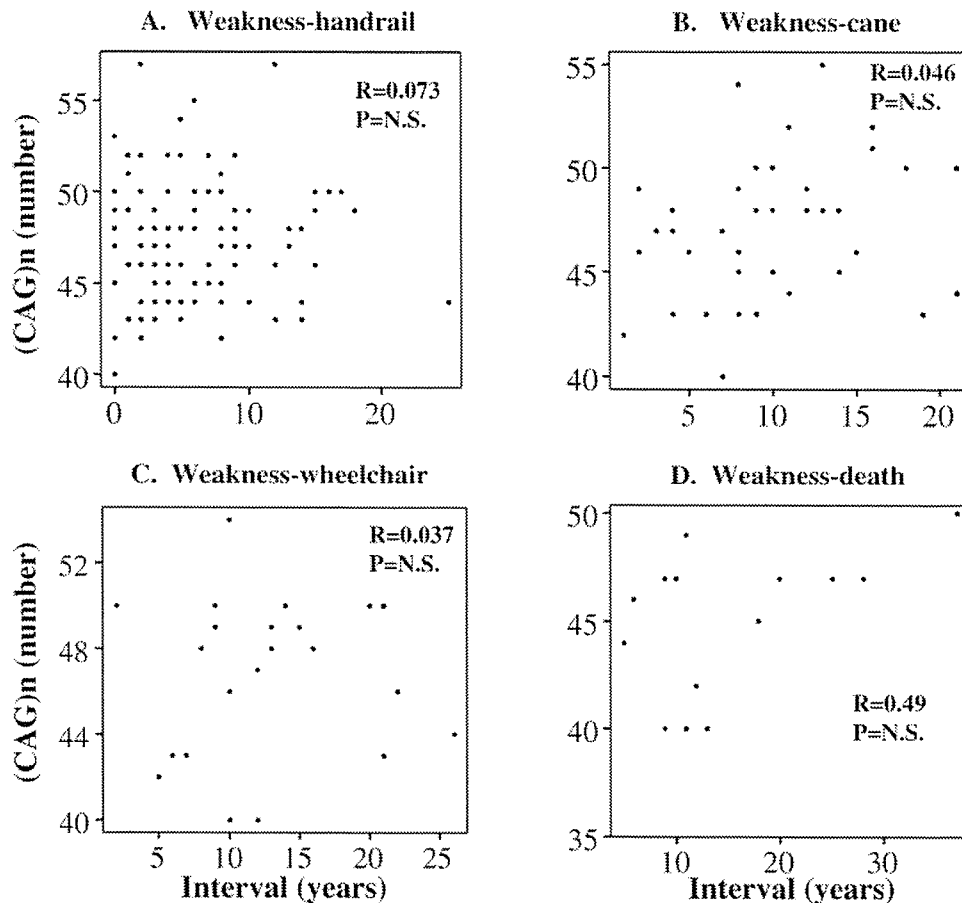


**Fig. 3 (A–C)** Kaplan–Meier analysis of age at onset of hand tremor, muscular weakness and requirement of a handrail. There was a highly significant difference between the patient group with  $\geq 47$  CAG repeats and the group with  $< 47$  CAG repeats, as compared by log rank tests.

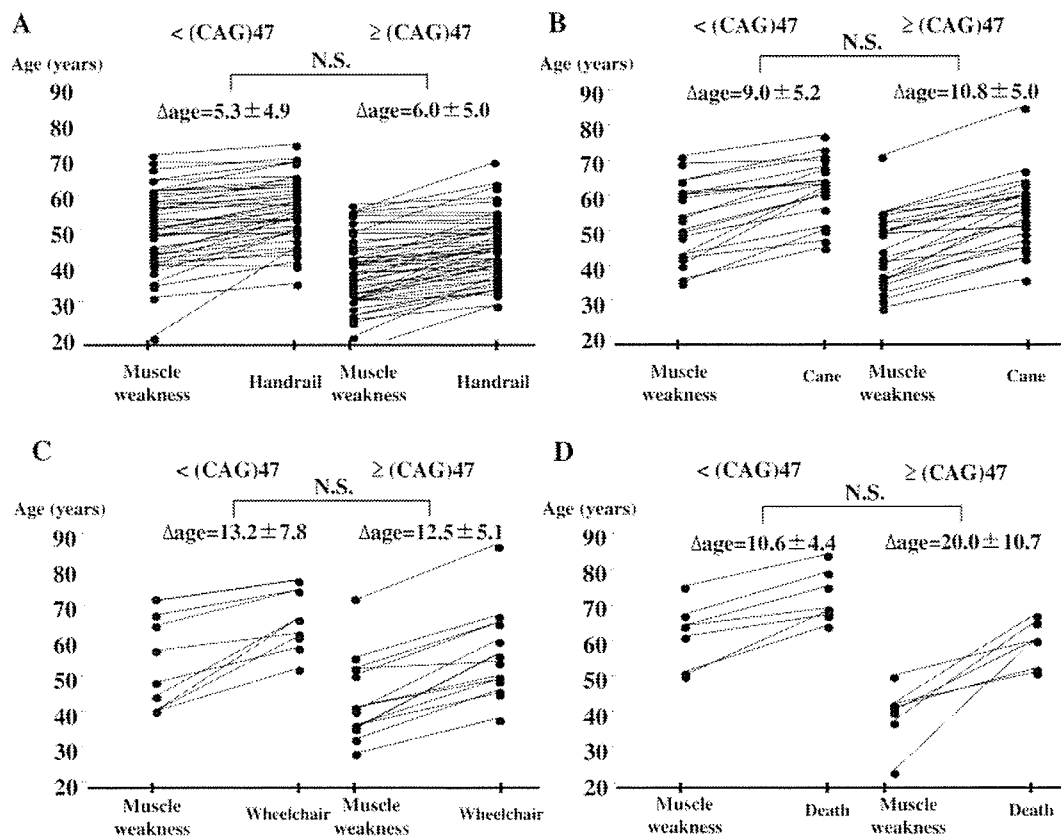


**Fig. 4 (A–D)** Correlation between the AR gene CAG number and the time interval between the ADL milestones. The time interval from the age at first awareness of muscular weakness to the age at requirement of a handrail, use of a cane, use of a wheelchair and death were compared with the CAG number by Pearson’s correlation coefficient. There were no significant correlations in any of the interval times.

**Discussion**

Our study elucidated the natural history of SBMA patients based on nine ADL milestones. SBMA progressed slowly to the end stage with a median duration from onset assessed by

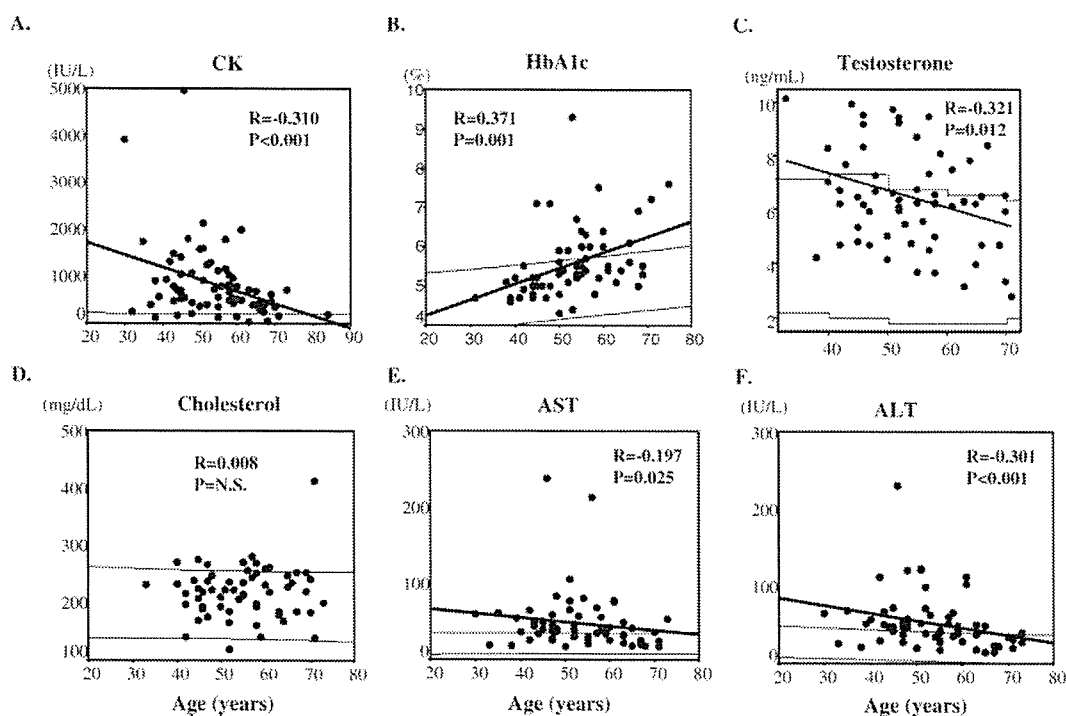
muscle weakness to the appearance of pneumonia of 16 years, and to death of 22 years whereas the median durations from age of onset to the age of requirement of a handrail, dysarthria and dysphagia were 5, 6 and 10 years, respectively,



**Fig. 5 (A–D)** Individual case presentation of the declining regression assessed by ADL milestones. The interval times from the age at first awareness of weakness to the age at requirement of a handrail, use of a cane, use of a wheelchair and death are described for individual patients from two groups, those with <47 CAG repeats and those with ≥47 repeats. These regression lines were divergent from each other, possibly owing to divergent CAG-repeat size, while the mean slopes of the regression lines were likely to be parallel among the two subgroups of patients. There were no significant differences between the interval times of the two groups of patients analysed by unpaired t-test.

indicating that the ADL deterioration leading to a decline in the quality of daily living during early phases of the diseases is significant, in spite of a relatively long lifespan. The lifespan of SBMA patients was previously speculated to be 10–15 years shorter than those of the general Japanese male population (Mukai, 1989). In this study, 15 of the 223 patients died at a median age of 65 years. Although there are too few data to make a reliable calculation, this is ~12 years shorter than that of the current lifespan of the normal Japanese male indicated by the abridged life table announced by the Japanese Ministry of Health, Labor and Welfare in 2003, and, thus, is consistent with the previous speculation (Mukai, 1989). Of these 15 patients, the most common cause of death was pneumonia due to aspiration and dysphagia. Thus, the bulbar symptoms, such as difficulty in proper articulation and mild dysphagia, were relatively mild in their early manifestations, but were serious symptoms in the late phase of the disease, when the patients were prone to death. The progression was apparently slower than that of ALS, another adult-onset motoneuron disease, which occasionally mimics SBMA phenotypes, particularly in the early phase (La Spada *et al.*, 1992; Parboosingh *et al.*, 1997; Traynor *et al.*, 2000).

The onset ages of each ADL milestone were extremely variable, but all were well correlated with the CAG-repeat size in the AR gene. Patients with longer CAG-repeat sizes showed an earlier onset age of each ADL milestone examined, including occurrence of pneumonia or death in the end stage. Several previous studies also documented the natural history of SBMA. They showed that the age of disease onset assessed by muscle weakness was strongly correlated with AR gene CAG-repeat size (Doyu *et al.*, 1992; Igarashi *et al.*, 1992; La Spada *et al.*, 1992; Shimada *et al.*, 1995), whereas the onset ages of other symptoms such as fatigue, tremor, occurrence of gynaecomastia and severity of muscle weakness were not significantly correlated with repeat size (La Spada *et al.*, 1992; Amato *et al.*, 1993; Mariotti *et al.*, 2000; Dejager *et al.*, 2002; Sperfeld *et al.*, 2002). It is not clear why the relations between the onset age of these symptoms and CAG repeat size were not apparent in these reports, since significant correlations with the onset age of hand tremor and muscular weakness were confirmed in the present study. One possibility may be the relatively small sample sizes in the previous studies (Amato *et al.*, 1993; Sperfeld *et al.*, 2002). An alternative explanation may be that very early symptoms, such as



**Fig. 6 (A–F)** Correlation between the levels of serum markers and the age at examination. A weak, but significant, correlation was seen between HbA1c and age, while weak, but significant, inverse correlations were seen between CK, testosterone, AST and ALT and age as analysed by Pearson's correlation coefficient. Cholesterol levels were not correlated with age. The thin lines in each plot indicate the 95% confidence intervals calculated from control subjects.

gynaecomastia and fatigue, are not very accurate or reliable markers for ADL milestones compared with later symptoms, especially in a retrospective study. Indeed, the correlation of the onset age of tremor with CAG-repeat size was weaker than that of the onset age of the use of a wheelchair or a cane, which were more advanced ADL milestones used in our study.

The relationship between CAG-repeat size, disease markers and rate of disease progression have also been assessed extensively in Huntington's disease (Illarioshkin *et al.*, 1994; Brandt *et al.*, 1996; Furtado *et al.*, 1996; Penney *et al.*, 1997; Rosenblatt *et al.*, 2003). Neuronal loss in the caudate nucleus and putamen, adjusted for age of death, correlated well with CAG-repeat length (Furtado *et al.*, 1996; Penney *et al.*, 1997; Rosenblatt *et al.*, 2003). The rate of progression assessed by symptom severity controlled by duration from onset (Illarioshkin *et al.*, 1994) also correlated strongly with the CAG-repeat size. In addition, we previously demonstrated that the extent of cerebellar atrophy and severity of muscle weakness, both adjusted by age at examination correlated well with CAG-repeat size in SCA3 and SBMA, respectively (Doyu *et al.*, 1992; Abe *et al.*, 1998). These observations suggested that longer CAG repeats resulted in an earlier age at onset and greater neuronal loss when compared with shorter repeats. There is also some evidence that they contribute to a faster rate of clinical decline.

In our present study, as documented in Figs 2 and 3, patients with longer CAG repeats reached each of the ADL

milestones such as hand tremor, muscle weakness or the requirement of a handrail when going up stairs much earlier than did the patients with shorter CAG repeats. Interestingly, however, the decline curves, as documented with Kaplan–Meier analyses, for these individual milestones were similar (Fig. 3), with an ~10-year difference between the patients with  $\geq 47$  repeats and those with  $< 47$  repeats. The earlier age at onset for each ADL milestone in patients with longer repeat lengths is similar to observations in cases of Huntington's disease.

The most striking observation in our study was that the interval periods between individual ADL milestones, such as between onset of muscle weakness and that of requirement of a handrail, use of a cane, being wheelchair-bound or death were not affected by the CAG-repeat length (Figs 4 and 5). Although patients with longer CAG-repeat size reached individual ADL milestones faster than those with shorter repeats, the decline rate from one ADL milestone to another was not influenced by the CAG-repeat size. These results suggest that the rate of disease progression assessed by ADL milestones is not influenced by CAG-repeat length.

Therefore, we may propose a view simulating the natural history of SBMA, in that, the decline curves of ADL in the SBMA patients with longer CAG-repeat size are shifted earlier than those in the SBMA patients with shorter CAG-repeat size, and the slopes of the decline curves are parallel to one another.

The phenotypic decline curves provided by mouse models of CAG-repeat diseases (Adachi *et al.*, 2003; von Horsten *et al.*, 2003) also support our view of the natural history of SBMA. The present findings are informative in understanding the pathophysiology of SBMA. CAG-repeat size is known to be a determinant factor for the entry of neuronal cells harbouring a mutant AR gene with expanded CAG repeats into the neuronal degeneration process *in vitro*, as well as *in vivo* (Mangiarini *et al.*, 1996). However, it is not known whether the rate of neuronal degeneration leading to subsequent cell death is dependent on CAG-repeat size. Once neuronal cell degeneration or neuronal cell dysfunction is initiated, the progression of degeneration to cell death may be determined by intrinsic factors such as a cell death processing system other than the CAG-repeat size. Thus, we suggest that the onset time of certain ADL milestones reflects how many neurons have entered into the neurodegeneration-dysfunction process, which is determined by CAG-repeat size, rather than the intrinsic cell death process.

Recently, we demonstrated that several interventions, anti-testosterone therapy with leuprorelin (Katsuno *et al.*, 2003), induction of Hsp70 (Adachi *et al.*, 2003; Katsuno *et al.*, 2005), inhibition of HDAC (Minamiyama *et al.*, 2004) or inhibition of Hsp90 (Waza *et al.*, 2005) showed potent therapeutic effects in improving the characteristic phenotypes and pathology in the SBMA transgenic mouse model. These observations strongly encourage the application of the therapeutics to human SBMA patients. Unlike the therapeutic approach commonly taken in neurodegenerative diseases of replacing lost substances such as neurotransmitters, these new therapeutics ameliorate the disease progression itself by preventing pathological molecular processes. Since the progression of SBMA is slow, clinical end-points will be useful for efficiently assessing the effectiveness of these therapies. The present study may indicate ADL milestones that can be clinical end-points in therapeutic trials. However, assessing the ADL milestones adopted in this study, such as the use of a cane or a wheelchair, would take years during clinical trials. Thus, we need to find a shorter-term surrogate marker that reflects the pathological process, although a genuine clinical therapeutic end-point should be examined to determine whether the ADL milestones are effectively delayed by the therapeutic intervention.

One interesting observation in this study is that serum testosterone levels were maintained at relatively high levels, even at advanced ages, although they did decrease with age (Fig. 6C). Since testosterone is an important triggering factor for polyglutamine-mediated motoneuron degeneration (Katsuno *et al.*, 2002, 2003), these findings suggest that anti-testosterone therapy with leuprorelin (Katsuno *et al.*, 2003) may be applicable even in aged SBMA patients.

The advantages of our study over previous studies are the large sample size and the employment of marked and apparent ADL milestones that the patients recognized easily. Nevertheless, several limitations are also present. One major limitation is that the study was retrospective in design

and the decline curve was not successively and prospectively assessed in individual patients. A prospective study that follows individual patients in assessing the ADL milestones is needed to ascertain the validity of this natural history of SBMA.

The ADL milestones that we adopted for this study were selected with the assumption that they could be accurately assessed by us, the patients or family members, even in a retrospective study. However, as we demonstrated, the development of pneumonia and death showed higher significant correlations with CAG-repeat size than did other earlier ADL milestones such as the appearance of hand tremor or dysarthria, suggesting that these critical end-stage events may be more accurately assessed in a retrospective manner. We need further long-standing prospective studies to assess the disease progression more properly.

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Review

# Pathogenesis, animal models and therapeutics in Spinal and bulbar muscular atrophy (SBMA)

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## Abstract

Spinal and bulbar muscular atrophy (SBMA) is a hereditary neurodegenerative disease characterized by slowly progressive muscle weakness and atrophy of bulbar, facial, and limb muscles. The cause of SBMA is expansion of a trinucleotide CAG repeat, which encodes the polyglutamine tract, in the first exon of the androgen receptor (AR) gene. SBMA chiefly occurs in adult males, whereas neurological symptoms are rarely detected in females having mutant AR gene. The cardinal histopathological finding of SBMA is loss of lower motor neurons in the anterior horn of spinal cord as well as in brainstem motor nuclei. Animal models carrying human mutant AR gene recapitulate polyglutamine-mediated motor neuron degeneration, providing clues to the pathogenesis of SBMA. There is increasing evidence that testosterone, the ligand of AR, plays a pivotal role in the pathogenesis of neurodegeneration in SBMA. The striking success of androgen deprivation therapy in SBMA mouse models has been translated into clinical trials. In addition, elucidation of pathophysiology using animal models leads to emergence of candidate drugs to treat this devastating disease: HSP inducer, Hsp90 inhibitor, and histone deacetylase inhibitor. Utilizing biomarkers such as scrotal skin biopsy would improve efficacy of clinical trials to verify the results from animal studies. Advances in basic and clinical researches on SBMA are now paving the way for clinical application of potential therapeutics.

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*Keywords:* Spinal and bulbar muscular atrophy; Polyglutamine; Androgen receptor; Testosterone; Luteinizing hormone-releasing hormone analog; Heat shock protein; Geranylgeranylacetone; 17-Allylamino geldanamycin; Histone deacetylase inhibitor; Axonal transport

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## History and nomenclature

More than a hundred years have elapsed since the first description of spinal and bulbar muscular atrophy (SBMA) from Hiroshi Kawahara, who described the clinical and hereditary characteristics of two Japanese brothers with progressive bulbar palsy (Kawahara, 1897). This work was followed by several reports on similar cases with or without X-linked pattern of inheritance (Katsuno et al., 2004). SBMA is also known as Kennedy disease (KD), named after William R. Kennedy, whose study on 11 patients from 2 families depicted the clinical, genetical, and pathological features of this disorder (Kennedy et al., 1968). Other names for this disease are bulbospinal neuronopathy and bulbospinal muscular atrophy.

In 1991, the cause of SBMA was identified as the expansion of a trinucleotide CAG repeat in the androgen receptor (AR) gene (La Spada et al., 1991). This was the first discovery of polyglutamine-mediated neurodegenerative diseases, and subsequent studies using transgenic animal models opened the door to development of pathogenesis-based therapies for this devastating disease.

## Clinical features

SBMA exclusively affects adult males. The prevalence of this disease is estimated to be 1–2 per 100,000, whereas a considerable number of patients may have been misdiagnosed as other neuromuscular diseases including amyotrophic lateral sclerosis (Fischbeck, 1997). Patients of various ethnic backgrounds have been reported around the world.

Major symptoms of SBMA are weakness, atrophy, and fasciculations of bulbar, facial and limb muscles (Sperfeld et al., 2002; Katsuno et al., 2004). In extremities, involvement is usually predominant in proximal musculature. The onset of weakness is usually between 30 and 60 years but is often preceded by nonspecific symptoms such as postural tremor and muscle cramps. Although fasciculations in the extremities are rarely present at rest, they are easily induced when patients hold their arms horizontally or bend their legs while lying on their backs. These contraction fasciculations are especially noticeable in the face, neck, and tongue and are usually present in the early stage. Fatigability after exercise might also be accompanied. Bilateral facial and masseter muscle weakness, poor uvula and soft palatal movements, and atrophy of the tongue with fasciculations are often encountered. Speech has a nasal quality in most cases due to reduced velopharyngeal closure. Advanced cases often develop dysphagia, eventually resulting in aspiration or choking. Muscle tone is usually hypotonic, and no pyramidal signs are detected. Deep tendon reflex is diminished or absent with no pathological reflex. Sensory involvement is largely restricted to vibration sense which is affected distally in the legs. Cerebellar symptoms, dysautonomia, and cognitive impairment are absent. Patients occasionally demonstrate signs of androgen insensitivity such as gynecomastia, testicular atrophy, dyserection, and decreased fertility, some of which are detected before the onset of motor symptoms. Abdominal obesity is common, whereas male pattern baldness is rare in patients with SBMA.

Electromyogram shows neurogenic abnormalities, and distal motor latencies are often prolonged in nerve conduction study. Both sensory nerve action potential and sensory evoked potential are reduced or absent. Endocrinological examinations frequently reveal partial androgen resistance with elevated serum testosterone level. Serum creatine kinase level is elevated in the majority of patients. Hyperlipidemia, liver dysfunction, and glucose intolerance are also detected in some patients. Profound facial fasciculations, bulbar signs, gynecomastia, and sensory disturbance are the main clinical features distinguishing SBMA from other motor neuron diseases, although gene analysis is indispensable for diagnosis. Female patients are usually asymptomatic, but some express subclinical phenotypes including high amplitude motor unit potentials on electromyography (Sobue et al., 1993).

The progression of SBMA is usually slow, but a considerable number of patients need assistance to walk in their fifties or sixties. Life-threatening respiratory tract infection often occurs in the advanced stage of the disease, resulting in early death in some patients. No specific therapy for SBMA has been established. Testosterone has been used in some patients, although it has no effects on the progression of SBMA.

## Etiology

The cause of SBMA is expansion of a trinucleotide CAG repeat, which encodes the polyglutamine tract, in the first exon of the androgen receptor (AR) gene (La Spada et al., 1991). The CAG repeat within AR ranges in size from 9 to 36 in normal subjects but from 38 to 62 in SBMA patients. Expanded polyglutamine tracts have been found to cause several neurodegenerative diseases including SBMA, Huntington's disease, several forms of spinocerebellar ataxia, and dentatorubral-pallidoluy-sian atrophy (Gatchel and Zoghbi, 2005). These disorders, known as polyglutamine diseases, share salient clinical features including anticipation and somatic mosaicism, as well as selective neuronal and nonneuronal involvement despite widespread expression of the mutant gene. There is an inverse correlation between the CAG repeat size and the age at onset or the disease severity adjusted by the age at examination in SBMA as documented in other polyglutamine diseases (Doyu et al., 1992). These observations explicitly suggest that common mechanisms underlie the pathogenesis of polyglutamine diseases.

AR, the causative protein of SBMA, is an 110-kDa nuclear receptor which belongs to the steroid/thyroid hormone receptor family (Poletti, 2004). AR mediates the effects of androgens, testosterone, and dihydrotestosterone, through binding to an androgen response element in the target gene to regulate its expression. AR is essential for major androgen effects including normal male sexual differentiation and pubertal sexual development, although AR-independent nongenomic function of androgen has been reported. AR is expressed not only in primary and secondary sexual organs but also in nonreproductive organs including the kidney, skeletal muscle, adrenal gland, skin, and nervous system, suggesting its far-reaching influence on a variety of mammalian tissues. In the central nervous system, the expression level of AR is relatively high in spinal and brainstem



motor neurons, the same cells which are vulnerable in SBMA. The AR gene is located on chromosome Xq11–12. This 90-kb DNA contains eight exons coding for the functional domains specific to the nuclear receptor family. The first exon codes for the N-terminal transactivating domain. Exons 2 and 3 code for the DNA-binding domain, whereas exons 4 through 8 code for the ligand-binding domain. The N-terminal transactivating domain, in which a CAG trinucleotide repeat locates, possesses a major transactivation function maintained by interaction with general transcriptional coactivators such as c-AMP response element binding protein-binding protein (CBP), TAFIII30, and steroid receptor coactivator-1 (SRC-1). The CAG repeat beginning at codon 58 in the first exon of AR encodes polyglutamine tract. The length of this repeat is highly variable because of the slippage of DNA polymerase upon DNA replication. Whereas its abnormal elongation causes SBMA, the shorter CAG repeat is likely to increase the risk of prostate cancer (Clark et al., 2003). Transcriptional coactivators also possess glutamine-rich regions modulating protein–protein interaction with the N-terminal transactivating domain of AR.

The expansion of a polyglutamine tract in AR has been implicated in the pathogenesis of SBMA in two different, but not mutually exclusive, ways: loss of normal AR function induces neuronal degeneration; and the pathogenic AR acquires toxic property damaging motor neurons. Since AR possesses trophic effects on neuronal cells, one can assume that loss of AR function may play a role in the pathogenesis of SBMA. Expansion of the polyglutamine tract mildly suppresses the transcriptional activities of AR, probably because it disrupts interaction between the N-terminal transactivating domain of AR and transcriptional coactivators (Poletti, 2004). Although this loss of function of AR may contribute to the androgen insensitivity in SBMA, the pivotal cause of neurodegeneration in SBMA has been believed to be a gain of toxic function of the

pathogenic AR due to expansion of the polyglutamine tract. This hypothesis is supported by the observation that motor impairment has never been observed in severe testicular feminization (Tfm) patients lacking AR function or in AR knockout mice. Moreover, a transgenic mouse model carrying an elongated CAG repeat driven by human AR promoter demonstrated motor impairment, suggesting that the expanded polyglutamine tract is sufficient to induce the pathogenic process of SBMA (Adachi et al., 2001).

Aggregation of abnormal protein has been considered to be central to the pathogenesis of neurodegenerative diseases such as Alzheimer disease, Parkinson disease, amyotrophic lateral sclerosis, and prion disease. An expanded polyglutamine stretch alters conformation of causative proteins, resulting in aggregation of the proteins. It is now widely accepted that aggregation of these abnormal proteins in neurons is the primary event in the pathogenesis of polyglutamine diseases. The rate-limiting step of aggregation has been proposed to be the formation of oligomeric nucleus, which may occur from after a repeat length-dependent conformational change of polyglutamine monomer from a random coil to a parallel, helical  $\beta$ -sheet (Wyttenbach, 2004). Several experimental observations indicate that formation of toxic oligomers, or intermediates, of abnormal polyglutamine-containing protein instigates a series of cellular events which lead to neurodegeneration (Muchowski and Wacker, 2005). This hypothesis is likely to be the case in SBMA.

### Pathology

Histopathological studies provide important information on the pathogenesis of polyglutamine-mediated neurodegeneration. The fundamental histopathological finding of SBMA is loss of lower motor neurons in the anterior horn of spinal cord

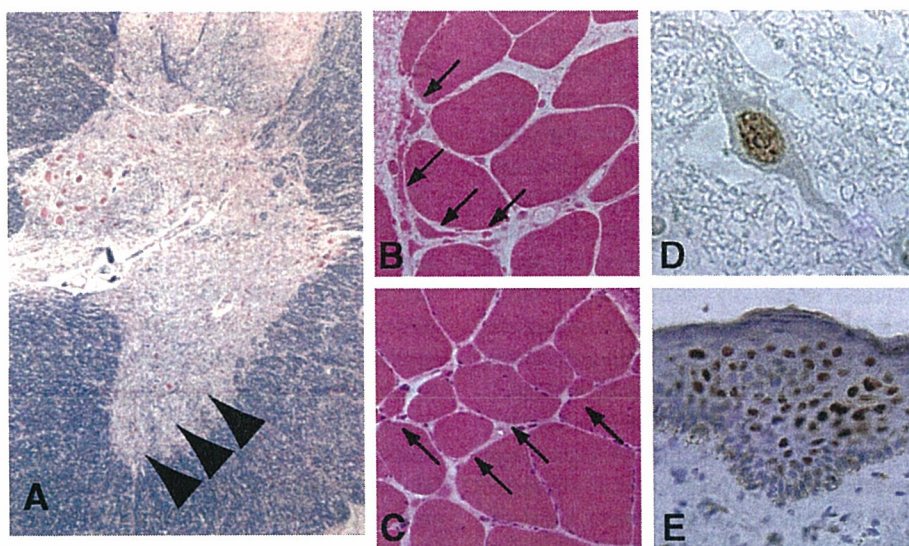


Fig. 1. Histopathology of SBMA. (A) A transverse section of spinal cord demonstrates marked depletion of motor neurons in the anterior horn. (B and C) HE staining of skeletal muscle shows both neurogenic (B, arrows) and myogenic changes (C, arrows). (D) A residual motor neuron in the lumbar anterior horn shows a diffuse nuclear accumulation of pathogenic androgen receptor detected by anti-polyglutamine antibody. (E) Nuclear accumulation of pathogenic AR is also detected in nonneuronal tissues such as scrotal skin (E).

as well as in brainstem motor nuclei except for the third, fourth and sixth cranial nerves (Fig. 1A) (Sobue et al., 1989). The number of nerve fibers is reduced in the ventral spinal nerve root, reflecting motor neuronopathy. Sensory neurons in the dorsal root ganglia were less severely affected, and large myelinated fibers demonstrate a distally accentuated sensory axonopathy in the peripheral nervous system. Neurons in the Onufrowicz nuclei, intermediolateral columns, and Clarke's columns of the spinal cord are generally well preserved. Muscle histopathology includes both neurogenic and myogenic findings: there are groups of atrophic fibers with a number of small angular fibers, fiber type grouping and clumps of pyknotic nuclei as well as variability in fiber size, hypertrophic fibers, scattered basophilic regenerating fibers, and central nuclei (Figs. 1B and C).

A pathologic hallmark of polyglutamine diseases is the presence of nuclear inclusions (NIs). In SBMA, NIs containing the pathogenic AR are found in the residual motor neurons in the brainstem and spinal cord as well as in nonneuronal tissues including prostate, testis, and skin (Li et al., 1998). These inclusions are detectable using antibodies recognizing a small portion of the N-terminus of the AR protein, but not by those against the C-terminus of the protein. This observation implies that the C-terminus of AR is truncated or masked upon formation of NI. A full-length AR protein with expanded polyglutamine tract is cleaved by caspase-3, liberating a polyglutamine-containing toxic fragment, and the susceptibility to cleavage is polyglutamine repeat length-dependent (Kobayashi et al., 1998). Thus, proteolytic cleavage is likely to enhance the toxicity of the pathogenic AR protein. Electron microscopic immunohistochemistry shows dense aggregates of AR-positive granular material without limiting membrane, both in the neural and nonneural inclusions, in contrast to the other polyglutamine diseases in which NIs take the form of filamentous structures. Although NI is a disease-specific histopathological finding, its role in pathogenesis has been heavily debated. Several studies have suggested that NI may indicate cellular response coping with the toxicity of abnormal polyglutamine protein (Arrasate et al., 2004). Instead, the diffuse nuclear accumulation of the mutant protein has been considered essential for inducing neurodegeneration in polyglutamine diseases including SBMA.

An immunohistochemical study on autopsied SBMA patients using an anti-polyglutamine antibody demonstrates that diffuse nuclear accumulation of the pathogenic AR is more frequently observed than NIs in the anterior horn of spinal cord (Adachi et al., 2005). Intriguingly, the frequency of diffuse nuclear accumulation of the pathogenic AR in spinal motor neurons strongly correlates with the length of the CAG repeat in the AR gene. No such correlation has been found between NI occurrence and the CAG repeat length. Similar findings have also been reported on other polyglutamine diseases. Taken together, it appears that the pathogenic AR containing an elongated polyglutamine tract principally accumulates within the nuclei of motor neurons in a diffusible form, leading to neuronal dysfunction and eventual cell death. In support of this hypothesis, neuronal dysfunction is halted by genetic modulation preventing nuclear import of the pathogenic polygluta-

mine-containing protein in cellular and animal models of polyglutamine diseases (Gatchel and Zoghbi, 2005).

Since human AR is widely expressed in various organs, nuclear accumulation of the pathogenic AR protein is detected not only in the central nervous system but also in nonneuronal tissues such as scrotal skin (Figs. 1D and E). The degree of pathogenic AR accumulation in scrotal skin epithelial cells tends to be correlated with that in the spinal motor neurons in autopsy specimens, and it is well correlated with CAG repeat length and inversely correlated with the motor functional scale (Banno et al., in press). These findings indicate that scrotal skin biopsy with anti-polyglutamine immunostaining is a potent biomarker with which to monitor SBMA pathogenic processes. Since SBMA is a slowly progressive disorder, appropriate biomarkers would help improve the power and cost effectiveness of longitudinal clinical treatment trials.

## Molecular pathogenesis and therapeutic strategies

### *Ligand-dependent pathogenesis in animal models of SBMA*

SBMA is unique among polyglutamine diseases in that the pathogenic protein, AR, has a specific ligand, testosterone, which alters the subcellular localization of the protein by favoring its nuclear uptake. AR is normally confined to a multi-heteromeric inactive complex in the cell cytoplasm and translocates into the nucleus in a ligand-dependent manner. This ligand-dependent intracellular trafficking of AR appears to play important roles in the pathogenesis of SBMA.

In order to investigate ligand effect in SBMA, we generated transgenic mice expressing the full-length human AR containing 24 or 97 CAGs under the control of a cytomegalovirus enhancer and a chicken  $\beta$ -actin promoter (Katsuno et al., 2002). This model recapitulated not only the neurologic disorder but also the phenotypic difference with gender which is a specific feature of SBMA. The mice with 97CAGs (AR-97Q) exhibited progressive motor impairment, although those with 24 CAGs did not show any manifested phenotypes. Affected AR-97Q mice demonstrated small body size, short life span, progressive muscle atrophy, and weakness as well as reduced cage activity, all of which were markedly pronounced and accelerated in the male AR-97Q mice, but either not observed or far less severe in the female AR-97Q mice. The onset of motor impairment detected by the rotarod task was at 8 to 9 weeks of age in the male AR-97Q mice while 16 weeks or more in the females. The 50% mortality ranged from 66 to 132 days of age in the male AR-97Q mice, whereas mortality of the female AR-97Q mice remained only 10 to 30% at more than 210 days. Western blot analysis revealed the transgenic AR protein smearing from the top of the gel in the spinal cord, cerebrum, heart, muscle, and pancreas. Although the male AR-97Q mice had more smearing protein than their female counterparts, the female AR-97Q mice had more monomeric AR protein. The nuclear fraction contained the most of smearing pathogenic AR protein. Diffuse nuclear staining and less frequent NIs detected by 1C2, an antibody specifically recognizing the expanded polyglutamine tract, were demonstrated in the neurons of spinal cord,

cerebrum, cerebellum, brainstem, and dorsal root ganglia as well as in nonneuronal tissues such as heart, muscle, and pancreas. Male AR-97Q mice showed markedly more abundant diffuse nuclear staining and NIs than females, in agreement with the symptomatic and Western blot profile differences with gender. Despite the profound sexual difference of the pathogenic AR protein expression, there was no significant difference in the expression of the transgene mRNA between the male and female AR-97Q mice. These observations indicate that the testosterone level plays important roles in the sexual difference of phenotypes, especially in the post-transcriptional stage of the pathogenic AR.

The dramatic sexual difference of phenotypes led us to hormonal interventions in our mouse model. First, we castrated male AR-97Q mice in order to decrease their testosterone level. Castrated male AR-97Q mice showed profound improvement of symptoms, histopathologic findings, and nuclear localization of the pathogenic AR compared with the sham-operated male AR-97Q mice. Body weight, motor function, and lifespan of male AR-97Q mice were significantly improved by castration. Western blot analysis and histopathology revealed diminished nuclear accumulation of the pathogenic AR in the castrated male AR-97Q mice. Next, we administered testosterone to the female AR-97Q mice. In contrast to castration of the male mice, testosterone caused significant aggravation of symptoms, histopathologic features, and nuclear localization of the pathogenic AR in the female AR-97Q mice. Since the nuclear translocation of AR is ligand-dependent, testosterone appears to show toxic effects in the female AR-97Q mice by accelerating nuclear translocation of the pathogenic AR. On the contrary, castration prevented the nuclear localization of the pathogenic AR by reducing the testosterone level. The nuclear accumulation of the pathogenic AR protein with an expanded polyglutamine tract is likely essential in inducing neuronal cell dysfunction and degeneration in the majority of polyglutamine diseases. It thus appears logical that reduction in testosterone level improves phenotypic expression by preventing nuclear localization of the pathogenic AR. In support of this hypothesis, the ligand-dependent neurodegeneration has also been revealed in a fruit fly model of SBMA (Takeyama et al., 2002). Alternatively, castration may enhance protective effects of molecular chaperones, which are normally associated with AR and dissociate upon ligand binding.

#### *Testosterone blockade therapy for SBMA*

Successful treatment of AR-97Q mice with castration inspired us testosterone blockade therapies using leuporelin and flutamide (Katsuno et al., 2003). Leuporelin is a potent luteinizing hormone-releasing hormone (LHRH) analog suppressing the releases of gonadotrophins, luteinizing hormone and follicle-stimulating hormone. This drug has been used for a variety of sex hormone-dependent diseases including prostate cancer, endometriosis, and prepuberty. The primary pharmacological target of leuporelin is the anterior pituitary. Through its agonizing effect on LHRH-releasing cells, it initially promotes the releases of gonadotrophins, resulting in transient increase in

the serum level of testosterone or estrogens. After this surge, the continued use of this drug induces desensitization of the pituitary by reducing LHRH receptor binding sites and/or uncoupling of receptors from intracellular processes. Within about 2 to 4 weeks of leuporelin administration, human serum testosterone level decreases to the extent achieved by surgical castration. The effects are maintained during the treatment, suggesting that continuous administration of leuporelin is required for its clinical use. This drug thus has been provided as sustained release depot taking the form of polymer microspheres. On the other hand, flutamide, the first discovered androgen antagonist, has highly specific affinity for AR and competes with testosterone for binding to the receptor. It has been used for the treatment of prostate cancer, usually in association with an LHRH analog, in order to block the action of adrenal testosterone. Although flutamide suppresses the androgen-dependent transactivation, it does not reduce the plasma levels of testosterone.

Leuporelin successfully inhibited nuclear accumulation of the pathogenic AR, resulting in marked amelioration of neuromuscular phenotypes seen in the male AR-97Q mice (Fig. 2). Leuporelin initially increased the serum testosterone level by agonizing the LHRH receptor but subsequently reduced it to undetectable levels. Androgen blockade effects were also confirmed by reduced weights of the prostate and seminal vesicle. The leuporelin-treated AR-97Q mice showed longer lifespan, larger body size, and better motor performance compared with vehicle-treated mice. Although leuporelin-induced infertility was prevented by dose reduction, the therapeutic effects on neuromuscular phenotypes were insufficient at a lower dose of leuporelin. In the Western blot analysis and anti-polyglutamine immunohistochemistry, the leuporelin-treated male AR-97Q mice demonstrated a markedly diminished amount of the pathogenic AR in the nucleus, suggesting that leuporelin successfully reduced nuclear AR accumulation. Testosterone, which was given from 13 weeks of age, markedly aggravated neurological symptoms and pathologic findings of leuporelin-treated male AR-97Q mice. Leuporelin appears to improve neuronal dysfunction by preventing ligand-dependent nuclear translocation of the pathogenic AR in the same way as castration. Given its minimal invasiveness and established safety, leuporelin appears to be a promising therapeutic agent for SBMA. In a preliminary open trial, 6-month treatment with leuporelin significantly diminished nuclear accumulation of pathogenic AR in the scrotal skin of patients, suggesting that androgen deprivation intervenes in the pathogenic process of human SBMA, as demonstrated in animal studies (Banno et al., in press). Another trial on a larger scale is currently underway to verify clinical benefits of leuporelin for SBMA patients.

Leuporelin-treated AR-97Q mice showed deterioration of body weight and rotarod task at the age of 8–9 weeks, when serum testosterone initially increased through the agonistic effect of leuporelin. This change was transient and followed by sustained amelioration along with consequent suppression of testosterone production. The foot print analysis also revealed temporary exacerbation of motor impairment. Immunostaining of tail specimen, sampled from the same individual mouse,



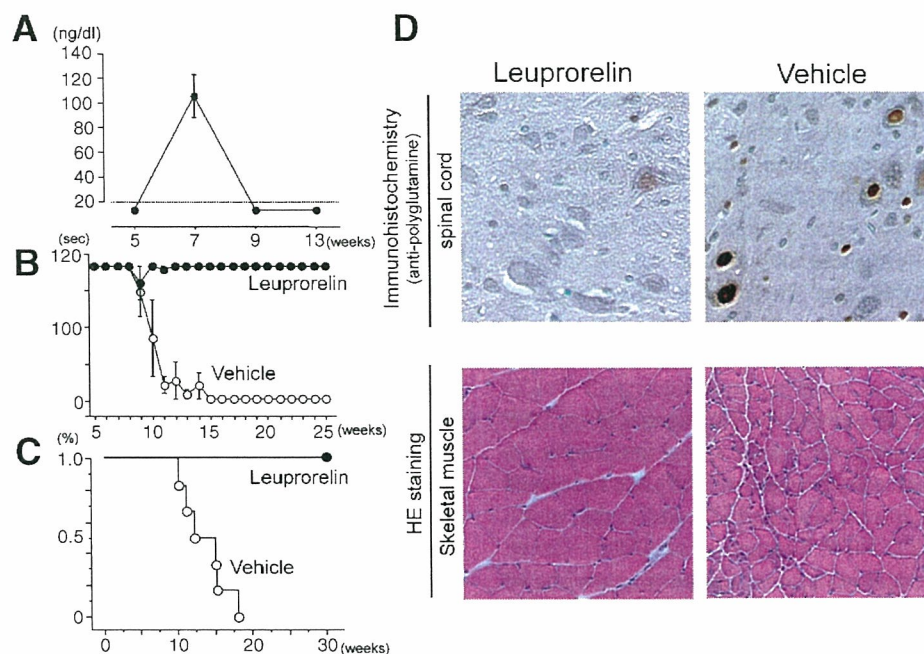


Fig. 2. Effects of leuporelin on mutant androgen receptor (AR) expression and neuropathology of male AR-97Q mice. (A) Serum testosterone level in AR-97Q mice. Leuporelin initially increased serum testosterone level but subsequently reduced it to undetectable levels. (B and C) Rotarod task (B) and survival rate (C) of the AR-97Q mice. Leuporelin markedly improved motor function of the mice at the dose. (D) Immunohistochemistry using 1C2 showed marked differences in diffuse nuclear staining and nuclear inclusions between the leuporelin-treated and vehicle-treated AR-97Q male mice in the spinal anterior horn. HE staining of the muscle in the vehicle-treated male mouse revealed apparent grouped atrophy and small angulated fibers, which were not seen in the leuporelin-treated mice.

demonstrated an increase in the number of the muscle fibers with nuclear 1C2 staining at 4 weeks of leuporelin administration, although this 1C2 staining was diminished by another 4 weeks of treatment. Our results indicate that preventing nuclear translocation of the pathogenic AR is enough to reverse both symptomatic and pathologic phenotypes in our AR-97Q mice. In support with these observations, testosterone deprivation by means of castration reverses motor dysfunction in another transgenic mouse model of SBMA showing fairly slow progression (Chevalier-Larsen et al., 2004). Since the pathophysiology of AR-97Q mice is neuronal dysfunction without neuronal cell loss, our results indicate that the pathogenesis of polyglutamine diseases is reversible at least in its initial stage. From therapeutic point of view, it is of importance to determine the early dysfunctional period in the natural history of human SBMA.

By contrast, flutamide, AR antagonist, did not ameliorate symptoms, pathologic features, or nuclear localization of the pathogenic AR in the male AR-97Q mice, although there was no significant difference in the androgen blockade effects between flutamide and leuporelin. Flutamide does not inhibit, but may even facilitate, the nuclear translocation of AR. In consistency with mouse study, this AR antagonist also promotes nuclear translocation of the pathogenic AR containing an expanded polyglutamine in cellular and fly models of SBMA (Takeyama et al., 2002; Walcott and Merry, 2002). This appears to be the reason why flutamide demonstrated no therapeutic effect in our transgenic mouse model of SBMA. Flutamide is not likely to be a proper therapeutic agent for SBMA.

The castrated or leuporelin-treated AR-97Q mice showed phenotypes similar to those seen in the female AR-97Q mice,

implying that motor impairment of SBMA patients can be reduced to the level in females. SBMA has been considered an X-linked disease, whereas other polyglutamine diseases show autosomal dominant inheritance. In fact, SBMA female patients hardly manifest clinical phenotypes, although they possess similar number of a CAG repeat in the disease allele of AR gene as their siblings with SBMA (Sobue et al., 1993; Mariotti et al., 2000). Reduction in the mutant AR expression due to X-inactivation may prevent females from disease manifestation, but hormonal intervention studies using mouse and fly models clearly suggest that low level of testosterone prevents nuclear accumulation of the pathogenic AR protein, resulting in a lack of neurological phenotypes in the females. This view is strongly supported by the observation that manifestation of symptoms is minimal even in homozygous females of SBMA (Schmidt et al., 2002). Therefore, it seems inappropriate to regard SBMA as an X-recessive inherited disease, but rather its neurological phenotype is likely to depend on testosterone concentration.

#### *Role of heat shock proteins in pathogenesis of SBMA*

Many components of ubiquitin-proteasome and molecular chaperones are known to colocalize with polyglutamine-containing NIs, implying that failure of cellular defense mechanism underlies neurodegeneration in polyglutamine diseases. Heat shock protein (HSP), a stress-inducible molecular chaperone, is another key to elucidation of the pathogenesis of SBMA. HSPs are classified into different families according to molecular size: Hsp100, Hsp90, Hsp70, Hsp60, Hsp40, and small HSPs (Macario and Conway de Macario, 2005). These HSPs are either constitutively expressed or inducibly

synthesized after cellular stress. HSPs play a crucial role in maintaining correct folding, assembly, and intracellular transport of proteins. For example, Hsp70 and Hsp90, essential components of AR-chaperone complex in the cell cytoplasm, regulate function, nuclear translocation, and degradation of AR (Heinlein and Chang, 2001). Under toxic conditions, HSP synthesis is rapidly upregulated, and nonnative proteins are refolded as a consequence. Therefore, HSPs have attracted a great deal of attention as cytoprotective agents against detrimental conditions including ischemia and malignancy.

Several studies suggest that polyglutamine elongation interferes with the protective cellular responses against cytotoxic stress (Wytenbach, 2004). Truncated AR with an expanded polyglutamine tract delays the induction of Hsp70 after heat shock (Cowan et al., 2003). The threshold of HSP induction is known to be relatively high in spinal motor neurons (Batulan et al., 2003). Expression levels of HSPs are decreased in the brain lesion of an animal model of HD and in that of the SBMA mouse (Hay et al., 2004; Katsuno et al., 2005). Taken together, impairment of HSP induction capability is implicated in the pathogenesis of motor neuron degeneration in SBMA. Not only are HSPs implicated in the pathogenesis of neurodegeneration, but they are also potent suppressors of polyglutamine toxicity. There is increasing evidence that HSPs abrogate polyglutamine-mediated cytotoxicity by refolding and solubilizing the pathogenic proteins (Wytenbach, 2004; Muchowski and Wacker, 2005). Hsp70 cooperates with Hsp40 in functioning as a molecular chaperone. These HSPs are proposed to prevent the initial conformation conversion of abnormal polyglutamine-containing protein from a random coil to a  $\beta$ -sheet, leading to attenuation of toxic oligomer formation (Wytenbach, 2004). Overexpression of Hsp70, together with Hsp40, inhibits toxic accumulation of abnormal polyglutamine-containing protein and suppresses cell death in a variety of cellular models of polyglutamine diseases including SBMA (Kobayashi et al., 2000). Hsp70 has also been shown to facilitate proteasomal degradation of abnormal AR protein in a cell culture model of SBMA (Bailey et al., 2002). The favorable effects of Hsp70 have been verified in studies using mouse models of polyglutamine diseases. Overexpression of the inducible form of human Hsp70 markedly ameliorated symptomatic and histopathological phenotypes of our transgenic mouse model of SBMA (Adachi et al., 2003). These beneficial effects are dependent on Hsp70 gene dosage and correlate with the reduction in the amount of nuclear-localized AR protein. It should be noted that the soluble form of the pathogenic AR was also significantly decreased in amount by Hsp70 overexpression, suggesting the degradation of the pathogenic AR may have been accelerated by overexpression of this molecular chaperone.

Favorable effects obtained by genetic modulation of HSP suggest that pharmacological induction of molecular chaperones might be a promising approach to SBMA and other polyglutamine diseases. Geranylgeranylacetone (GGA), an acyclic isoprenoid compound with a retinoid skeleton, has been shown to strongly induce HSP expression in various tissues (Hirakawa et al., 1996). Oral administration of GGA

upregulates the levels of Hsp70, Hsp90, and Hsp105 via activation of heat shock factor-1 in the central nervous system and inhibits nuclear accumulation of the pathogenic AR protein, resulting in amelioration of polyglutamine-dependent neuromuscular phenotypes of SBMA transgenic mice (Katsuno et al., 2005). Given its extremely low toxicity, this compound has been used as an oral anti-ulcer drug. Although a high dose appears to be needed for clinical effects, GGA appears to be a safe and promising therapeutic candidate for polyglutamine-mediated neurodegenerative diseases including SBMA.

Inhibition of Hsp90 is also demonstrated to arrest the neurodegeneration in SBMA mouse (Waza et al., 2005). Hsp90 functions in a multi-chaperone complex, assisting proper folding, stabilization, and assembly of so-called client proteins including various oncoproteins and AR (Pratt and Toft, 2003). The Hsp90-client protein complex is stabilized when it is associated with p23, a cochaperone interacting with Hsp90. Treatment with 17-allylamino geldanamycin (17-AAG), a potent Hsp90 inhibitor, dissociated p23 from the Hsp90-AR complex, and thus facilitated proteasomal degradation of the pathogenic AR in cellular and mouse models of SBMA. 17-AAG thereby inhibits nuclear accumulation of this protein, leading to marked amelioration of motor phenotypes of the SBMA mouse model without detectable toxicity (Fig. 3). Of interest is the finding that the pathogenic AR is preferentially targeted to proteasomal degradation in the presence of 17-AAG compared with wild-type AR. Given a high association between p23 and the AR containing expanded polyglutamine, it appears logical that the pathogenic AR is more dependent on Hsp90 to maintain folding and function than wild-type AR and thus is particularly susceptible to Hsp90 inhibition. 17-AAG is also capable of inducing Hsp70 in cellular and mouse models of SBMA. Collectively, 17-AAG, which is now under clinical trials for a wide range of malignancies, would be a good candidate for treatment of SBMA.

#### *Transcriptional dysregulation in SBMA*

Disruption of transcriptional machinery has also been hypothesized to underlie the pathogenesis of polyglutamine diseases (Sugars and Rubinsztein, 2003). Gene expression analysis indicates that transcriptional disruption is an early change in the pathogenesis of mouse models of polyglutamine diseases. Transcriptional coactivators such as CBP are sequestered into the polyglutamine-containing NIs through protein–protein interaction in mouse models and patients with polyQ diseases (Nucifora et al., 2001). Alternatively, the interaction between transcriptional coactivators and soluble pathogenic protein has also been demonstrated in animal models of polyglutamine diseases as well as in postmortem tissues of patients (Steffan et al., 2001). The expression of genes regulated through CBP-mediated transcription is decreased in mouse models of polyglutamine diseases (Sugars and Rubinsztein, 2003). CBP functions as histone acetyltransferase, regulating gene transcription and chromatin structure. It has been indicated that the histone acetyltransferase activity of CBP is suppressed in cellular models of polyglutamine diseases. Taken together, transcriptional dysregulation due to decrease in histone acetylation is likely to



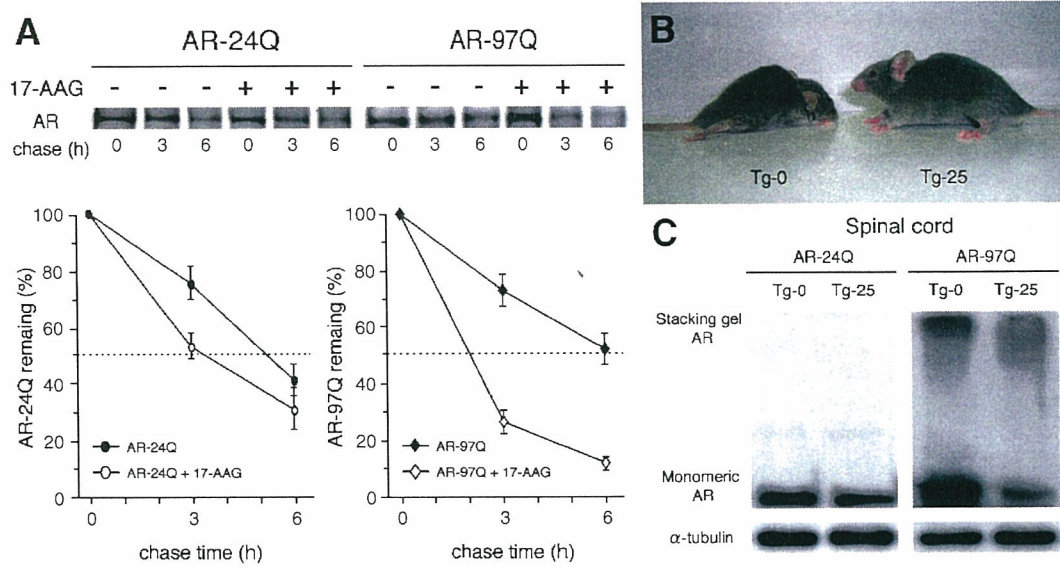


Fig. 3. Effects of 17-AAG on mutant androgen receptor (AR) turnover. (A) Pulse-chase analysis of two forms of AR. The pathogenic AR containing prolonged polyglutamine is degraded more rapidly than the wild-type AR in the presence of 17-AAG. (B) Muscle wasting is obvious in a vehicle-treated SBMA mouse (Tg-0), whereas it is hardly detected in an age-matched SBMA mouse treated with 25 mg/kg of 17-AAG (Tg-25). (C) Western blot analysis of total homogenates from the spinal cord of transgenic mice probed with an anti-AR antibody. 17-AAG decreases the amount of AR in mice bearing the pathogenic AR (AR-97Q), but this effect is only slightly observed in those expressing wild-type AR (AR-24Q).

underlie the pathogenesis of neurodegeneration in polyglutamine diseases. This hypothesis is exemplified by our experimental observation that acetylation of nuclear histone H3 is significantly diminished in SBMA mice (Minamiyama et al., 2004). Additionally, dysfunction of CBP results in a decreased expression of vascular endothelial growth factor in another mouse model of

SBMA, indicating the transcriptional alteration is a trigger of neurodegeneration in this disease (Sopher et al., 2004).

Histone acetylation level is determined by interplay between histone acetyltransferase and histone deacetylase (HDAC). Recruitment of HDAC to target genes represses transcription, leading to aberrant cellular function. Since cancellation of

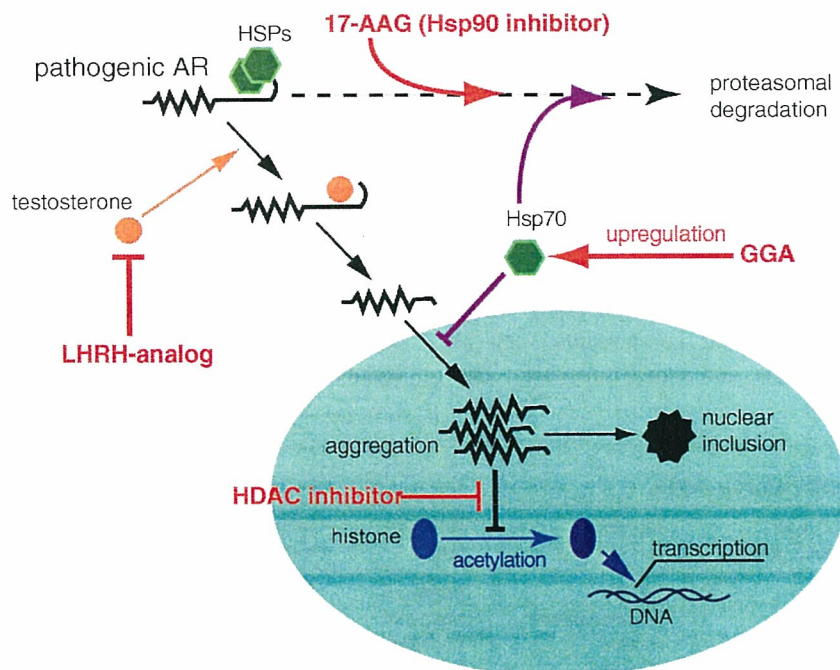


Fig. 4. Pathogenesis-targeting therapeutic approaches to SBMA. In the absence of ligand, the pathogenic AR is confined to a multi-heteromeric inactive complex with heat shock proteins (HSPs) in the cell cytoplasm. Ligand binding facilitates its dissociation from this complex and translocation into the nucleus. In the nucleus, the pathogenic AR forms aggregate and impairs histone acetylation, resulting in transcriptional dysregulation. Several candidates of therapies have been identified on the basis of insights into the molecular mechanisms of the neurodegeneration in SBMA.

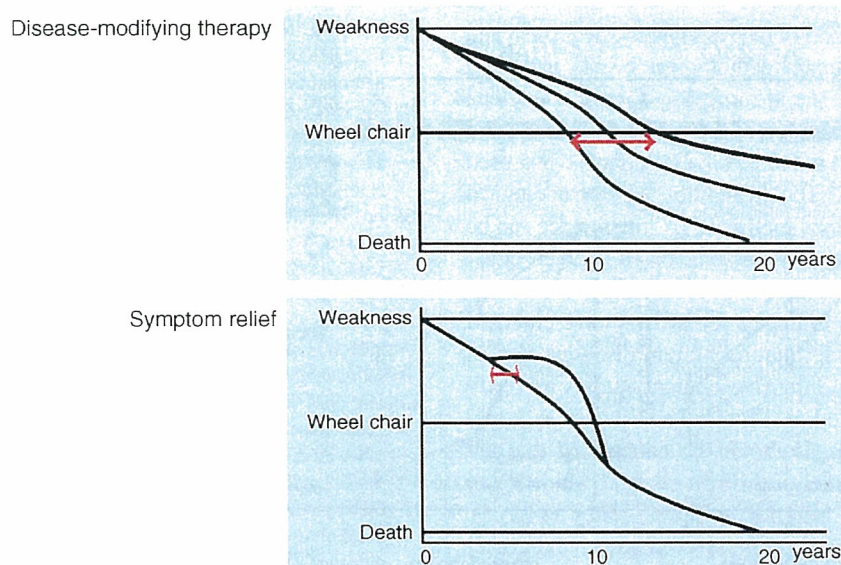


Fig. 5. Disease-modifying therapy and symptom relief. Since the goal of disease-modifying therapy is to inhibit pathogenic progression, long-term trials need to be carried out in order to evaluate drug effects by targeting certain clinical events as the primary endpoint. On the other hand, symptom relief, such as replacement of neurotransmitter, is used to ameliorate symptoms resulting from neurodegeneration. Although trial duration tends to be short, the effect of this therapy is often transient. Arrows indicate trial duration required for each therapy.

HDAC activity results in augmentation of histone acetylation and subsequent restoration of gene transcription, HDAC inhibitors have been considered to be of therapeutic benefit in polyglutamine diseases (Steffan et al., 2001, Hockly et al., 2003). Butyrate is the first HDAC inhibitor to be discovered, and the related compound, phenylbutyrate, has been successfully employed in experimental cancer therapy. Oral administration of sodium butyrate ameliorates symptomatic and histopathological phenotypes of our mouse model of SBMA through upregulation of histone acetylation in nervous tissues (Minamiyama et al., 2004). Although sodium butyrate is likely to be a promising treatment of SBMA, this compound yielded beneficial effects only within a narrow therapeutic window of dosage in the mouse model. Careful dose determination is mandatory when using HDAC inhibitors for treatment of polyglutamine diseases.

#### *Axonal trafficking defects in SBMA*

Motor neurons possess extremely long axon along which molecular motors transport essential components such as organelles, vesicles, cytoskeletons, and signal molecules. This

implies that axonal trafficking plays a fundamental role in maintenance of normal function of motor neurons. Obstruction of axonal transport has gained attention as a cause of neuronal dysfunction in a variety of neurodegenerative diseases including SBMA (Gunawardena and Goldstein, 2005). A mutation in the gene of proteins regulating axonal trafficking, dynein and dynactin 1, has been shown to cause motor neuron degeneration in both human and rodent (Puls et al., 2003; Hafezparast et al., 2003). Experimental data suggest that axonal transport might be retarded by pathogenic polyglutamine-containing AR (Szebenyi et al., 2003). Although this notion is intriguing, its contribution to the pathogenesis of SBMA should be further investigated, since aggregation of the pathogenic AR is rarely found within the axon of motor neurons in patients or model mice.

#### **Clinical application of potential therapeutics**

Analysis of cellular and animal models provides insight into mechanisms involved in neurodegeneration of SBMA and indicates retinal therapeutic approaches to this disease (Fig. 4). Therapeutic agent candidates for SBMA are grossly classified into two groups:

Table 1  
Summary of therapeutic trials in SBMA mouse models

Treatment	Number of mice (per treatment group)	Increase in survival (%)	Rotarod task	Body weight	AR accumulation	Reference
Castration	6	>120%	Improved	Improved	Decreased	Katsuno et al. (2002)
Castration <sup>a</sup>	8–9	Not determined	Improved	No effect	Decreased	Chevalier-Larsen et al. (2004)
Leuprorelin	6	>130%	Improved	Improved	Decreased	Katsuno et al. (2003)
Flutamide	6	No effect	No effect	No effect	No effect	Katsuno et al. (2003)
Sodium butyrate	12–15	56%	Improved	Improved	No effect	Minamiyama et al. (2004)
17-AAG	27	>60%	Improved	Improved	Decreased	Waza et al. (2005)
GGA	12–15	>60%	Improved	Improved	Decreased	Katsuno et al. (2005)

<sup>a</sup> Treatment is initiated after the onset of motor impairment. AR, androgen receptor; 17-AAG, 17-allylamino geldanamycin; GGA, geranylgeranylacetone.



(i) drugs inhibiting accumulation of the pathogenic AR protein and (ii) drugs mitigating downstream pathological events including transcriptional dysregulation. The ideal therapy for polyglutamine diseases appears to be a combination of these potential therapeutic strategies, since each drug has potential adverse effects when used in a long term (Agrawal et al., 2005). In addition to pharmacological approaches, genetic interventions such as RNA interference can be applied if safety and delivery problems are solved (Caplen et al., 2002).

Since various therapeutic strategies for SBMA have emerged thanks to animal models recapitulating human diseases, it is of utmost importance to pursue intensive clinical studies to verify the results from animal studies (Table 1). When we apply candidate agents for patients, it should be considered that the majority of therapeutics emerging from animal studies are disease-modifying therapy, but not symptom-relief (Fig. 5). Given that SBMA is a slowly progressive disease, extremely long-term clinical trials are likely necessary to verify clinical benefits of disease-modifying therapies by targeting clinical endpoints such as occurrence of aspiration pneumonia or becoming wheelchair-bound. Suitable surrogate endpoints, which reflect the pathogenesis and severity of SBMA, are thus substantial to assess the therapeutic efficacy in drug trials. To this end, appropriate biomarkers should be identified and validated in translational researches.

## Acknowledgments

Fig. 2 is reproduced from Katsuno et al., “Leuprorelin rescues polyglutamine-dependent phenotypes in a transgenic mouse model of spinal and bulbar muscular atrophy (SBMA)” *Nat. Med.* 9: 768–773, 2003. Fig. 3 is reproduced from Waza et al., “17-AAG, an Hsp90 inhibitor, ameliorates polyglutamine-mediated motor neuron degeneration” *Nat. Med.* 11: 1088–1095, 2005. This work was supported by a Center-of-Excellence (COE) grant from the Ministry of Education, Culture, Sports, Science and Technology, Japan, and grants from the Ministry of Health, Labour and Welfare, Japan.

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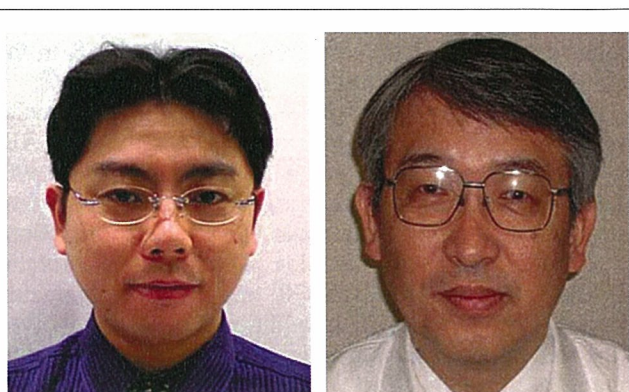
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## Modulation of Hsp90 function in neurodegenerative disorders: a molecular-targeted therapy against disease-causing protein

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**Abstract** Abnormal accumulation of disease-causing protein is a commonly observed characteristic in chronic neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease, and polyglutamine (polyQ) diseases. A therapeutic approach that could selectively eliminate would be a promising remedy for neurodegenerative disorders. Spinal and bulbar muscular atrophy (SBMA), one of the polyQ diseases, is a late-onset motor neuron disease characterized by proximal muscle atrophy, weakness, contraction fasciculations, and bulbar involvement. The pathogenic gene product is polyQ-expanded androgen receptor (AR), which belongs to the heat shock protein (Hsp) 90 client protein family. 17-Allylamino-17-demethoxygeldanamycin (17-AAG), a novel Hsp90 inhibitor, is a new derivative of geldanamycin that shares its important biological activities but shows less toxicity. 17-AAG is now in phase II clinical trials as a potential anti-cancer agent because of its ability to selectively degrade several oncoproteins. We have recently demonstrated the efficacy and safety of 17-AAG in a mouse model of SBMA. The administration of 17-AAG significantly ameliorated polyQ-mediated motor neuron degeneration by reducing the total amount of mutant AR. 17-AAG accomplished the preferential reduction of mutant AR mainly through Hsp90 chaperone complex formation and subsequent proteasome-dependent degradation. 17-AAG induced Hsp70 and Hsp40 in vivo as previously reported; however, its ability to induce HSPs was limited, suggesting that the HSP induction might support the degradation of mutant protein. The ability of 17-AAG to preferentially



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degrade mutant protein would be directly applicable to SBMA and other neurodegenerative diseases in which the disease-causing proteins also belong to the Hsp90 client protein family. Our proposed therapeutic approach, modulation of Hsp90 function by 17-AAG treatment, has emerged as a candidate for molecular-targeted therapies for neurodegenerative diseases. This review will consider our research findings and discuss the possibility of a clinical application of 17-AAG to SBMA and other neurodegenerative diseases.

**Keywords** Hsp90 inhibitor · Hsp90–client protein complex · Proteasomal degradation · Polyglutamine · Neurodegenerative diseases

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## Introduction

Polyglutamine (polyQ) diseases are caused by the expansion of a trinucleotide CAG repeat encoding glutamine in the causative genes and, to date, nine disorders have been identified as polyQ diseases [1]. Spinal and bulbar muscular atrophy (SBMA), also known as Kennedy's disease, was the first polyQ disease to be identified [2] and is characterized by premature muscular exhaustion, slow progressive muscular weakness, atrophy, and fasciculation in bulbar and limb muscles [3]. In SBMA, the pathogenic gene product is the androgen receptor (AR), which contains an abnormally expanded polyQ. The number of polymorphic CAG repeats in the *AR* gene is normally 14 to 32, but it is expanded to 40 to 62 CAGs in SBMA patients [4]. A correlation exists between the number of CAG repeats and disease severity [5]. The pathologic features of SBMA are motor neuron loss in the spinal cord and brainstem [3], and diffuse nuclear accumulations and nuclear inclusions (NIs) containing the mutant AR in the residual motor neurons and certain visceral organs [6].

Heat shock protein (Hsp) 90, one of the molecular chaperones, is essential for function and stability of the AR, the C-terminus of which has a high affinity for Hsp90, inducing the conformational change required for its nuclear translocation after ligand activation [7–9]. Hsp90 functions in a multi-component complex of chaperone proteins including Hsp70, Hop (Hsp70 and Hsp90 organizing protein), Cdc37, and p23. In addition, Hsp90 is involved in the folding, activation, and assembly of several proteins, known as Hsp90 client proteins [10]. As numerous oncoproteins belonging to the Hsp90 client protein family are selectively degraded in the ubiquitin–proteasome system (UPS) by Hsp90 inhibitors, 17-allylamino-17-demethoxygeldanamycin (17-AAG), a first-in-class Hsp90 inhibitor, is now under clinical trials as a novel molecular-targeted agent for a wide range of malignancies [11]. AR also belongs to the Hsp90 client protein family and is degraded in the presence of Hsp90 inhibitors [12–14].

In view of this ability of Hsp90 inhibitors to degrade Hsp90 client proteins, we have recently demonstrated that 17-AAG markedly ameliorated polyQ-mediated motor neuron degeneration through degradation of mutant AR [15]. This is apparently different from previous strategies employed against polyQ diseases, which unavoidably allowed abnormal protein to remain and placed much value mainly on the inhibition of protein aggregation. We consider that the ability to facilitate degradation of disease-causing protein by modulation of Hsp90 function would be of value when applied to SBMA and other related neurodegenerative diseases. In this paper, we review our research findings compared with previous studies and discuss the clinical application of Hsp90 inhibitors to neurodegenerative diseases.

## Development of Hsp90 inhibitors

The most classical Hsp90 inhibitor is geldanamycin (GA), a natural product that was developed as an antifungal agent [16]. Later, GA was also found to have a potent and selective anti-tumor effect against a wide range of malignancies [17]. Although GA showed potential as a novel anti-cancer agent [18], this agent was also found to have intolerable liver toxicity [19]. To overcome this GA-induced liver toxicity, scientists at the US National Cancer Institute succeeded in developing a new derivative of GA, 17-AAG, that shares its important biological activities [20] but shows less toxicity [21]. Owing to this promising derivative 17-AAG, Hsp90 inhibitors have taken a major developmental leap in their clinical applications, and 17-AAG is now in phase clinical trials with encouraging results as an anti-cancer agent [22–26]. To generate more selective and less toxic derivatives than 17-AAG, further development of Hsp90 inhibitors is also being pursued [27–29].

The anti-tumor effect of Hsp90 inhibitors was previously thought to be due to the inhibition of tyrosine kinase [30]. The mechanism subsequently proved to be based on their ability to specifically bind to the Hsp90 ATP-binding site, thereby modulating Hsp90 function [31, 32] and proteasomal degradation of Hsp90 client proteins. As numerous oncoproteins were shown to belong to the family of Hsp90 client proteins [10]. Hsp90 inhibitors are expected to become a new strategy in anti-tumor therapy [18]. Hsp90 inhibitors including GA and 17-AAG have been shown to have an advantageously higher selectivity for tumor cells compared with general anti-tumor agents [17]. Studies by Kamal et al. suggest a mechanism for this selectivity; Hsp90 in tumor cells is more likely to be incorporated in the Hsp90 multi-chaperone complex than the Hsp90 in normal cells is, thereby increasing their binding affinity to 17-AAG by more than 100-fold [33, 34].

We thought that this selectivity of Hsp90 inhibitors would also be advantageous for the treatment of neurodegenerative diseases. However, as neurodegenerative diseases generally follow a chronic progression and the medical treatment is long compared with that for malignancy, the toxic side effects of the treatments would need to be extensively suppressed. Therefore, we decided to explore the possibility of using 17-AAG as a therapeutic agent for neurodegenerative diseases by examining its effects on mutant AR in cultured cells and in a mouse model of SBMA.

## A model mouse of SBMA and a potent hormonal therapy

We had previously generated transgenic mice expressing the full-length human AR containing either 24 or 97 CAG repeats under the control of a cytomegalovirus enhancer and a chicken actin promoter [35]. The mice with 97 CAG repeats (AR-97Q) exhibited progressive motor impairment, while none of those with 24 CAG repeats (AR-24Q) showed abnormal phenotypes [35]. Other laboratories have



also generated various animal models of SBMA, almost all of which display phenotypic expressions of motor dysfunction [36]. For researching a truly effective molecular-targeted therapy, it is imperative to do so in a model that approximates the native state and metabolism of the disease-causing protein *in vivo*. We therefore consider that our SBMA mice carrying full-length AR are more beneficial for investigating therapeutic agents than those carrying the truncated one. Our transgenic mice indeed have a very severe phenotype, which is different to some extent from the human form of the disease, but they demonstrate polyQ-induced motor neuron degeneration and provide a beneficial tool to screen therapeutic agents to rescue this condition as we previously described [37]. For further details about the clinical features and our mouse model of SBMA, please refer to Katsuno et al. [38, 39].

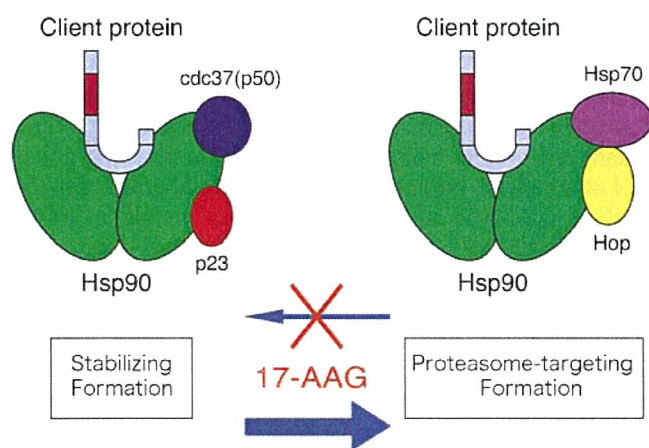
We have already experimentally demonstrated several therapeutic approaches using this model [35, 37, 40, 41] and consequently confirmed that leuprorelin, a luteinizing hormone-releasing hormone agonist that reduces testosterone release from the testis, significantly rescued motor dysfunction and nuclear accumulation of mutant AR in our SBMA mice. Due to its minimal invasiveness established in human and its powerful efficacy demonstrated in the above model [37], this hormonal therapy has already been in human clinical trials with encouraging results [42]. However, it is an extremely specialized therapy for SBMA and cannot be applied to other polyQ diseases [35, 37, 38]. In contrast to this hormonal therapy, 17-AAG would be a potential therapeutic agent for SBMA as well as for other related diseases [15].

### 17-AAG alters the form of the Hsp90 complex, leading to proteasomal degradation of mutant AR

Hsp90 functions in a multi-component complex of chaperone proteins, including Hsp70, Hop, Cdc37, and p23, leading to the folding, activation, and assembly of Hsp90 client proteins [10]. In addition, the Hsp90 complexes are thought to exist in two main forms: one complex is a proteasome-targeting form associated with Hsp70 and Hop, and the other is a stabilizing form with Cdc37 and p23 [14, 43–45] (Fig. 1). Hop is known to independently bind to both Hsp90 and Hsp70, which promotes the Hsp90/Hsp70 linkage, and is thought to direct the triage decision for client proteins by bridging the Hsp90–Hsp70 interaction [45]. On the other hand, p23 is thought to modulate Hsp90 activity in the last stages of the chaperoning pathway, leading to the stabilization of Hsp90 client proteins in an ATP-dependent manner [46]. Hsp90 inhibitors, including 17-AAG, inhibit the ATP-dependent progression of the Hsp90 complex toward the stabilizing form and shift it to the proteasomal-targeting form, resulting in proteasomal degradation of the Hsp90 client protein [47, 48]. Steroid receptors, including the progesterone receptor and the glucocorticoid receptor, were the first Hsp90 client proteins to be identified [49, 50]. As for AR, Hsp90 is essential to maintain its high ligand-binding affinity and its stabilization [7, 12]. In practice, Hsp90 inhibitors reduce androgen ligand-binding affinity and induce the degradation of AR [12, 13].

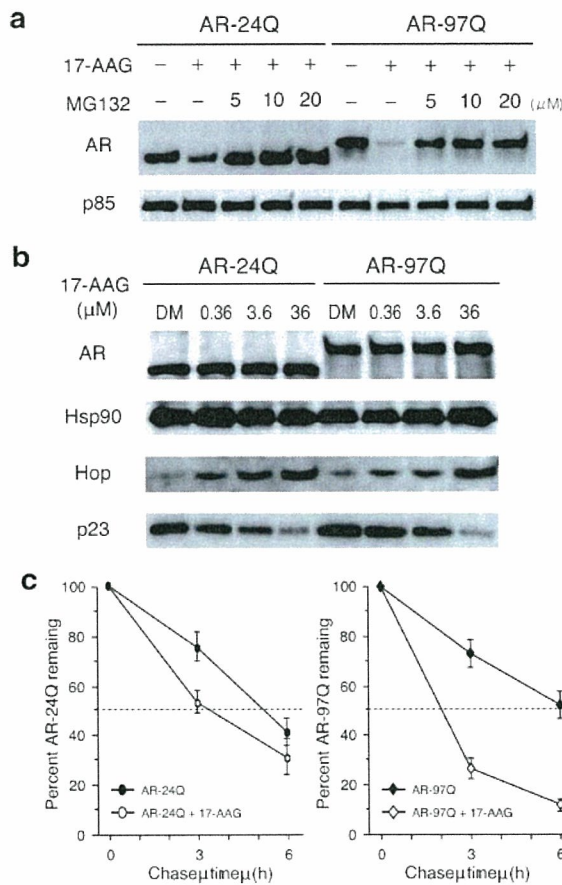
To address the question of whether 17-AAG also promotes the degradation of the disease-causing protein of SBMA, polyQ-expanded mutant AR, we treated SH-SY5Y cells highly expressing the wild-type (AR-24Q) or mutant (AR-97Q) AR for 6 h with 36  $\mu$ M 17-AAG or with dimethyl sulfoxide (DMSO) as control, in the absence or presence of the proteasome inhibitor, MG132. Immunoblot analysis demonstrated that the monomeric mutant AR decreased significantly more than the wild type did, suggesting that the mutant AR is more sensitive to 17-AAG than the wild type is. The degradation of wild-type and mutant AR by 17-AAG was completely blocked by the proteasome inhibitor, MG132 (Fig. 2a), suggesting that 17-AAG-facilitated degradation was dependent on the proteasome system as previously reported [47, 48].

Next, we examined changes in the Hsp90 chaperone complex in wild-type and mutant AR-expressing cultured cells after 17-AAG treatments. Immunoprecipitation with anti-AR antibody revealed that Hsp90-chaperone-complex-associated Hop was markedly increased, and p23 decreased in a 17-AAG dose-dependent manner, suggesting that 17-AAG resulted in the shifting of the AR-Hsp90 chaperone complex from a mature stabilizing form with p23 to a proteasome-targeting form with Hop. This chaperone complex shift appears to be very rapid as has been suggested previously [50, 51]. The loss of p23 from the mutant AR-Hsp90 complex was significantly greater than that from the wild-type one (Fig. 2b). Furthermore, these studies also strongly suggested that the mutant AR is more prone to be in the multi-chaperone complexes of



**Fig. 1** Hsp90-inhibitor-induced change in Hsp90 complex. Hsp90 inhibitor (17-AAG) specifically binds ATP-binding site of Hsp90, resulting in a shift of the Hsp90 complex. To exert its effects on client proteins, Hsp90 functions in a multi-component complex of co-chaperone proteins including Hsp70, Hop, Cdc37, and p23. Two main forms of this complex exist. One complex is a proteasome-targeting form associated with Hsp70 and Hop, and the other is a stabilizing form with Cdc37 and p23. Hsp90 inhibitors block the progression of the Hsp90 complex toward the stabilizing form and shift it to the proteasomal-targeting form. This figure is modified from a model proposed by Neckers [14]





**Fig. 2 a-c** 17-AAG-induced changes in the AR-Hsp90 complex: correlation to proteasomal degradation. **a** 17-AAG treatment (36  $\mu$ M, 6 h) of transfected SHSY5Y cells reduced the levels of mutant AR (AR-97Q) significantly more than the wild-type AR (AR-24Q); however, both decreases were completely blocked by the proteasomal inhibitor, MG132. **b** Immunoblots of lysates from transfected cells treated for 30 min with 17-AAG and immunoprecipitated with AR-specific antibody. The short time exposure to 17-AAG did not decrease the amount of mutant AR, but there were dose-dependent changes in both Hop and p23. There were no changes in the amounts of Hsp90 complexed with mutant AR. There were no changes in the expression of Hop, p23, and Hsp90 in whole lysates in the presence of 17-AAG (data not shown) **c** The effects of 17-AAG on the half-life of wild-type and mutant AR assessed from pulse-chase experiments. The amounts of AR-24Q remaining in the absence and presence of 17-AAG are indicated by *closed circles* ( $\bullet$ ) and *open circles* ( $\circ$ ), respectively. The amounts of AR-97Q remaining in the absence and presence of 17-AAG are indicated by *closed diamonds* ( $\blacklozenge$ ) and *open diamonds* ( $\diamond$ ), respectively. Mutant AR was degraded more rapidly than the wild-type AR in the presence of 17-AAG. Values are expressed as means $\pm$ SE ( $n=4$ )

Hsp90 with p23, which eventually enhances 17-AAG-dependent proteasomal degradation of mutant AR.

To determine whether the decrease in AR was due to protein degradation or to changes in RNA expression, the turnover of wild-type and mutant AR were then assessed with a pulse-chase labeling assay. In the presence of 17-AAG, the mutant AR and the wild-type AR had half-lives of 2 h and 3.5 h, respectively (Fig. 2c), while the mRNA levels for both the wild-type and mutant AR were quite similar [15]. These data indicate that 17-AAG preferentially degrades the mutant AR protein without altering

mRNA levels. These in vitro studies indicated that the mutant AR was a good target protein of 17-AAG. To determine if it would also be preferentially degraded in vivo, we next examined the effects of 17-AAG in SBMA transgenic mice.

### 17-AAG ameliorates the phenotype in a mouse model of SBMA mouse without detectable toxicity

We administered 17-AAG at doses of 2.5 or 25 mg/kg to males of the transgenic mouse model carrying full-length human AR with either 24Q or 97Q. The disease progression of AR-97Q mice treated with 25 mg/kg 17-AAG (Tg-25) was significantly ameliorated, and that of mice treated with the 2.5 mg/kg 17-AAG (Tg-2.5) was also mildly ameliorated (Fig. 3a). The AR-97Q mice treated with vehicle only (Tg-0) showed motor impairment assessed by the Rotarod task as early as 9 weeks after birth while the Tg-25 mice showed initial impairment only 18 weeks after birth and with less deterioration than the Tg-0 mice ( $P<0.005$ ; Fig. 3a). Tg-2.5 mice showed intermediate levels of impairment in Rotarod performance (Fig. 3a). 17-AAG also significantly prolonged the survival rate of the Tg-2.5 and Tg-25 mice compared with the Tg-0 mice ( $P=0.004$  and  $P<0.001$ , respectively; Fig. 3a). No lines were distinguishable in terms of body weight at birth; however, by 16 weeks, the Tg-0 mice showed obvious differences in body size, muscular atrophy, and kyphosis compared with the Tg-25 mice (Fig. 3b).

When mouse tissues were immunohistochemically stained for mutant AR using the 1C2 antibody, which specifically recognizes expanded polyQ, quantitative analysis revealed marked reductions in 1C2-positive nuclear accumulation in the spinal motor neurons and muscles of the Tg-25 mice compared with those of the Tg-0 mice (Fig. 3c).

Western blot analysis from lysates of the spinal cord and muscle of AR-97Q mice revealed high molecular weight mutant AR protein complex retained in the stacking gel as well as a band of monomeric mutant AR, whereas only the band of wild-type monomeric AR was visible in tissue from the AR-24Q mice (Fig. 3d). 17-AAG treatments significantly diminished both the high molecular weight complex and the monomer of mutant AR in the spinal cord and muscle of the AR-97Q mice but only slightly diminished the wild-type monomeric AR in AR-24Q mice (Fig. 3d). 17-AAG treatments decreased the amount of monomeric AR in AR-97Q mice by 64.4% in the spinal cord and 45.0% in the skeletal muscle, but only 25.9 and 12.5%, respectively, in AR-24Q mice (Fig. 3d). Thus, the reduction rate of the monomeric mutant AR was significantly higher than that of the wild-type AR in both spinal cord and skeletal muscle ( $P<0.001$  and  $P<0.01$  respectively). The levels of wild-type and mutant AR mRNA were similar in the respective mice treated with 17-AAG [15]. These observations indicate that 17-AAG markedly reduces not only the high molecular weight