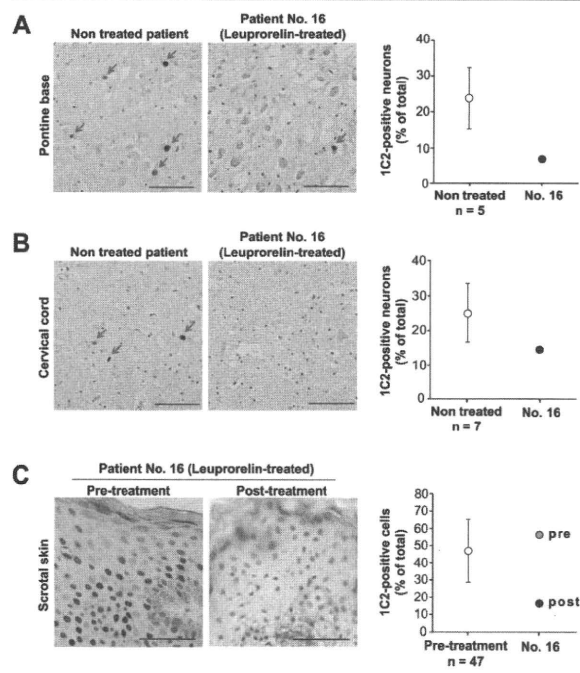

Fig 4. Effects of leuprorelin acetate on nuclear accumulation of mutant androgen receptor (AR). (A, B) Accumulation of mutant AR in neurons was remarkable both in the pontine base and in the spinal anterior horn of all the control, non-treated autopsied cases, but the number of 1C2-positive neurons was relatively small in the leuprorelin-treated patient (Patient 16). Scale bars = 100μm. (C) Mutant AR accumulation in scrotal skin epithelial cells that underwent biopsy was markedly reduced by leuprorelin acetate in Patient 16 (Patient 16 was excluded from this mean.) Scale bars = 50μm. Data are expressed as means ± standard deviation.

Table 3. Adverse Events in 48-Week Randomized Controlled Trial

AEs	Leuprorelin (n = 25)	Placebo (n = 25)
At least one AE	21 (84%)	9 (36%)
At least one AE other than ED	16 (64%)	6 (24%)
ED[a]	13 (52%)	4 (16%)
Hypertriglyceridemia	7 (28%)	0
Lumbago	5 (20%)	1 (4%)
Headache	5 (20%)	1 (4%)
Numbness	3 (12%)	2 (8%)
Hand arthralgia	4 (16%)	0
Fatigue	3 (12%)	0
Hot flush	3 (12%)	0
Injection site lump	3 (12%)	0
Hypertension	2 (8%)	0
Fracture	0	2 (8%)

[a]Number was calculated by questionnaire on every visit. AE = adverse event; ED = erectile dysfunction.

neurons in the anterior horn and brainstem of Patient 16 were less than those in non-treated SBMA patients (Figs 4A, B). By way of comparison, the pretreatment frequency of 1C2-positive cells in the biopsied scrotal skin of Patient 16 was a little higher than the mean value of other study participants at week 0 but decreased after 48 weeks of leuprorelin treatment in the RCT (see Fig 4C). Hence, this patient's pretreatment frequency of 1C2-positive cells in the anterior horn and brainstem were presumed to also be greater than the posttreatment levels.[17]

Discussion

Recent research on neurodegenerative diseases has repeatedly shown that abnormal protein accumulation in neuronal cells is important in the molecular pathogenesis of neurodegeneration.[41] In polyglutamine diseases including SBMA, the aberrant proteins that contain an extended polyglutamine tract accumulate chiefly in the nucleus, resulting in the disruption of cellular functions such as transcription.[14,42] To date, no disease-modifying therapies for polyglutamine diseases have proved beneficial in clinical trials. The results of this interventional trial suggest that androgen deprivation therapy for SBMA is a promising therapy targeting the molecular pathogenesis of polyglutamine diseases.

In this study, we demonstrated that leuprorelin acetate suppressed toxic accumulation of the mutant AR protein, and thereby slowed down the progression of SBMA. As shown previously in animal and human studies, leuprorelin-mediated androgen deprivation significantly decreased mutant AR accumulation in scrotal skin.[17,21] Furthermore, our histopathological analysis in the autopsied case suggests that leuprorelin treatment also attenuates the nuclear accumulation of pathogenic AR within neuronal cells. AR did not aggregate even in the cytoplasm of scrotal epithelial cells or in that of spinal motor neurons, presumably because androgen deprivation destabilizes AR and facilitates degradation of the protein.[43] Alternatively, androgen deprivation may enhance the protective effects of heat shock proteins, which are normally associated with AR and dissociate on ligand binding.

The 48-week treatment with leuprorelin acetate significantly extended cricopharyngeal opening duration, indicating that this therapy blocked disease progression measured with the most reliable VF parameter. The opening of the cricopharyngeal sphincter is triggered by the motion of the larynx and is widened by pharyngeal pressure.[44] Therefore, cricopharyngeal opening duration reflects the strength of deglutition and has been used as a quantitative parameter of swallowing function in disease conditions such as stroke and inflammatory myopathy.[45,46] Moreover, in patients with ALS, cricopharyngeal opening duration is shortened as a consequence of delayed opening or premature closure of the cricopharyngeal sphincter, or both, and this shortening correlates well with the severity of dysphagia.[47] The amelioration of dysphagia by androgen deprivation is also supported by the 96-week follow-up trial, in which leuprorelin treatment significantly prolonged cricopharyngeal opening duration and improved the bulbar subscores of the ALSFRS-R. Given that pneumonia and respiratory distress are the main causes of death in this disease, leuprorelin treatment appears to be beneficial for the prognosis of SBMA patients.[7]

Although the effect of leuprorelin acetate on general motor function was not clear in the 48-week RCT, the total ALSFRS-R score was significantly greater in patients who received androgen deprivation therapy for 144 or 96 weeks than in those who received no therapy throughout the trial. Although the total ALSFRS-R score is a reliable marker of the progression of ALS, this score is less sensitive for SBMA.[39,48] This study suggests that the ALSFRS-R score is not an appropriate end point in a short-term trial but may be useful in a long-term clinical trial on SBMA.

No unexpected or serious safety issues associated with the long-term use of leuprorelin acetate were identified during this study. The adverse effects of leuprorelin acetate did not differ from those in trials for prostate cancer.[49,50] Although erectile dysfunction after leuprorelin administration was more frequent in this trial than in previous trials for prostate cancer, this is likely because of pre-existing androgen insensitivity in

Banno et al: Leuprorelin for SBMA **147**

Table 4. Adverse Events during Leuprorelin Administration (48-Week Randomized Controlled Trial and 96-Week Follow-up)

AEs	Group A (n = 19)[a]	Group B (n = 15)	Group D (n = 10)
At least one AE	18 (95%)	15 (100%)	7 (70%)
At least one AE other than ED	15 (79%)	11 (73%)	5 (50%)
ED[b]	13 (68%)	9 (60%)	2 (20%)
Numbness	7 (37%)	3 (20%)	2 (20%)
Arthralgia	5 (26%)	5 (33%)	1 (10%)
Hot flush	5 (26%)	4 (27%)	0
Injection-site lump	5 (26%)	4 (27%)	0
Lumbago	5 (26%)	1 (7%)	1 (10%)
Myalgia	2 (11%)	2 (13%)	0
Edema	3 (16%)	0	0
Headache	3 (16%)	0	1 (10%)
Fatigue	2 (11%)	1 (7%)	0
Hyperglycemia	2 (11%)	1 (7%)	1 (10%)
Hypertension	1 (5%)	1 (7%)	0
Death	1 (5%)	0	0
Neuralgia	1 (5%)	0	0
Pollakiuria	1 (5%)	0	0
Depression	0	1 (7%)	0
Fracture	0	1 (7%)	1 (10%)
Hyperlipidemia	0	1 (7%)	1 (10%)

[a]All patients were analyzed including Patient 16. [b]Number was calculated by questionnaire on every visit. AE = adverse event; ED = erectile dysfunction.

SBMA.[51] The high tolerability of leuprorelin acetate was also supported by the low dropout rate in this trial.

An important limitation in this study is the trial duration. SBMA is a slowly progressive disease, with a disease duration of approximately 20 years.[7] Given that leuprorelin acetate did not suppress the decline in ALSFRS-R scores in our 48-week RCT, a long-term, placebo-controlled trial may be necessary to evaluate the efficacy of leuprorelin acetate on general motor function in SBMA. Based on this study, cricopharyngeal opening duration in VF appears to be a practical biomarker to evaluate therapy efficacy for SBMA in short-term trials.

In conclusion, the results of this study suggest that leuprorelin acetate administration suppresses nuclear accumulation, stabilization, or both of mutant AR, the causative protein of SBMA, and appears to inhibit functional deterioration of the patients. The results of this phase 2 trial support the start of large-scale clinical trials of androgen deprivation for 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. Sperfeld AD, Karitzky J, Brummer D, et al. X-linked bulbospinal neuronopathy: 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(pt 1):209–232.

4. Katsuno M, Adachi H, Tanaka F, Sobue G. Spinal and bulbar muscular atrophy: ligand-dependent pathogenesis and therapeutic perspectives. J Mol Med 2004;82:298–307.

5. Fischbeck KH. Kennedy disease. J Inherit Metab Dis 1997;20:152–158.

6. Katsuno M, Adachi H, Waza M, et al. Pathogenesis, animal models and therapeutics in spinal and bulbar muscular atrophy (SBMA). Exp Neurol 2006;200:8–18.

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

8. 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.

9. Tanaka F, Doyu M, Ito Y, et al. Founder effect in spinal and bulbar muscular atrophy (SBMA). Hum Mol Genet 1996;5:1253–1257.

10. Andrew SE, Goldberg YP, Hayden MR. Rethinking genotype and phenotype correlations in polyglutamine expansion disorders. Hum Mol Genet 1997;6:2005–2010.

11. 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.

12. 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.

13. Zoghbi HY, Orr HT. Glutamine repeats and neurodegeneration. Annu Rev Neurosci 2000;23:217–247.

14. Ross CA. Polyglutamine pathogenesis: emergence of unifying mechanisms for Huntington's disease and related disorders. Neuron 2002;35:819–822.

15. Gatchel JR, Zoghbi HY. Diseases of unstable repeat expansion: mechanisms and common principles. Nat Rev Genet 2005;6:743–755.

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. Banno H, Adachi H, Katsuno M, et al. Mutant androgen receptor accumulation in spinal and bulbar muscular atrophy scrotal skin: a pathogenic marker. Ann Neurol 2006;59:520–526.

18. 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.

19. 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.

20. 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.

21. 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.

22. Takeyama K, Ito S, Yamamoto A, et al. Androgen-dependent neurodegeneration by polyglutamine-expanded human androgen receptor in Drosophila. Neuron 2002;35:855–864.

23. 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.

24. 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.

25. Wilson AC, Meethal SV, Bowen RL, Atwood CS. Leuprolide acetate: a drug of diverse clinical applications. Expert Opin Investig Drugs 2007;16:1851–1863.

26. Pocock SJ, Simon R. Sequential treatment assignment with balancing for prognostic factors in the controlled clinical trial. Biometrics 1975;31:103–115.

27. Niijima T, Aso Y, Akaza H, et al. [Clinical phase I and phase II study on a sustained release formulation of leuprorelin acetate (TAP-144-SR), an LH-RH agonist, in patients with prostatic carcinoma. Collaborative++ Studies on Prostatic Carcinoma by the Study Group for TAP-144-SR.]. Hinyokika Kiyo 1990;36:1343–1360.

28. Plosker GL, Brogden RN. Leuprorelin. A review of its pharmacology and therapeutic use in prostatic cancer, endometriosis and other sex hormone-related disorders. Drugs 1994;48:930–967.

29. Ohashi Y, Tashiro K, Itoyama Y, et al. [Study of functional rating scale for amyotrophic lateral sclerosis: revised ALSFRS(ALSFRS-R) Japanese version]. No To Shinkei 2001;53:346–355.

30. Chahin N, Klein C, Mandrekar J, Sorenson E. Natural history of spinal-bulbar muscular atrophy. Neurology 2008;70:1967–1971.

31. Morin LG. Creatine kinase: re-examination of optimum reaction conditions. Clin Chem 1977;23:1569–1575.

32. Logemann JA, Pauloski BR, Rademaker AW, et al. Temporal and biomechanical characteristics of oropharyngeal swallow in younger and older men. J Speech Lang Hear Res 2000;43:1264–1274.

33. Logemann JA. Evaluation and treatment of swallowing disorders. 2nd ed. Austin, TX: Pro-Ed, 1998.

34. 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.

35. 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 1996;92:109–114.

36. Suzuki K, Katsuno M, Banno H, et al. CAG repeat size correlates to electrophysiological motor and sensory phenotypes in SBMA. Brain 2008;131:229–239.

37. Nishie M, Mori F, Yoshimoto M, et al. A quantitative investigation of neuronal cytoplasmic and intranuclear inclusions in the pontine and inferior olivary nuclei in multiple system atrophy. Neuropathol Appl Neurobiol 2004;30:546–554.

38. Tanaka F, Reeves MF, Ito Y, et al. Tissue-specific somatic mosaicism in spinal and bulbar muscular atrophy is dependent on CAG-repeat length and androgen receptor–gene expression level. Am J Hum Genet 1999;65:966–973.

39. Takeuchi Y, Katsuno M, Banno H, et al. Walking capacity evaluated by the 6-minute walk test in spinal and bulbar muscular atrophy. Muscle Nerve 2008;38:964–971.

40. Fowler JE Jr, Gottesman JE, Reid CF, et al. Safety and efficacy of an implantable leuprolide delivery system in patients with advanced prostate cancer. J Urol 2000;164:730–734.

41. Taylor JP, Hardy J, Fischbeck KH. Toxic proteins in neurodegenerative disease. Science 2002;296:1991–1995.

42. Orr HT, Zoghbi HY. Trinucleotide repeat disorders. Annu Rev Neurosci 2007;30:575–621.

43. Zhou ZX, Lane MV, Kemppainen JA, et al. Specificity of ligand-dependent androgen receptor stabilization: receptor domain interactions influence ligand dissociation and receptor stability. Mol Endocrinol 1995;9:208–218.

44. Jacob P, Kahrilas PJ, Logemann JA, et al. Upper esophageal sphincter opening and modulation during swallowing. Gastroenterology 1989;97:1469–1478.

45. Lazarus CL, Logemann JA, Rademaker AW, et al. Effects of bolus volume, viscosity, and repeated swallows in nonstroke subjects and stroke patients. Arch Phys Med Rehabil 1993;74:1066–1070.

46. Williams RB, Grehan MJ, Hersch M, et al. Biomechanics, diagnosis, and treatment outcome in inflammatory myopathy presenting as oropharyngeal dysphagia. Gut 2003;52:471–478.

47. Ertekin C, Aydogdu I, Yuceyar N, et al. Pathophysiological mechanisms of oropharyngeal dysphagia in amyotrophic lateral sclerosis. Brain 2000;123(pt 1):125–140.

48. A double-blind placebo-controlled clinical trial of subcutaneous recombinant human ciliary neurotrophic factor (rHCNTF) in amyotrophic lateral sclerosis. ALS CNTF Treatment Study Group. Neurology 1996;46:1244–1249.

49. Leuprolide versus diethylstilbestrol for metastatic prostate cancer. The Leuprolide Study Group. N Engl J Med 1984;311:1281–1286.

50. Higano CS. Side effects of androgen deprivation therapy: monitoring and minimizing toxicity. Urology 2003;61:32–38.

51. Dejager S, Bry-Gauillard H, Bruckert E, et al. A comprehensive endocrine description of Kennedy's disease revealing androgen insensitivity linked to CAG repeat length. J Clin Endocrinol Metab 2002;87:3893–3901.

- 147 -

Efficacy and safety of leuprorelin in patients with spinal and bulbar muscular atrophy (JASMITT study): a multicentre, randomised, double-blind, placebo-controlled trial

Masahisa Katsuno*, Haruhiko Banno*, Keisuke Suzuki*, Yu Takeuchi, Motoshi Kawashima, Ichiro Yabe, Hidenao Sasaki, Masashi Aoki, Mitsuya Morita, Imaharu Nakano, Kazuaki Kanai, Shoichi Ito, Kinya Ishikawa, Hidehiro Mizusawa, Tomotaka Yamamoto, Shoji Tsuji, Kazuko Hasegawa, Takayoshi Shimohata, Masatoyo Nishizawa, Hiroaki Miyajima, Fumio Kanda, Yasuhiro Watanabe, Kenji Nakashima, Akira Tsujino, Taro Yamashita, Makoto Uchino, Yasushi Fujimoto, Fumiaki Tanaka, Gen Sobue, for the Japan SBMA Interventional Trial for TAP-144-SR (JASMITT) study group†

Summary

Background Spinal and bulbar muscular atrophy is a hereditary motor neuron disease caused by the expansion of a polyglutamine tract in the androgen receptor. At present there are no treatments for spinal and bulbar muscular atrophy, although leuprorelin suppressed the accumulation of pathogenic androgen receptors in a phase 2 trial. We aimed to assess the efficacy and safety of leuprorelin for spinal and bulbar muscular atrophy.

Methods The Japan SBMA Interventional Trial for TAP-144-SR (JASMITT) was a 48-week, randomised, double-blind, placebo-controlled trial done at 14 hospitals between August, 2006, and March, 2008. Patients with spinal and bulbar muscular atrophy were randomly assigned (1:1) by minimisation to subcutaneous 11·25 mg leuprorelin or identical placebo every 12 weeks. Patients and investigators were masked to treatment allocation. The primary endpoint was pharyngeal barium residue, which indicates incomplete bolus clearance, measured at week 48 by videofluorography. All patients who were randomly assigned and who were assessed with videofluorography at least once were included in the analyses. This study is registered with the JMACCT clinical trials registry, number JMA-IIA00009, and the UMIN clinical trials registry, number UMIN000000465.

Findings 204 patients were randomly assigned and 199 started treatment: 100 with leuprorelin and 99 with placebo. At week 48, the pharyngeal barium residue after initial swallowing had changed by −5·1% (SD 21·0) in the leuprorelin group and by 0·2% (18·2) in the placebo group (difference between groups −5·3%; 95% CI −10·8 to 0·3; p=0·063). The mean difference in pharyngeal barium residue after piecemeal deglutition at week 48 was −3·2% (−6·4 to 0·0; p=0·049), but there was no significant difference between the groups after covariate adjustment for the baseline data (−4·1 to 1·6; p=0·392). In a predefined subgroup analysis, leuprorelin treatment was associated with a greater reduction in barium residue after initial swallowing than was placebo in patients with a disease duration less than 10 years (difference between groups −9·8, −17·1 to −2·5; p=0·009). There were no significant differences in the number of drug-related adverse events between groups (57 of 100 in the leuprorelin group and 54 of 99 in the placebo group; p=0·727).

Interpretation 48 weeks of treatment with leuprorelin did not show significant effects on swallowing function in patients with spinal and bulbar muscular atrophy, although it was well tolerated. Disease duration might influence the efficacy of leuprorelin and thus further clinical trials with sensitive outcome measures should be done in subpopulations of patients.

Funding Large Scale Clinical Trial Network Project, Japan and Takeda Pharmaceuticals.

Introduction

Spinal and bulbar muscular atrophy (SBMA), also known as Kennedy's disease, is an adult-onset, X-linked motor neuron disease characterised by muscle atrophy; limb, trunk, and facial weakness; contraction fasciculations; and bulbar involvement.[1-3] The prevalence of SBMA has been estimated to be 1–2 per 100 000, although a substantial number of patients with the disorder might have been misdiagnosed with other neuromuscular diseases such as amyotrophic lateral sclerosis.[4] Disease progression is usually slow, but life-threatening respiratory tract infections often occur in

the advanced stage, resulting in premature death.[5] SBMA is caused by the expansion of a CAG triplet repeat, which encodes a polyglutamine tract, within the first exon of the androgen receptor gene.[6] Patients with SBMA have 38–62 CAG repeats, whereas individuals without the disorder have 9–36 CAG repeats.[4,6] Accumulation of the pathogenic androgen receptor protein in the nuclei of lower motor neurons is thought to lead to induction of neuronal cell dysfunction and eventual degeneration.[7] Deposition of pathogenic androgen receptors also occurs in non-neuronal tissues such as scrotal skin and can be used as a histopathological

Department of Neurology
(M Katsuno MD, H Banno MD,
K Suzuki MD, Y Takeuchi MD,
M Kawashima MD, F Tanaka MD,
Prof G Sobue MD) and
Department of
Otorhinolaryngology
(Y Fujimoto MD), Nagoya
University Graduate School of
Medicine, Nagoya, Japan;
Institute for Advanced
Research, Nagoya University,
Nagoya, Japan (M Katsuno,
H Banno); Department of
Neurology, Hokkaido
University Graduate School of
Medicine, Kita-ku, Sapporo,
Japan (I Yabe MD,
Prof H Sasaki MD); Department
of Neurology, Tohoku
University School of Medicine,
Sendai, Miyagi, Japan
(M Aoki MD); Division of
Neurology, Department of
Medicine, Jichi Medical School,
Shimotsuke, Tochigi, Japan
(M Morita MD,
Prof I Nakano MD); Department
of Neurology, Graduate School
of Medicine, Chiba University,
Chiba, Japan (K Kanai MD,
S Ito MD); Department of
Neurology and Neurological
Science Graduate School, Tokyo
Medical and Dental University,
Tokyo, Japan (K Ishikawa MD,
Prof H Mizusawa MD);
Department of Neurology,
Division of Neuroscience,
Graduate School of Medicine,
University of Tokyo, Tokyo,

Japan (T Yamamoto MD,
Prof S Tsuji MD); Department of
Neurology, National Hospital
Organization, National
Sagamihara Hospital,
Sagamihara, Kanagawa, Japan
(K Hasegawa MD); Department
of Neurology, Brain Research
Institute, Niigata University,
Niigata, Japan
(T Shimohata MD,
Prof M Nishizawa MD); First
Department of Medicine,
Hamamatsu University School
of Medicine, Hamamatsu,
Shizuoka, Japan
(H Miyajima MD); Department
of Neurology, Kobe University
Hospital, Kobe, Japan
(Prof F Kanda MD); Division of
Neurology, Department of
Brain and Neurosciences,
Tottori University Faculty of
Medicine, Yonago, Tottori,
Japan (Y Watanabe MD,
Prof K Nakashima MD); First
Department of Internal
Medicine, Graduate School of
Biomedical Sciences, Nagasaki
University, Nagasaki, Japan
(A Tsujino MD); and
Department of Neurology,
Graduate School of Medical
Sciences, Kumamoto
University, Kumamoto, Japan
(T Yamashita MD,
Prof M Uchino MD)

Correspondence to:
Prof Gen Sobue, Department of
Neurology, Nagoya University
Graduate School of Medicine,
65 Tsurumai-cho, Showa-ku,
Nagoya 466-8550, Japan
sobueg@med.nagoya-u.ac.jp

biomarker for SBMA.[8] Most patients also have high serum concentrations of creatine kinase.[1,3]

SBMA is a male-specific disease; even homozygous females do not display symptoms.[9,10] The sex dependency of the disorder seems to stem from the testosterone-dependent toxicity of the pathogenic androgen receptor.[10–14] In mouse models of SBMA, surgical castration delays disease onset and progression and reverses the neuromuscular phenotype.[11,14] Similar effects emerge when the mice are treated with leuprorelin, a luteinising hormone-releasing hormone (LH-RH) agonist that reduces testosterone release from the testes by downregulating LH-RH receptors in the pituitary gland.[12] A phase 2 randomised controlled trial of leuprorelin in patients with SBMA suggested a short-term improvement of swallowing function and long-term suppression of deterioration in motor function with a high tolerability.[15] These promising results, together with the well known tolerability of LH-RH agonists, led us to undertake a randomised, placebo-controlled clinical trial of leuprorelin in SBMA.

Methods

Patients

The Japan SBMA Interventional Trial for TAP-144-SR (JASMITT) study was a randomised, double-blind, placebo-controlled, parallel-group, multicentre trial at 14 hospitals in Japan. Patients were enrolled between August, 2006, and March, 2007; the study ended in March, 2008. Inclusion criteria were a clinical diagnosis of SBMA with more than one motor symptom (muscle weakness, muscle atrophy, bulbar palsy, and hand tremor); confirmation of androgen receptor CAG repeat expansion (>38 repeats); age 30–70 years at the time of informed consent; no desire to father a child; serum aspartate aminotransferase less than four times the upper limit of normal; serum alanine aminotransferase less than four times the upper limit of normal; ability to stand for 6 min with or without support; and ability to attend ambulatory hospital visits. Exclusion criteria were treatment with LH-RH agonists, testosterone drugs, 5-alpha-reductase inhibitors, antiandrogen drugs, anabolic-androgenic steroids, progesterone, or oestrogen drugs within 48 weeks before informed consent; previous treatment with LH-RH agonists for more than 48 weeks; history of surgical androgen deprivation (eg, orchiectomy); depression diagnosed by the mini-international neuropsychiatric interview Japanese version 5.0.0 major depression episode; coexisting severe disease besides SBMA; known allergy to leuprorelin, synthetic LH-RH, or LH-RH derivatives; and participation in other clinical trials within 12 weeks before informed consent.

Patients provided written informed consent before enrolment. The protocol was approved by the institutional review board at each participating centre and the Japanese regulatory authority (Pharmaceuticals and Medical Devices Agency, Japan). The study was done in accordance with the Declaration of Helsinki and good clinical practice.[16,17]

Randomisation and masking

Patients were randomly assigned (1:1) to receive either leuprorelin or an identical placebo by an independent registration centre (Clinical Trial Coordinating Center, Research Center for Clinical Pharmacology, The Kitasato Institute, Tokyo, Japan). Dynamic random allocation was done with minimisation on the basis of the patients' age (≤54 years or ≥55 years) and CAG repeat length (≤49 repeats or ≥50 repeats) to reduce bias.[18] The cutoff values were chosen on the basis of the mean age and CAG repeat length of the patients enrolled in the phase 2 trial.[15] Patients were assigned to a computer-generated randomisation list. Patients and investigators were masked to treatment allocation. An independent safety monitoring committee could request the unmasking of trial participants if necessary. The drug codes were broken and made available for data analysis when the study was completed and the data files had been verified.

Procedures

Leuprorelin (leuprorelin acetate) or placebo was subcutaneously injected at a dose of 11·25 mg every 12 weeks. Placebo was supplied as a vial containing microcapsule powder without leuprorelin, which was suspended in the same solution as the active drug for injection. We did not do a dose-response study because previous studies suggested that leuprorelin-mediated androgen deprivation is incomplete at doses lower than 3·75 mg every 4 weeks in adult men.[19] In a phase 2 trial of leuprorelin for prostate cancer, doses higher than 3·75 mg every 4 weeks led to a greater occurrence of adverse effects with no further reduction in serum testosterone concentrations.[20] A 3-month formulation of leuprorelin was effective at reducing testosterone concentration in patients with prostate cancer.[21] Therefore, we gave patients 11·25 mg every 12 weeks, which corresponds to 3·75 mg every 4 weeks.

The primary endpoint was pharyngeal barium residue at 48 weeks, visualised by videofluorography according to a standardised method.[22,23] This variable was selected because, although there is no established endpoint for clinical trials of SBMA, previous studies suggested that dysphagia and aspiration most strongly affect prognosis.[5] Among the various parameters of videofluorography, pharyngeal barium residue, which indicates incomplete bolus clearance, is directly associated with aspiration, and is the most common abnormal finding in patients with SBMA. Pharyngeal barium residue visualised by videofluorography is a predictive factor for aspiration and is associated with residues that are quantified by scintigraphy, suggesting that this measurement can be used to assess swallowing function reliably in patients with neuromuscular disorders.[24,25] In the phase 2

- 150 -

randomised controlled trial, only 22% of patients had dysphagia but 62% had pharyngeal residue.[15] In addition, swallowing function decreased as the disease progressed. The results of initial analyses of the phase 2 randomised trial, which involved videofluorography quantification by one investigator, suggested that the effect size would be big enough with pharyngeal barium residue for the effect to be obvious with fewer patients than it would with other measures such as the revised amyotrophic lateral sclerosis functional rating scale (ALSFRS-R). Neither the results of subsequent analyses by two independent videofluorography assessors nor the data from the follow-up study of the phase 2 trial[15] were available when we planned the present trial and chose pharyngeal barium residue as the primary outcome measure.

Secondary outcome measures were frequency of anti-polyglutamine (1C2) antibody-positive cells in scrotal skin biopsies; serum concentrations of creatine kinase; motor function, as measured by the ALSFRS-R, Japanese edition,[26] five components of the quantitative myasthenia gravis (QMG) score (without ptosis or diplopia sections),[27] and the 6-min walk test (6MWT);[28,29] temporal parameters of videofluorography, such as stage transition duration, duration of maximum laryngeal elevation, and duration of cricopharyngeal opening; and quality of life (amyotrophic lateral sclerosis assessment questionnaire 5 [ALSAQ-5], Japanese edition).[30] To assess safety, standard laboratory parameters were checked every 12 weeks and bone mineral density was monitored at weeks 0 and 48. Primary and secondary endpoints were measured at weeks 0, 24, and 48.

We measured the frequency of anti-polyglutamine (1C2) antibody-positive cells in scrotal skin biopsies because this parameter is associated with the number of 1C2-positive spinal motor neurons in autopsy specimens and is inversely associated with the limb Norris scale.[8] Three scrotal punch biopsies were taken from each patient at each timepoint under local anaesthesia and were processed for immunohistochemical analysis using the 1C2 antibody to detect accumulation of the pathogenic androgen receptor (webappendix p 1). We used the QMG and 6MWT to assess motor function because these parameters can be measured in a multicentre setting without any particular equipment.

In the videofluorography examinations, patients were instructed to swallow 3 mL of 40% weight/volume barium sulphate twice while standing. Pharyngeal barium residue was measured by the first 3 mL swallowed because the first residue directly affects the second one. All of the parameters were measured by three masked independent investigators according to standard procedures.[22,31] Briefly, pharyngeal residue was measured using a semi-quantitative scale: 0, 2, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100%. We trained the evaluators to use this method, and the kappa statistic in the validation of pharyngeal barium residue was 0·80 before the trial, 1·0 during, and 1·0 after the end of the trial. Previous studies have also

shown high intra-rater and inter-rater reliabilities for measurement of videofluorographic swallowing,[32] although little is known about the reproducibility of this parameter. Piecemeal deglutition—multiple repeated swallows to empty a bolus from the oral cavity—is often observed with videofluorography in patients with SBMA, and thus we measured pharyngeal residues not only after
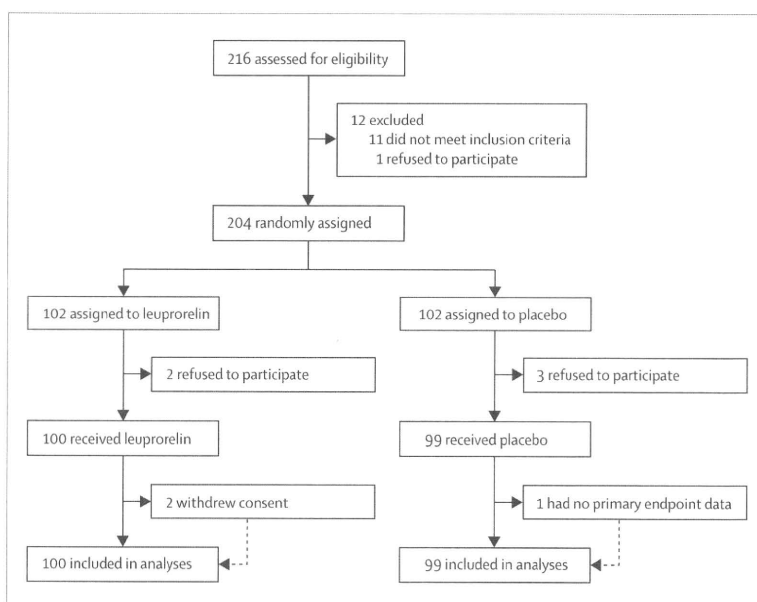
See Online for webappendix

Figure 1: Trial profile

	Total study population		Subset group (duration of disease <10 years)	
	Leuprorelin (n=100)	Placebo (n=99)	Leuprorelin (n=44)	Placebo (n=37)
Age (years)	53·6 (9·2)	54·2 (9·2)	52·2 (9·6)	52·6 (9·9)
Duration of disease (years)	12·7 (8·4)	13·3 (7·3)	6·1 (2·4)	6·1 (2·6)
CAG repeat length	48·6 (4·0)	48·2 (3·2)	48·3 (4·4)	47·3 (2·6)
Weight (kg)	62·7 (9·4)	63·8 (11·2)	62·8 (10·5)	65·8 (12·0)
Residue after piecemeal deglutition (%)	10·6 (13·5)	6·7 (7·2)	9·6 (10·7)	4·6 (4·7)
Residue after initial swallowing (%)	20·3 (27·1)	18·7 (26·6)	21·5 (28·9)	12·0 (19·9)
Duration of cricopharyngeal opening (s)	0·4 (0·1)	0·4 (0·1)	0·4 (0·1)	0·4 (0·1)
Stage transition duration (s)	0·1 (0·3)	0·2 (0·3)	0·1 (0·2)	0·1 (0·1)
Laryngeal elevation duration (s)	0·2 (0·1)	0·3 (0·1)	0·3 (0·1)	0·3 (0·2)
ALSFRS-R score	40·8 (3·6)	41·0 (3·7)	42·2 (2·6)	42·5 (3·3)
6MWT (m)	323·2 (141·9)	303·7 (132·0)	378·4 (109·1)	372·2 (111·9)
Modified QMG score	7·2 (3·0)	6·8 (2·8)	6·5 (2·8)	5·6 (2·7)
ALSAQ-5 score	10·1 (3·4)	10·7 (3·8)	9·8 (3·4)	9·6 (4·0)
1C2-positive cells (%)	20·6 (15·0)	20·7 (14·0)	22·0 (16·4)	19·9 (12·8)
Creatine kinase (IU/L)	754·2 (443·2)	784·4 (479·8)	765·8 (488·7)	1019·5 (533·0)
Testosterone (ng/mL)	7·8 (3·0)	7·7 (2·6)	8·3 (2·9)	7·8 (2·6)

Data are mean (SD). ALSFRS-R=revised amyotrophic lateral sclerosis functional rating scale. 6MWT=6-min walk test. QMG=quantitative myasthenia gravis. ALSAQ-5=amyotrophic lateral sclerosis assessment questionnaire 5.

Table 1: Demographics and baseline characteristics

- 151 -

initial swallowing but also after piecemeal deglutition. The stage transition duration (also known as pharyngeal delay time) was defined as the interval from the bolus passing the base of the tongue to the onset of laryngeal elevation, and the duration of maximum laryngeal elevation was the length of time during which the larynx was maximally raised from its rest position. The duration of cricopharyngeal opening (also known as the duration of opening of the upper oesophageal sphincter) was defined as the length of time for which the cricopharyngeal sphincter was open. The mean values of the first and the second doses of barium sulphate were calculated for all the temporal parameters of videofluorography.

	n	Baseline	48 weeks	Difference	Between-group difference (95% CI)	p value*
Primary endpoint						
Pharyngeal barium residue after initial swallowing (%)						
Leuprorelin	98	20·3 (27·1)	15·2 (20·4)	−5·1 (21·0)	..	..
Placebo	96	18·7 (26·6)	18·8 (24·6)	0·2 (18·2)	−5·3 (−10·8 to 0·3)	0·063
Pharyngeal barium residue after piecemeal deglutition (%)						
Leuprorelin	97	10·6 (13·5)	9·0 (11·3)	−1·6 (12·9)	..	..
Placebo	96	6·7 (7·2)	8·3 (11·2)	1·7 (9·3)	−3·2 (−6·4 to 0·0)	0·049†
Secondary endpoints						
Duration of cricopharyngeal opening (s)						
Leuprorelin	98	0·43 (0·07)	0·46 (0·08)	0·03 (0·07)	..	..
Placebo	98	0·43 (0·09)	0·46 (0·08)	0·02 (0·06)	0·00 (−0·02 to 0·02)	0·68
Stage transition duration (s)						
Leuprorelin	98	0·14 (0·25)	0·19 (0·26)	0·04 (0·25)	..	..
Placebo	98	0·17 (0·28)	0·17 (0·28)	−0·01 (0·19)	0·05 (−0·01 to 0·11)	0·12‡
Laryngeal elevation duration (s)						
Leuprorelin	98	0·24 (0·11)	0·21 (0·09)	−0·03 (0·11)	..	..
Placebo	98	0·25 (0·12)	0·23 (0·11)	−0·02 (0·11)	−0·01 (−0·04 to 0·02)	0·52
ALSFRS-R score						
Leuprorelin	100	40·8 (3·6)	40·5 (4·1)	−0·4 (2·8)	..	..
Placebo	99	41·0 (3·7)	40·8 (3·4)	−0·1 (2·4)	−0·2 (−1·0 to 0·5)	0·54
6MWT (m)						
Leuprorelin	100	323·2 (141·9)	298·9 (144·6)	−24·2 (48·8)	..	..
Placebo	99	303·7 (132·0)	289·7 (139·1)	−14·0 (46·8)	−10·2 (−23·6 to 3·1)	0·13
Modified QMG score						
Leuprorelin	100	7·2 (3·0)	7·1 (3·1)	−0·1 (1·9)	..	..
Placebo	99	6·8 (2·8)	7·0 (2·9)	0·2 (1·8)	−0·3 (−0·8 to 0·2)	0·20
ALSAQ-5 score						
Leuprorelin	100	10·1 (3·4)	11·1 (3·8)	1·0 (2·9)	..	..
Placebo	99	10·7 (3·8)	10·9 (3·7)	0·1 (2·9)	0·9 (0·1 to 1·7)	0·033
1C2-positive cells (%)						
Leuprorelin	100	20·6 (15·0)	8·7 (8·3)	−11·9 (13·0)	..	..
Placebo	98	20·6 (14·0)	23·4 (14·3)	2·7 (12·1)	−14·7 (−18·2 to −11·2)	<0·0001§
Creatine kinase (IU/L)						
Leuprorelin	100	754·2 (443·2)	632·7 (398·7)	−121·5 (245·1)	..	..
Placebo	98	786·3 (481·9)	766·7 (494·9)	−20·0 (273·2)	−101·9 (−174·6 to −29·2)	0·006§

Data are mean (SD). ALSFRS-R=revised amyotrophic lateral sclerosis functional rating scale. 6MWT=6-min walk test. QMG=quantitative myasthenia gravis. ALSAQ-5=amyotrophic lateral sclerosis assessment questionnaire 5. *Two sample t test. †Wilcoxon rank sum test p=0·044. ‡Wilcoxon rank sum test p=0·012. §Wilcoxon rank sum test p<0·0001.

Table 2: **Primary and secondary endpoints**

All patients gave written informed consent for the genetic analyses. The results of genetic analyses of CAG repeat number were reported to the registration centre for dynamic allocation but were not disclosed to patients or investigators. Serum creatine kinase and testosterone concentrations were assessed in a central laboratory so that the patients and investigators were masked to the results.

All data related to the trial were recorded on case report forms that were reviewed with the patients' examination results by the independent trial monitor. After validation, all results were double entered into an SQL Server 2005 database. Data entry was controlled for consistency by use of SAS (version 9.1.3), according to the protocol and data management plan. An independent safety monitoring committee reviewed the adverse events that occurred during the trial.

Statistical analysis

In analyses of the phase 2 randomised trial of leuprorelin in patients with SBMA that were done 6 months after the last patient completed the trial, the standard deviation (SD) of pharyngeal barium residue was 9·34% and the standardised difference was −3·8%. We calculated that a sample size of 76 patients per group would provide 80% power to detect a −3·8% difference for pharyngeal barium residue between treatment groups (two sample t test, with a two-sided α level of 0·10 and an SD of 9·34% in both groups). We chose this α level on the basis of advice from the Japanese regulatory authority during our planning of the trial, which took into consideration the severity and rarity of SBMA. There is no effective treatment or established outcome measure for this disease; therefore the regulatory authority used the International Conference on Harmonization E9 guideline statement[33] that alternative values to the conventional level of type I error (5%) might be acceptable in some cases. The number of patients needed was estimated to be 85 per group (total 170 patients) to allow for dropouts. All patients who were randomised and who were assessed with videofluorography at least once were included in the analyses. Patients who discontinued treatment prematurely were encouraged to attend assessments, and the results of these assessments were used for efficacy analyses; we used the last-observation-carried-forward method to impute values that were not available at the final assessment.

The primary endpoint was assessed by use of the two sample t test. We used analysis of covariance to adjust for baseline differences of the respective covariates.[34] For the secondary endpoints, we used the two sample t test and the Wilcoxon rank sum test to calculate the differences between the groups. We noted adverse events and other safety information (laboratory tests and bone mineral density) for safety analyses. Each adverse event was coded to a preferred term and associated organ system according to an established and validated adverse reaction dictionary (MedDRA/J,

version 11.0). The endpoints for adverse events were the number of patients with at least one event or an event under each recorded preferred term.

We also investigated the efficacy of leuprorelin in a prespecified subgroup analysis of patients who had a disease duration of less than 10 years, because previous studies have suggested that motor function in patients with SBMA is inversely associated with disease duration.[5,29,35] The cutoff of 10 years was chosen because the mean disease duration was 11·9 years in the previous phase 2 randomised controlled trial[15] and the median duration from the onset of weakness to death was about 20 years in a large retrospective study on the natural history of SBMA.[5] All primary and secondary endpoints were assessed in the subgroup analyses and differences between groups were calculated by use of the two sample *t* test and the Wilcoxon rank sum test.

Statistical analyses were done using SAS (version 9.1.3). Two-sided p values less than 0·05 were deemed statistically significant. This study is registered in the JMACCT clinical trials registry, number JMA-IIA00009 and the UMIN clinical trials registry, number UMIN000000465.

Role of the funding source

The sponsors of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report. All authors had full access to all the data in the study and had final responsibility for the decision to submit for publication.

Results

204 patients were randomly assigned to leuprorelin or placebo (figure 1). 199 received at least one dose of the study drug (100 received leuprorelin, 99 placebo). 196 patients completed 48 weeks of treatment; two patients in the leuprorelin group refused to participate and two withdrew consent, and three patients in the placebo group refused to participate and one had no primary endpoint data. Demographics and baseline characteristics were similar between the two groups (table 1), except for barium residue after piecemeal deglutition (p=0·013). To examine the reliability of outcome measures and the effects of serum testosterone concentrations on these measures, we did cross-sectional analyses of the baseline data. ALSFRS-R score was associated with the duration of disease (p<0·0001), 6MWT (p<0·0001), modified QMG score (p<0·0001), ALSAQ-5 (p=0·0057), and pharyngeal barium residue (after initial swallowing p=0·0278; after piecemeal deglutition p=0·0409; webappendix p 3). However, the pretreatment serum concentrations of testosterone were not significantly associated with outcome measures of motor or swallowing function.

In the leuprorelin group, one patient's testosterone concentration was accidentally measured at week 48 on site and two investigators noticed the result. After the incident, the record was deleted from the hospital database and other investigators examined this patient.
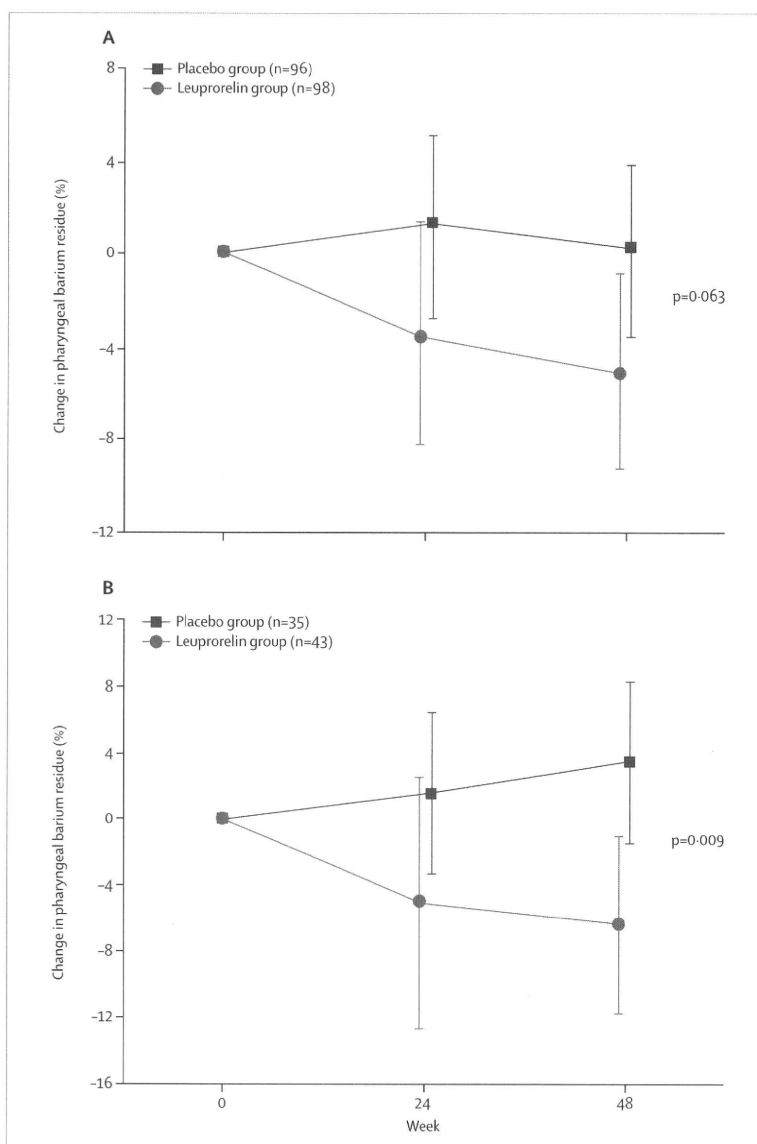

Figure 2: Mean change in pharyngeal barium residue after initial swallowing
(A) All patients. (B) Patients with disease duration <10 years. Bars=95% CI.

The two investigators who knew the concentration of testosterone had no contact with the patient and did not reveal the result until the end of the statistical analyses, to keep other investigators and the patient masked to treatment allocation.

At week 48, the difference in pharyngeal barium residue after initial swallowing between the leuprorelin group and the placebo group was −5·3% (95% CI −10·8 to 0·3; 90% CI −9·92 to −0·60; p=0·063; table 2; figure 2). The difference in pharyngeal barium residue after piecemeal deglutition was −3·2% (95% CI −6·4 to 0·0; 90% CI −5·89 to −0·52; p=0·049), but there was no

	Leuprorelin (n=100)		Placebo (n=99)	
	Patients	Events	Patients	Events
Any	76	380	77	273
Nasopharyngitis	36	55	32	41
Pharyngitis	5	5	3	4
Dizziness	5	7	1	1
Headache	7	8	3	3
Hot flush	10	10	2	3
Upper respiratory tract inflammation	4	5	6	6
Constipation	10	11	5	6
Gastritis	4	4	5	5
Abnormal hepatic function	6	6	3	3
Arthralgia	8	10	3	3
Back pain	8	10	10	11
Myalgia	5	5	3	3
Injection site induration	7	11	5	8
Increased blood triglycerides	5	5	2	2
Contusion	13	16	4	6
Post-procedural haemorrhage	3	3	6	6

Table 3: Adverse events reported in at least 5% of patients in either group, irrespective of cause

significant difference between the groups after covariate adjustment for baseline data (95% CI −4·09 to 1·62; 90% CI −4·13 to 0·59; p=0·392).

There were significant differences between the groups for the mean change in frequency of 1C2-positive cells (p<0·0001), the mean change in serum creatine kinase concentrations (p=0·006), and the mean change in ALSAQ-5 score (p=0·033; table 2). For the other secondary outcomes, no significant differences were seen between the groups at week 48. In patients who received leuprorelin, mean serum testosterone concentration decreased from 7·8 (SD 3·0) ng/mL to 0·3 (0·2) ng/mL in the first 12 weeks. Mean serum testosterone concentration at week 48 was 0·5 (1·3) ng/mL in the leuprorelin group compared with 7·6 ng/mL in the placebo group (2·7; p<0·0001).

Drug treatment was well tolerated and the incidence of adverse events was similar between the groups (76% in the leuprorelin group and 78% in the placebo group; table 3). There were no significant differences between the groups for drug-related adverse events (57 of 100 in the leuprorelin group and 54 of 99 in the placebo group; p=0·727). Six patients had serious adverse events that required admission to hospital: four in the placebo group (contusion, gastrointestinal neoplasm, dyspnoea, and foot fracture) and two in the leuprorelin group (dyspnoea and neoplasm). The drug-related adverse event hot flush was seen more often in the leuprorelin group compared with the placebo group (p=0·03). Although bone mineral density decreased by 6% in the leuprorelin group (data not shown), there were no marked exacerbations compared with previous reports of leuprorelin treatment

(3–6%).[36–38] No drug-related adverse events were reported as the reason for treatment withdrawal.

In patients with disease duration less than 10 years, the baseline characteristics did not differ between the leuprorelin and placebo subgroups (table 1), except for barium residue after piecemeal deglutition (p=0·011) and serum creatine kinase (p=0·028). The mean difference in pharyngeal barium residue after initial swallowing between the groups was −9·8% (95% CI −17·1 to −2·5; p=0·009; table 4; figure 2). This difference was significant after covariate adjustment for the baseline data (p=0·037), although there was no significant difference between the patients included in the subgroup analysis and patients with a disease duration of 10 years or more for this endpoint by test for interaction (p=0·210). In a test of interaction, quality of life seemed to be less affected by leuprorelin in patients with a disease duration of less than 10 years than in those with a disease duration of 10 years or more (p=0·075). Secondary endpoints did not differ between groups in this subgroup analysis, except for the frequency of 1C2-positive cells (between-group difference −15·5, 95% CI −21·4 to −9·6; p<0·0001; table 4).

Discussion

Pharyngeal barium residue after piecemeal deglutition decreased by 1·6% between baseline and week 48 in the leuprorelin group and increased by 1·7% in the placebo group. Although this finding suggests that leuprorelin might improve swallowing function, the change in this primary outcome measure was not significant after covariate adjustment for the difference in baseline data between groups. Pharyngeal barium residue after initial swallowing also seemed to decrease in the leuprorelin group, but no significant difference was detected between the groups. Among the secondary outcome measures, the frequency of 1C2-positive cells and the serum concentrations of creatine kinase decreased by more in the leuprorelin group than in the placebo group, but we did not observe any significant differences in the temporal parameters of videofluorography, ALSFRS-R, 6MWT, or the modified QMG score. The ALSAQ-5 score showed a greater increase in patients in the leuprorelin group than in those in the placebo group. There was no difference in adverse events between the groups except for hot flush, which was more common in the leuprorelin group, and all of the drug-related adverse events had already been documented in the treatment of prostate cancer with leuprorelin.[19] No drug-related adverse effects were given as reasons for patients withdrawing from the study.

The results of all of the previous clinical trials that are relevant to this study are summarised in table 5. Phase 2 trials of leuprorelin reported a decreased frequency of 1C2-positive cells in scrotal skin, reduced serum concentrations of creatine kinase, and extended cricopharyngeal opening in patients with SBMA who were treated with leuprorelin compared with those given

placebo, together with a high tolerability.[15] The present study confirmed that leuprorelin treatment reduces accumulation of the pathogenic androgen receptor proteins and reduces serum concentrations of creatine, a marker of SBMA. We also confirmed that leuprorelin was well tolerated in patients with SBMA, but we were unable to verify the previous observation that leuprorelin extends the duration of cricopharyngeal opening.[15] This discrepancy might be because the phase 2 trial included only 50 patients in total and therefore the positive result could have been due to chance. The result of the previous trial should also be interpreted with caution because the duration of cricopharyngeal opening seemed to differ between the treatment groups at baseline.[15]

Several factors probably contribute to the absence of a significant effect of leuprorelin on the primary endpoint in our study. First, the videofluorography parameters we chose might not have been sensitive enough. Although standard videofluorography parameters for outcome measures in clinical trials have not been formally established, pharyngeal barium residue better represents overall swallowing function than do other temporal measurements.[23,41] Moreover, analyses of baseline data suggest a weak association between ALSFRS-R and the pharyngeal barium residue after initial swallowing. However, our data suggest that pharyngeal barium residue is highly variable between patients, which leads to a decreased sensitivity in the detection of disease-modifying effects of leuprorelin. Furthermore, in piecemeal deglutition—a possible compensatory mechanism against slowly progressive bulbar palsy—patients repeat multiple swallows and thus pharyngeal residue might be measured after each swallow, leading to multiple measurements for one patient; in this situation, the decision of which residue to quantify can vary among investigators. This suggests that the method of examination and evaluation of pharyngeal barium residue should be improved if it is to be used as the primary endpoint in future multicentre clinical trials. Specifically, calculation of the mean value of repetitive measurements or development of effective quantification methods might provide more accurate measurements.

Second, the antianabolic effects of leuprorelin might interfere with the improvement of motor function in patients with SBMA. Although a previous study reported a potential risk of androgen deprivation treatment for patients with SBMA because of an association between muscle strength and serum testosterone concentrations,[35] in our study, none of the baseline values for outcome measures were associated with hormone concentrations. Therefore, the balance between potential neuroprotection by leuprorelin and negative effects on muscles caused by androgen deprivation should be investigated in future studies.

Third, 48 weeks might not be long enough to assess the therapeutic effects of leuprorelin in SBMA. The neuromuscular symptoms of this disease progress for

	n	Baseline	48 weeks	Difference	Between-group difference (95% CI)	p value*
Primary endpoint						
Pharyngeal barium residue after initial swallowing (%)						
Leuprorelin	43	21·5 (28·9)	15·1 (23·4)	−6·4 (17·4)	..	..
Placebo	35	12·0 (20·2)	15·4 (22·4)	3·4 (14·2)	−9·8 (−17·1 to −2·5)	0·009
Pharyngeal barium residue after piecemeal deglutition (%)						
Leuprorelin	42	9·6 (10·7)	9·1 (14·5)	−0·6 (12·3)	..	..
Placebo	35	4·4 (4·7)	7·4 (10·3)	2·9 (8·6)	−3·5 (−8·4 to 1·4)	0·16†
Secondary endpoints						
Duration of cricopharyngeal opening (s)						
Leuprorelin	43	0·42 (0·07)	0·45 (0·09)	0·03 (0·07)	..	..
Placebo	36	0·44 (0·10)	0·46 (0·09)	0·02 (0·07)	0·01 (−0·02 to 0·04)	0·59
Stage transition duration (s)						
Leuprorelin	43	0·11 (0·15)	0·18 (0·20)	0·08 (0·17)	..	..
Placebo	36	0·09 (0·13)	0·12 (0·16)	0·04 (0·14)	0·04 (−0·03 to 0·11)	0·26
Laryngeal elevation duration (s)						
Leuprorelin	43	0·25 (0·12)	0·21 (0·08)	−0·05 (0·11)	..	..
Placebo	36	0·28 (0·16)	0·27 (0·14)	−0·02 (0·14)	−0·03 (−0·09 to 0·03)	0·28
ALSFRS-R score						
Leuprorelin	44	42·2 (2·6)	42·2 (2·9)	0·1 (2·7)	..	..
Placebo	37	42·5 (3·3)	42·4 (2·8)	0·0 (2·6)	0·1 (−1·1 to 1·3)	0·87
6MWT (m)						
Leuprorelin	44	378·4 (109·1)	363·5 (115·9)	−14·8 (52·2)	..	..
Placebo	37	372·2 (111·9)	362·7 (114·9)	−9·5 (53·0)	−5·3 (−28·7 to 18·0)	0·65
Modified QMG score						
Leuprorelin	44	6·5 (2·8)	6·0 (2·9)	−0·5 (1·9)	..	..
Placebo	37	5·6 (2·7)	5·6 (2·6)	0·0 (1·5)	−0·5 (−1·3 to 0·3)	0·19
ALSAQ-5 score						
Leuprorelin	44	9·8 (3·4)	10·4 (3·7)	0·6 (3·1)	..	..
Placebo	37	9·6 (4·1)	10·2 (3·7)	0·5 (3·0)	0·1 (−1·3 to 1·4)	0·94
1C2-positive cells (%)						
Leuprorelin	44	22·0 (16·4)	9·0 (8·2)	−13·0 (13·7)	..	..
Placebo	36	19·5 (12·8)	22·0 (15·3)	2·5 (12·6)	−15·5 (−21·4 to −9·6)	<0·0001‡
Creatine kinase (IU/L)						
Leuprorelin	44	765·8 (488·7)	666·9 (426·3)	−98·9 (242·1)	..	..
Placebo	36	1031·1 (535·8)	993·5 (602·9)	−37·6 (25·6)	−61·3 (−174·5 to 51·8)	0·28

Data are mean (SD). ALSFRS-R=revised amyotrophic lateral sclerosis functional rating scale. 6MWT=6-min walk test. QMG=quantitative myasthenia gravis score. ALSAQ=amyotrophic lateral sclerosis assessment questionnaire. *Two sample t test. †Wilcoxon rank sum test p=0·022. ‡Wilcoxon rank sum test p<0·0001.

Table 4: **Subgroup analyses in patients with disease duration <10 years**

15–20 years and thus the power of short-term trials is probably limited.[5] Patients who completed this double-blind trial will be followed up for 96 weeks in an open-label study. Trials with a longer follow-up period would be of benefit, but such studies could face problems such as poor patient recruitment and financial support.

Fourth, the disease duration of the patients might have influenced the results. Although we excluded patients with severe disease, the period from disease onset ranged from 4 months to 38 years in the enrolled patients. A disease-modifying treatment that prevents the accumulation of abnormal proteins might be more powerful before downstream molecular events have