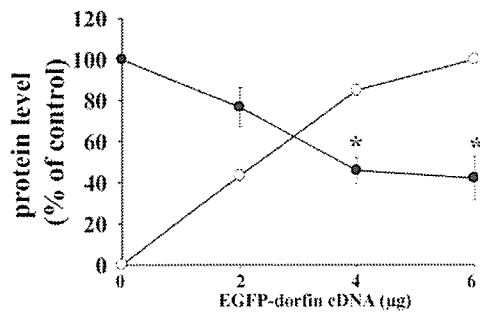
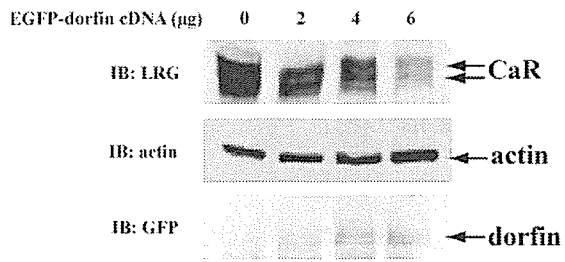
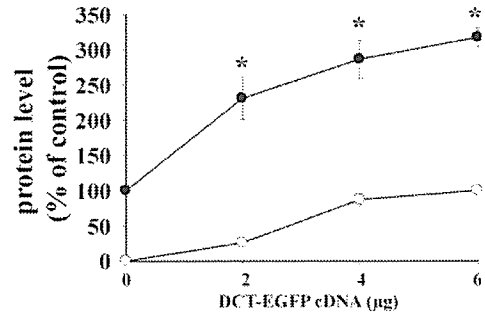
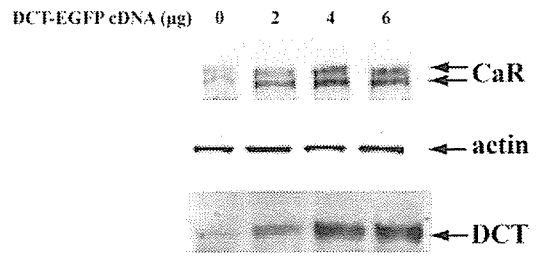


Figure 4

A.



B.



C.

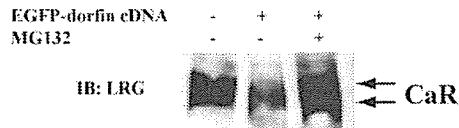


Figure 5

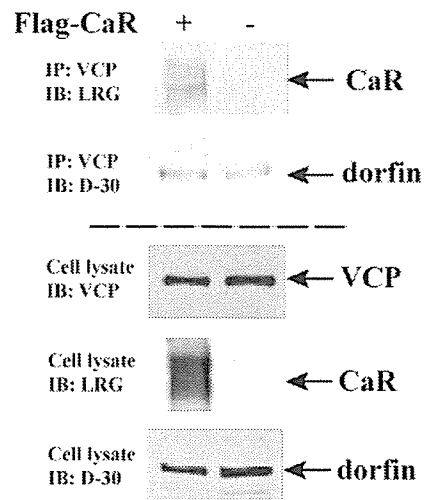


Figure 6

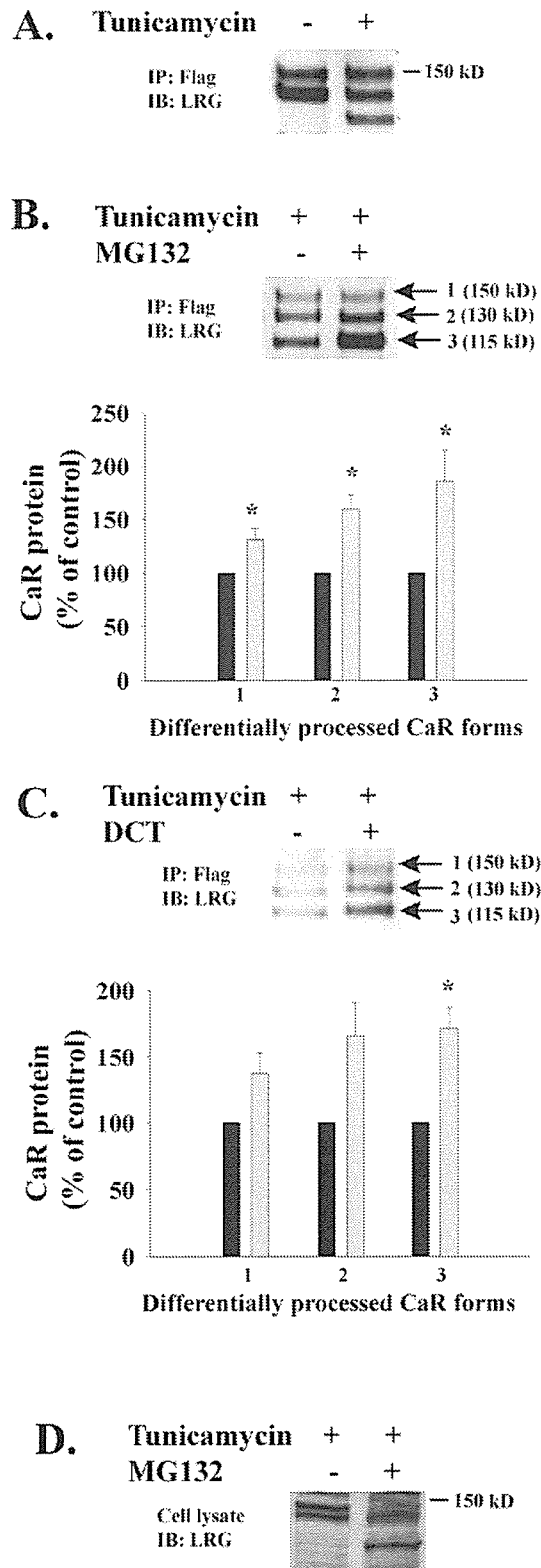


Figure 7



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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

Contents

History and nomenclature	0
Clinical features	0
Etiology	0
Pathology	0
Molecular pathogenesis and therapeutic strategies	0
Ligand-dependent pathogenesis in animal models of SBMA	0
Testosterone blockade therapy for SBMA	0
Role of heat shock proteins in pathogenesis of SBMA	0
Transcriptional dysregulation in SBMA	0
Axonal trafficking defects in SBMA	0
Clinical application of potential therapeutics	0
Acknowledgments	0
References	0

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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-pallidolucian 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), TAFII130, 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 form after a repeat length-dependent conformational change of polyglutamine monomer from a random coil to a parallel, helical β -sheet (Wytenbach, 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 as well as in brainstem motor nuclei except for the third, fourth and sixth cranial nerves (Fig. 1A) (Sobue et al., 1989). The

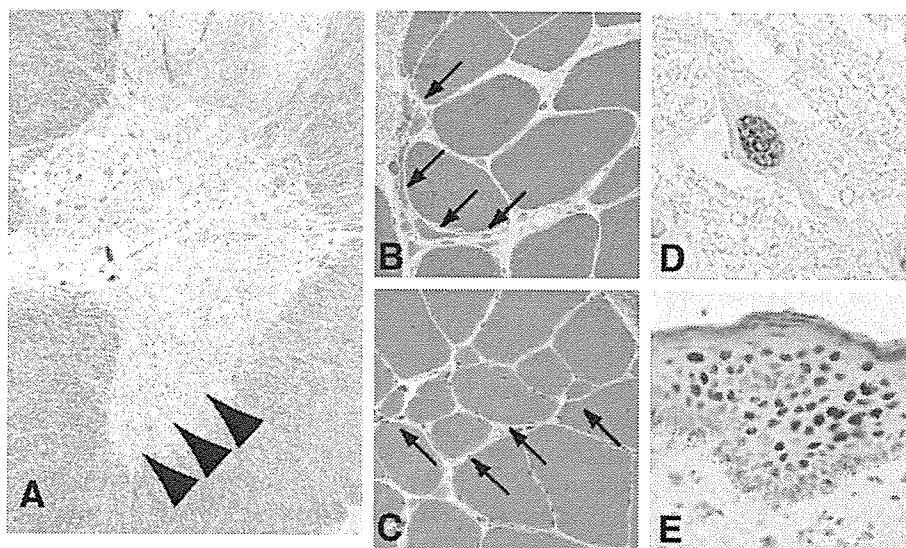


Fig. 1. Histopathology of SBMA. (A) A transverse section of spinal cord demonstrates marked depletion of motor neurons in the anterior horn. (B and C) HE staining of skeletal muscle shows both neurogenic (B, arrows) and myogenic changes (C, arrows). (D) A residual motor neuron in the lumbar anterior 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).

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 (Atrasate 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 polyglutamine-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 β -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, demonstrated an increase in the number of the muscle fibers with nuclear 1C2 staining at 4 weeks of leuporelin administration,

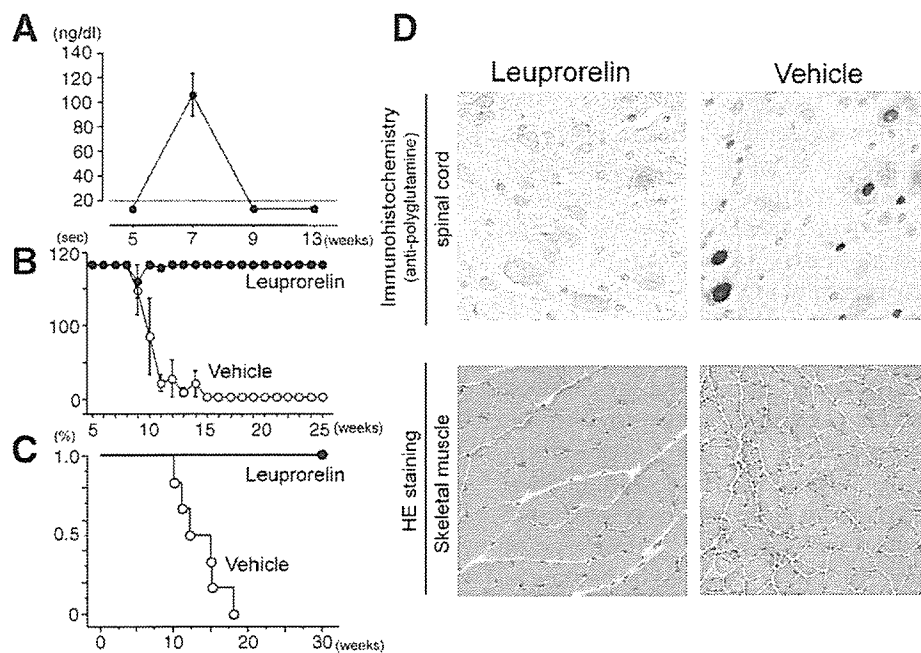


Fig. 2. Effects of leuprorelin on mutant androgen receptor (AR) expression and neuropathology of male AR-97Q mice. (A) Serum testosterone level in AR-97Q mice. Leuprorelin initially increased serum testosterone level but subsequently reduced it to undetectable levels. (B and C) Rotarod task (B) and survival rate (C) of the AR-97Q mice. Leuprorelin 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 leuprorelin-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 leuprorelin-treated mice.

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 leuprorelin. 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 leuprorelin-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 β -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 Nls 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 underlie the pathogenesis of neurodegeneration in polyglutamine diseases. This hypothesis is exemplified by our experimental

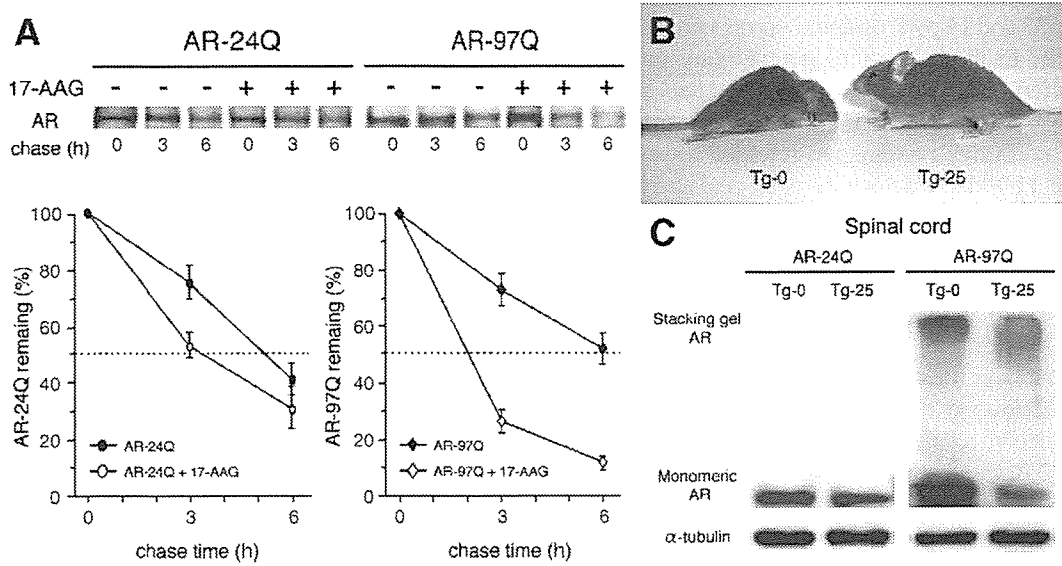


Fig. 3. Effects of 17-AAG on mutant androgen receptor (AR) turnover. (A) Pulse-chase analysis of two forms of AR. The pathogenic AR containing prolonged polyglutamine is degraded more rapidly than the wild-type AR in the presence of 17-AAG. (B) Muscle wasting is obvious in a vehicle-treated SBMA mouse (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).

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 HDAC activity results in augmentation of histone acetylation and subsequent restoration of gene transcription, HDAC

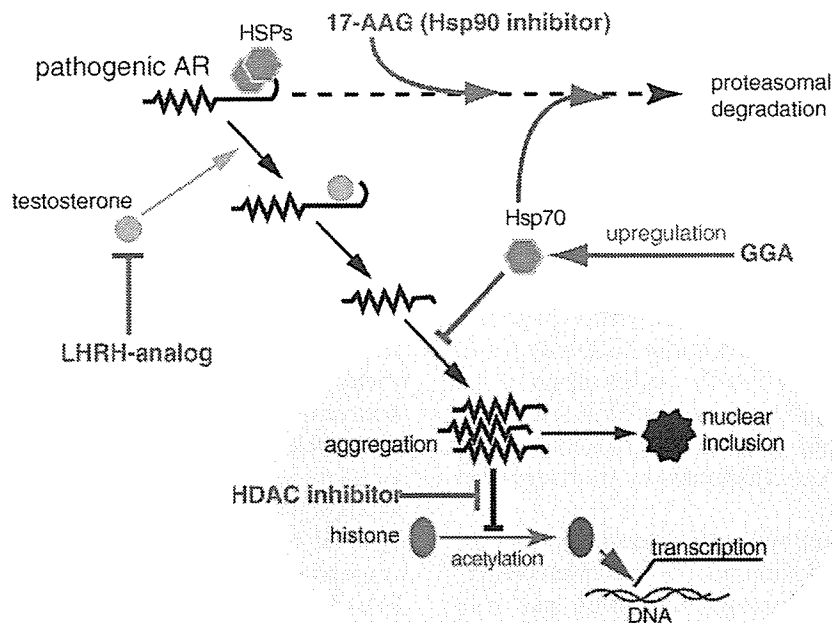


Fig. 4. Pathogenesis-targeting therapeutic approaches to SBMA. In the absence of ligand, the pathogenic AR is confined to a multi-heteromeric inactive complex with heat shock proteins (HSPs) in the cell cytoplasm. Ligand binding facilitates its dissociation from this complex and translocation into the 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.

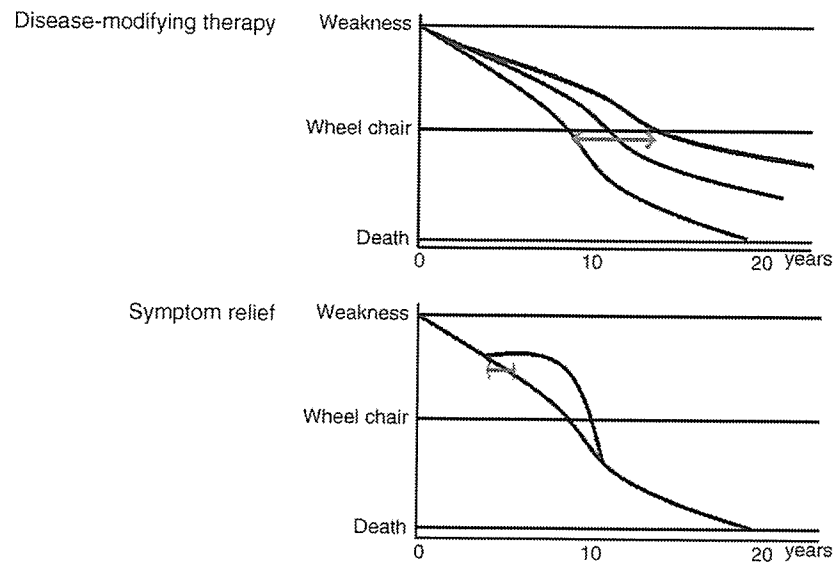


Fig. 5. Disease-modifying therapy and symptom relief. Since the goal of disease-modifying therapy is to inhibit pathogenic progression, long-term trials need to be carried out in order to evaluate drug effects by targeting certain clinical events as the primary endpoint. On the other hand, symptom relief, 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.

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: (i) drugs inhibiting accumulation of the pathogenic AR protein and (ii) drugs mitigating downstream pathological events including

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 ^a	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)

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

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

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

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

Histopathologically, lower motor neurons are markedly depleted in the spinal cord and brainstem, and nuclear inclusions (NIs) containing the mutant and truncated AR with expanded polyQ are present in the residual motor neurons, as well as in cells of the scrotal skin and other visceral organs.^{3,11,12} Although NIs are

a disease-specific pathological marker, they may reflect a cellular protective response against the toxicity of abnormal polyQ-containing protein.¹³ In contrast, the therapeutic effect of testosterone deprivation in our SBMA transgenic mouse model suggested that diffuse nuclear accumulation of mutant AR is a cardinal pathogenic process underlying neurological manifestations.^{14,15} This hypothesis has also been clearly illustrated by the observation that the extent of diffuse nuclear accumulation of mutant AR, but not NIs, in the motor neurons of the spinal cord was closely related to CAG repeat length in autopsied SBMA cases.¹⁶ Nuclear localization of the mutant protein has now been considered essential for inducing neuronal cell dysfunction and degeneration in the majority of polyQ diseases.¹⁰

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

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

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

Patients and Methods

Patients

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

For each of the 18 patients who underwent biopsy of the scrotal skin, three scrotal skin specimens were made by punch biopsy using a 3mm diameter Dermapunch (Nipro, Tokyo, Japan) under 10ml lidocaine acetate local anesthesia. All patients who underwent biopsy sterilized the wound for several days by themselves and received 4 days of cefaclor (250mg three times a day) antibiotic therapy after the procedure. The 13 patients who underwent biopsy who were

not enrolled in the leuporelin trial were also assessed on the anyotrophic lateral sclerosis functional scale (limb Norris score), as described previously.²²

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

Leuporelin Administration

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

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

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

Table. Patient Characteristics

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

The anyotrophic lateral sclerosis functional rating scale-revised.

SD = standard deviation; (CAG)n = number of expanded CAG repeats in the SBMA allele; NA = not applicable; ADL = activities of daily living.

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

Quantification of Cell Population with Diffuse Nuclear Staining

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

Statistical Analysis

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

Results

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

In the five autopsied cases, mutant AR nuclear accumulations were clearly visualized with anti-expanded polyQ immunostaining with 1C2 in the scrotal skin and spinal cord specimens (Fig 1A). Pathological accumulation of mutant AR was distributed in all layers of the epithelium. Diffuse nuclear accumulations were predominantly observed, and the occurrence of NIs was less frequent. This was also the case in the spinal cord specimens. Electron microscopic immunohistochemistry with the 1C2 antibody demonstrated granular dense and amorphous aggregates corresponding to diffuse nuclear staining in both spinal motor neurons and epithelial cells of scrotal skin (see Figs 1B, C). Filamentous structures such as those reported in Huntington's disease,²⁵ dentatorubal-pallidoluyisian atrophy (DRPLA),²⁶ and Machado-Joseph disease²⁷ were not seen. No diffuse nuclear staining was seen in the control subjects. The extent of mutant AR accumulation in the scrotal skin epithelial cells showed a tendency to correlate with that in the anterior horn cells ($r = 0.84$; $p = 0.08$; see Fig 1D). Mutant AR accumulation was remarkable in both the spinal motor neurons and the

scrotal skin of Patient 1, but was far less remarkable in Patient 2 (see Figs 1A, D).

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

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

Leuporelin Treatment Depletes Mutant Androgen Receptor Accumulation in the Scrotal Skin

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

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

Discussion

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

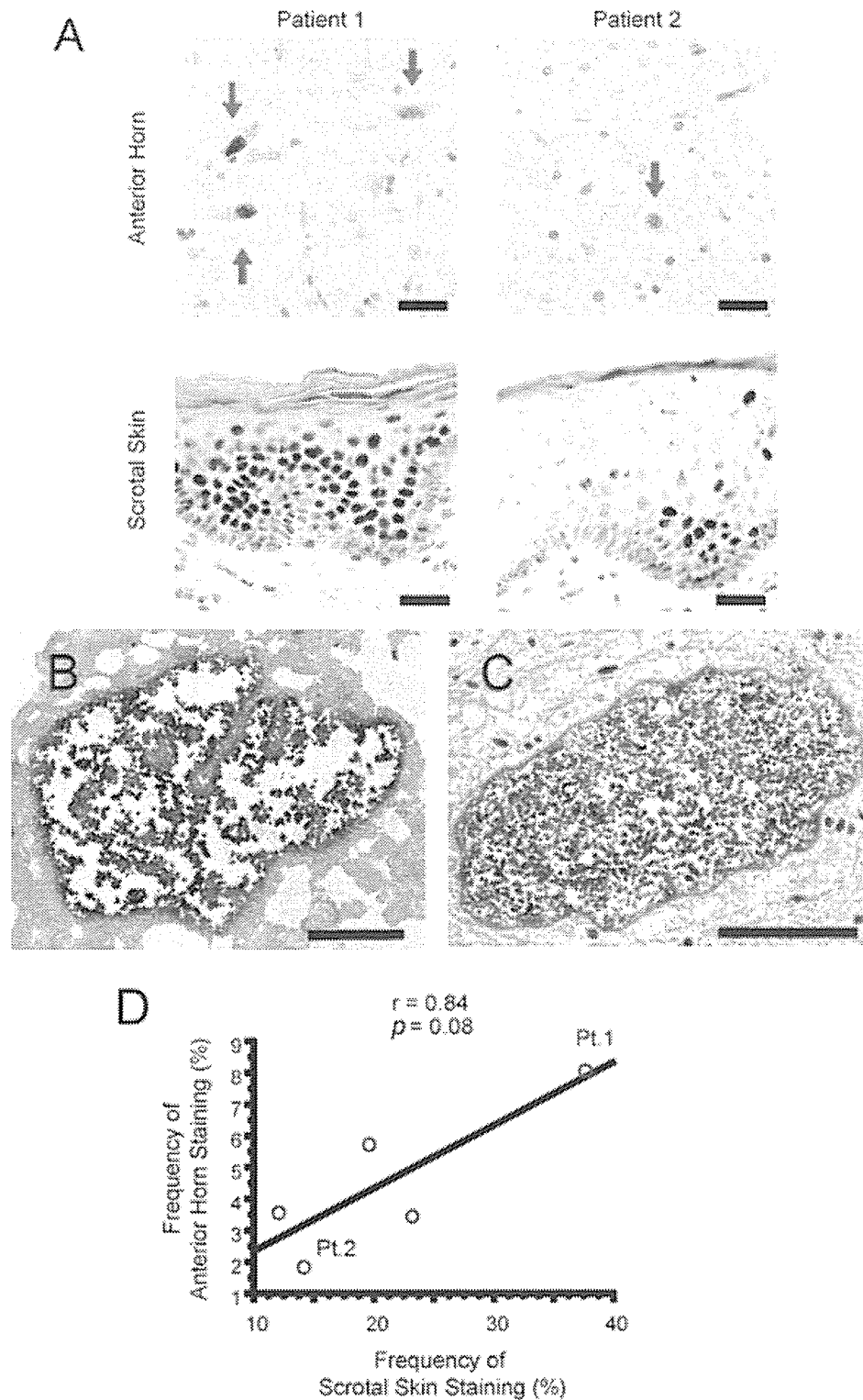


Fig 1. Mutant androgen receptor (AR) nuclear accumulation in scrotal skin and spinal motor neurons (A) Mutant AR accumulation was remarkable in both spinal motor neurons (arrows) and scrotal skin of Patient 1, but was less remarkable in both motor neurons (arrows) and skin in Patient 2. Bar = 30 μ m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

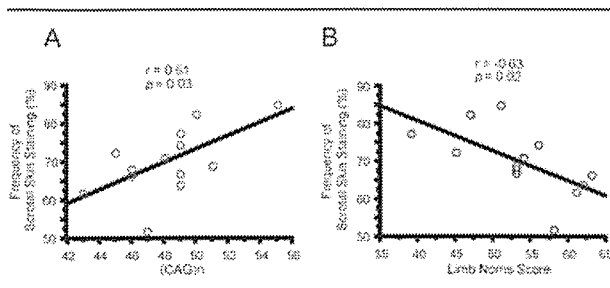


Fig 2. Correlation of the frequency of scrotal skin staining to CAG repeats and limb Norris score. The frequency of 1C2-positive cells in the scrotal skin biopsies correlated significantly with (A) CAG repeat length and (B) inversely correlated with the amyotrophic lateral sclerosis functional scale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

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

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

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

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

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