

As for the death signals and inflammatory factors, we previously have reported that these genes were significantly upregulated in the spinal cord of mutant SOD1 transgenic mice,^{15,17} suggesting that these inflammatory and apoptotic death signals play a crucial role in concert with motor neuron degeneration and inflammatory cellular reactions, including microglial activation.^{7,47} However, in sporadic ALS spinal cords, the expression profiles of inflammation- and death signal-related genes are somewhat different from those in the Tg mouse model. Death receptor 5 (TNF receptor 10b, TNFR10b), TNF receptor-associated factor 6 (TRAF 6), interleukin-1 receptor antagonist, and ephrin A1 were overexpressed in ALS motor neurons, whereas the expression levels of the respective ligands or inducers, TNF- α , TNF superfamily member 10 (TRAIL) and IL-1 β , were not markedly changed in either motor neurons or ventral horn homogenates. Because TNF- α was prominently upregulated in the Tg mouse spinal cords,¹⁷ its almost unchanged expression level in sporadic ALS was a surprising observation. Many other inflammation-related genes were also not significantly upregulated in human ALS spinal cords, in contrast with the findings in animal models. Far less invasion and activation of microglia, a major source of TNF- α , IL-1 β , and many other inflammatory factors, were seen in human ALS spinal cords at the terminal autopsy stage as compared with those of Tg mice,⁴⁸ which could explain these differences.

Genes related to apoptotic pathways, caspase-1, -3, -9, caspase, and RIP adaptor with death domain (CRADD), were upregulated in ALS motor neurons in SOM analysis (SOM24 and SOM25), and an anti-apoptotic factor, NF- κ B, was markedly upregulated. Although Bax, a proapoptotic Bcl-2 family member, has been reported to increase in ALS motor neurons,^{49,50} and another member, Bak, was underexpressed in this study, the expression of Bcl-2 and Bcl-xL, antiapoptotic Bcl-2 family members was not significantly altered in motor neurons, possibly suggesting that Bcl-2 family members are not primarily involved in motor neuron degeneration in sporadic ALS. Cyclin A1 and C were upregulated (SOM25) and cyclin E was downregulated (SOM1) in ALS motor neurons. These cell cycle regulators are specific to G1/S phase transition, and upregulation of these cyclins enhances arrest in G1/S phase, preventing entry into S phase. Our finding on cyclin expression support the recently reported view that G1/S phase is aberrantly activated in ALS motor neurons, eventually inducing motor neuron death.⁵¹ The subcellular localization of cyclin C in the nucleus may trigger cell death signaling mechanisms. These factors related to the cell death signaling pathway, TNFR, TRAF6, CRADD, caspases, cyclins, Bak, and NF- κ B may be involved in the motor neuron degeneration process in sporadic ALS, although

we cannot simply state that an apoptotic process is present in ALS motor neurons, as has been suggested by many histological analyses.⁶ Because neuronal cell degeneration and the eventual neuronal cell death process are the results of interactions of complex pathways involving many factors and signaling molecules, we need to further elucidate the pathophysiological significance of these factors with altered expression levels in ALS motor neurons.

Microarray analysis on the laser-captured motor neurons provided us with significant information about motor neuron degeneration and dysfunction in sporadic ALS patients. Such information cannot be obtained by whole spinal cord tissue microarray assay,⁵² as discussed above. Although this study was performed on postmortem patients' tissues, the remaining individual motor neurons would express ongoing or even early molecular events in the neurodegeneration process, because motor neurons in the remaining motor neuron population randomly enter into the degeneration process among up to the terminal stage in ALS.⁵³ We need to study larger numbers of ALS patients, and to understand the pathophysiological roles of candidate genes identified by the combined methodology of DNA microarray analysis and LCM, compared with other neurodegeneration processes. This methodology provides crucial clues about candidate genes whose related products might hamper the disease process of ALS.

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Widespread nuclear and cytoplasmic accumulation of mutant androgen receptor in SBMA patients

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Summary

Spinal and bulbar muscular atrophy (SBMA) is an inherited adult onset motor neuron disease caused by the expansion of a polyglutamine (polyQ) tract within the androgen receptor (AR), affecting only males. The characteristic pathological finding is nuclear inclusions (NIs) consisting of mutant AR with an expanded polyQ in residual motor neurons, and in certain visceral organs. We immunohistochemically examined 11 SBMA patients at autopsy with 1C2, an antibody that specifically recognizes expanded polyQ. Our study demonstrated that diffuse nuclear accumulation of mutant AR was far more frequent and extensive than NIs being distributed in a

wide array of CNS nuclei, and in more visceral organs than thus far believed. Mutant AR accumulation was also present in the cytoplasm, particularly in the Golgi apparatus; nuclear or cytoplasmic predominance of accumulation was tissue specific. Furthermore, the extent of diffuse nuclear accumulation of mutant AR in motor and sensory neurons of the spinal cord was closely related to CAG repeat length. Thus, diffuse nuclear accumulation of mutant AR apparently is a cardinal pathogenetic process underlying neurological manifestations, as in SBMA transgenic mice, while cytoplasmic accumulation may also contribute to SBMA pathophysiology.

Keywords: polyglutamine; spinal and bulbar muscular atrophy; diffuse nuclear accumulation; nuclear inclusion, cytoplasmic accumulation

Abbreviations: polyQ = polyglutamine; SBMA = spinal and bulbar muscular atrophy; AR = androgen receptor; NIs = nuclear inclusions; DRPLA = dentatorubral-pallidolusian atrophy; CBP = CREB-binding protein

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Introduction

Polyglutamine (polyQ) diseases are inherited neurodegenerative disorders caused by expansion of a trinucleotide CAG repeat in the causative genes. To date, nine polyQ diseases have been identified (Ross, 2002). Spinal and bulbar muscular atrophy (SBMA) is a polyQ disease involving mainly spinal and brainstem motor neurons (Kennedy *et al.*, 1968; Sobue *et al.*, 1989). In SBMA, a polymorphic CAG repeat ordinarily consisting of 14–32 CAGs is expanded to 40–62 CAGs in the first exon of the androgen receptor (AR) gene (La Spada *et al.*, 1991; Tanaka *et al.*, 1996), and shows somatic mosaicism (Tanaka *et al.*, 1999). An inverse correlation exists between CAG repeat size and age at onset as well as disease severity in SBMA (Doyu *et al.*, 1992; Igarashi *et al.*, 1992; La Spada *et al.*,

1992). SBMA patients develop premature muscular exhaustion, and subsequently slowly progressive muscular weakness, atrophy and fasciculations in bulbar and limb muscles (Kennedy *et al.*, 1968; Sobue *et al.*, 1989; Sperfeld *et al.*, 2002). SBMA patients may also have mild sensory impairment, which usually remains subclinical (Sobue *et al.*, 1989; Li *et al.*, 1995; Mariotti *et al.*, 2000). Besides these symptoms of neuronal degeneration, androgen insensitivity symptoms such as gynaecomastia, testicular atrophy and reduced fertility are common (Arbizu *et al.*, 1983). Elevated serum creatine kinase concentrations, impaired glucose tolerance, hepatic dysfunction and hyperlipidaemia are frequent (Sobue *et al.*, 1989; Li *et al.*, 1995). These findings show that involvement of

SBMA is not restricted to motor neurons, but extends to several visceral organs.

The cardinal pathological findings of SBMA are motor neuron loss in the spinal cord and brainstem (Sobue *et al.*, 1989) and the presence of the nuclear inclusions (NIs), representing mutant AR, in residual motor neurons in brainstem motor nuclei, in spinal motor neurons (Li *et al.*, 1998a) and in certain visceral organs (Li *et al.*, 1998b). However, diffuse nuclear accumulation of the mutant protein has been detected in a more widespread distribution than NIs in a transgenic mouse model of SBMA (Katsuno *et al.*, 2002, 2003; Adachi *et al.*, 2003) and in models of other polyQ diseases (Schilling *et al.*, 1999; Yvert *et al.*, 2000; Lin *et al.*, 2001). Such accumulation has been found to be relevant to neuronal dysfunction and eventual symptom appearance. Indeed, in dentatorubral-pallidoluysian atrophy (DRPLA), tissue distribution of diffuse nuclear accumulation of the responsible mutant protein was more widespread and more relevant to the disease severity and symptoms than that of NIs (Yamada *et al.*, 2001a, b).

Recently we demonstrated in our transgenic mouse model that diffuse nuclear mutant AR accumulation can be prevented by reduction of circulating testosterone with castration or with an anti-androgenic agent such as leuproterin; in treated animals, motor function and survival rate were dramatically improved (Katsuno *et al.*, 2002, 2003), suggesting that disease manifestation in SBMA is highly testosterone dependent (Lieberman *et al.*, 2002; Walcott and Merry, 2002a; Chevalier-Larsen *et al.*, 2004). Indeed, a female carrier of SBMA, even if homozygous, does not express disease phenotypes (Sobue *et al.*, 1993; Schmidt *et al.*, 2002), presumably because circulating testosterone concentrations are low. These observations indicate that nuclear translocation and nuclear accumulation of mutant AR, detected as diffuse nuclear accumulation, is closely linked to the phenotypic expressions and that diffuse nuclear mutant AR accumulation is of major pathogenetic importance in neuronal dysfunction (Katsuno *et al.*, 2002, 2003).

In this study, to understand better the pathophysiology of SBMA, we examined neural and non-neural tissue distributions of mutant AR accumulation in 11 SBMA patients at autopsy, using 1C2, an antibody specific for the expanded polyQ tract, as well as antibodies against AR. First, diffuse nuclear accumulation of mutant AR was far more extensive than that of NIs. Secondly, mutant AR accumulation was also present in cytoplasm, specifically in the Golgi apparatus, with predominance of nuclear or cytoplasmic accumulation being tissue specific. Thirdly, the extent of diffuse nuclear accumulation was closely related to CAG repeat length. Our present results strongly suggested that diffuse nuclear accumulation of mutant AR is of critical pathogenetic importance for motor symptoms as in the SBMA transgenic mouse model, although cytoplasmic accumulation may also contribute to the pathophysiology of SBMA.

Subjects and methods

Patients

Eleven patients with clinicopathologically and genetically confirmed SBMA (age at death, 51–84 years; mean, 66) were examined in this study (Table 1). These patients had been hospitalized and followed-up at Nagoya University Hospital and its affiliated hospitals during the past 25 years. Age at onset ranged between 20 and 75 years, and muscle weakness and bulbar symptoms had progressed for 6–53 years. Elevated serum creatine kinase and glucose was observed in many patients. Causes of death included respiratory failure related to pneumonia in seven patients, lung cancer and colon cancer in one patient each, and tuberculosis and suffocation in one patient each. At autopsy, the brain, spinal cord, dorsal root ganglia, thoracic sympathetic ganglia and various visceral organs were removed and fixed in 10% buffered formalin solution. CAG repeat length in the AR gene ranged between 40 and 50. Five other subjects (age 60–74 years, mean 67.3) who died of non-neurological diseases served as controls.

Tissue preparation and immunohistochemistry

We prepared 5 µm thick, formalin-fixed, paraffin-embedded sections of various portions of the cerebrum, brainstem, cerebellum,

Table 1 Clinical features of 11 SBMA patients

Patient	Age at death (years)	Onset of limb weakness (years)	CK (normal range)	Glucose (mg/dl)	(CAG) _n	Cause of death
1	74	20	455 (57–197)	84*	48	Pneumonia
2	60	27	477 (36–203)	276	50	Pneumonia
3	71	50	995 (53–288)	141	48	Pneumonia
4	60	40	191 (32–197)	134	44	Lung cancer
5	78	25	411 (30–170)	362	42	Pneumonia
6	84	75	75 (<110)	100	40	Tuberculosis, silicosis
7	51	41	712 (30–170)	96*	47	Pneumonia
8	66	41	471 (<120)	163	48	Pneumonia
9	72	39	45 (<25)	101*	43	Colon cancer
10	59	53	301 (8–80)	105*	ND	Suffocation
11	51	27	173 (20–100)	101	ND	Pneumonia

CK = serum creatine kinase; (CAG)_n = number of expanded CAG repeats in the AR allele. (CAG)_n was determined on the DNA from blood samples (patients 1–7) or from stored tissue samples (patients 8 and 9, liver). *Impaired glucose tolerance assessed with 75 g oral glucose tolerance test. ND = not determined.

spinal cord, dorsal root ganglia, sympathetic ganglia, pituitary gland, peripheral nerve, muscle and non-neural visceral organs from SBMA and control subjects. Sections then were deparaffinized and rehydrated through a graded series of alcohol-water solutions. For the mutant AR immunohistochemical study, sections were pre-treated with immersion in 98% formic acid for 5 min and then with microwave oven heating for 10 min in 10 mM citrate buffer at pH 6.0. Sections were blocked with normal serum from the animal species in which each second antibody was raised (1:20), and then incubated with a mouse anti-expanded polyQ antibody (Trottier *et al.*, 1995) (1C2; Chemicon, Temecula, CA; 1:10 000); a mouse anti-Golgi 58K protein antibody (Sigma, St. Louis, MO; 1:100); rabbit polyclonal antibody N-20 (Santa Cruz Biotechnology, Santa Cruz, CA; 1:200); rabbit polyclonal antibody PG-21 (Affinity BioReagents, Golden, CO; 1:200); rabbit polyclonal antibody H-280 (Santa Cruz; 1:200); rabbit polyclonal antibody C-19 (Santa Cruz; 1:200); or a mouse monoclonal antibody (Ab-1; Neomarkers, Fremont, CA; ready-to-use) against human AR protein. Then the sections were incubated with biotinylated IgG raised against the species used for each primary antibody (Vector Laboratories, Burlingame, CA). Immune complexes were visualized using streptavidin-horseradish peroxidase (Dako, Glostrup, Denmark) and 3,3'-diaminobenzidine (Dojindo, Kumamoto, Japan) substrate. Sections were counterstained with methyl green or Mayer's haematoxylin. As a negative control, primary antibodies were replaced with normal rabbit or mouse serum. The population of labelled neurons was analysed semi-quantitatively in all 11 SBMA patients, and non-neural visceral organs in nine patients (patients 1-8, and 11) by counting the positive and negative cells for labelling in the region of interest and graded as - to +++.

To assess the co-localization of cytoplasmic mutant AR accumulation and cell organelles, five selected SBMA patients (patients 1, 2, 6, 8 and 10) were analysed by double immunofluorescence staining. The sections were blocked with 5% normal serum and then sequentially incubated at 4°C overnight with any antibody to lysosomal markers, anti-cathepsin B antibody (Ab-3; Oncogene, Cambridge, MA; 1:20), anti-cathepsin D antibody (Ab-2; Oncogene; 1:20), anti-cathepsin K antibody (N-20; Santa Cruz Biotechnology; 1:50), anti-cathepsin L antibody (S-20; Santa Cruz Biotechnology; 1:50), antibody to Golgi apparatus, anti-human TGN46 antibody (Serotec, Oxford, UK; 1:1000), antibody to endoplasmic reticulum marker, anti-GRP78 antibody (N-20; Santa Cruz; 1:200), or antibody to mitochondria, anti-mitochondria antibody (Chemicon; 1:50), and 1C2 antibody (Chemicon; 1:10 000). Sections were incubated with Alexa 488-conjugated anti-mouse IgG (Molecular Probes, Leiden, The Netherlands; 1:1300) and Alexa 568-conjugated IgG raised against the species used for each primary antibody (Molecular Probes; 1:1000). For double immunofluorescence staining using anti-human TGN46 antibody, sections were incubated with biotinylated anti-sheep IgG (Vector Laboratories; 1:400) for 8 h at 4°C, the sections were incubated with Alexa 568-conjugated streptavidin (Molecular Probes; 1:1000) and Alexa 488-conjugated anti-mouse IgG (Molecular Probes; 1:1300) for 2 h at 4°C. Sections then were examined and photographed using a confocal laser scanning microscope (MRC 1024; Bio-Rad Laboratories, Hercules, CA).

For electron microscopic immunohistochemistry, buffered formalin-fixed, paraffin-embedded tissue sections were deparaffinized, rehydrated, immunostained with 1C2 antibody (Chemicon, 1:10 000), and then incubated with biotinylated anti-mouse IgG (Vector Laboratories; 1:1300). Immunoreactivity in tissue sections was visualized using streptavidin-horseradish peroxidase (Dako)

and 3,3'-diaminobenzidine substrate (Dojindo), fixed with 2% osmium tetroxide in 0.1 mol/l phosphate buffer at pH 7.4, dehydrated in graded alcohol-water solutions, and embedded in epoxy resin. Ultrathin sections then were cut for observation under an electron microscope (H-7100; Hitachi High-Technologies Corporation, Tokyo, Japan).

Quantification of diffuse nuclear- and NI-positive cell populations

For quantitative assessment, we prepared at least 100 transverse sections each from the cervical, thoracic and lumbar spinal cord for staining with 1C2 antibody as above. The numbers of 1C2-positive and -negative cells in the ventral and dorsal horn on both right and left sides were counted on every 10th section under the light microscope with a computer-assisted image analyser (Luzex FS; Nikon, Tokyo, Japan). For the purposes of counting, a cell was defined by the presence of its nucleus in a given 5 µm thick section. Diffuse nuclear staining and NI-positive neurons were assessed separately. Neurons showing both diffuse nuclear staining and NIs were counted in both categories. The area of the ventral and dorsal horn of each spinal cord section was determined as described previously (Terao *et al.*, 1996; Adachi *et al.*, 2001). Populations of 1C2-positive cells were expressed as percentages of the total neuronal count. For statistical analysis, mean values of these percentages in sections examined from each of the cervical, thoracic and lumbar spinal segments for each patient were obtained.

Statistical analysis

We analysed the data by Pearson's correlation coefficient and Spearman's rank correlation as appropriate using Statview software (version 5; Hulinks, Tokyo, Japan), considering *P* values <0.05 to be indicative of significance.

Results

Immunohistochemical localization of mutant androgen receptor in the neural tissues

In all 11 patients with SBMA, NIs were visualized clearly with 1C2 (Fig. 1). In addition to NIs, diffusely distributed staining with 1C2 was observed in neuronal nuclei (Fig. 1). Among nuclei with diffuse staining, some showed punctate, granular or web-like patterns, while others showed intense diffuse staining (Fig. 1). In some neurons, NIs and diffuse nuclear staining co-existed (Fig. 1). Moreover, occasional neurons showed granular or punctate 1C2-positive accumulation in the cytoplasm (Fig. 1). As reported previously (Li *et al.*, 1998a, b), NIs were observed frequently in lower motor neurons, which are known to be affected in this disease. However, we found that neuronal nuclear and cytoplasmic accumulations extended to various regions of the nervous system previously reported to be spared (Li *et al.*, 1998a, b), including the striatum, caudate nucleus, mammillary body, thalamus, hypothalamus, reticular formation, red nucleus, substantia nigra, locus coeruleus, nucleus raphe pontis, pontine nuclei, cuneate nucleus, nucleus ambiguus, gracile nucleus, supraspinal nucleus, cerebellar dentate nucleus, Clarke's

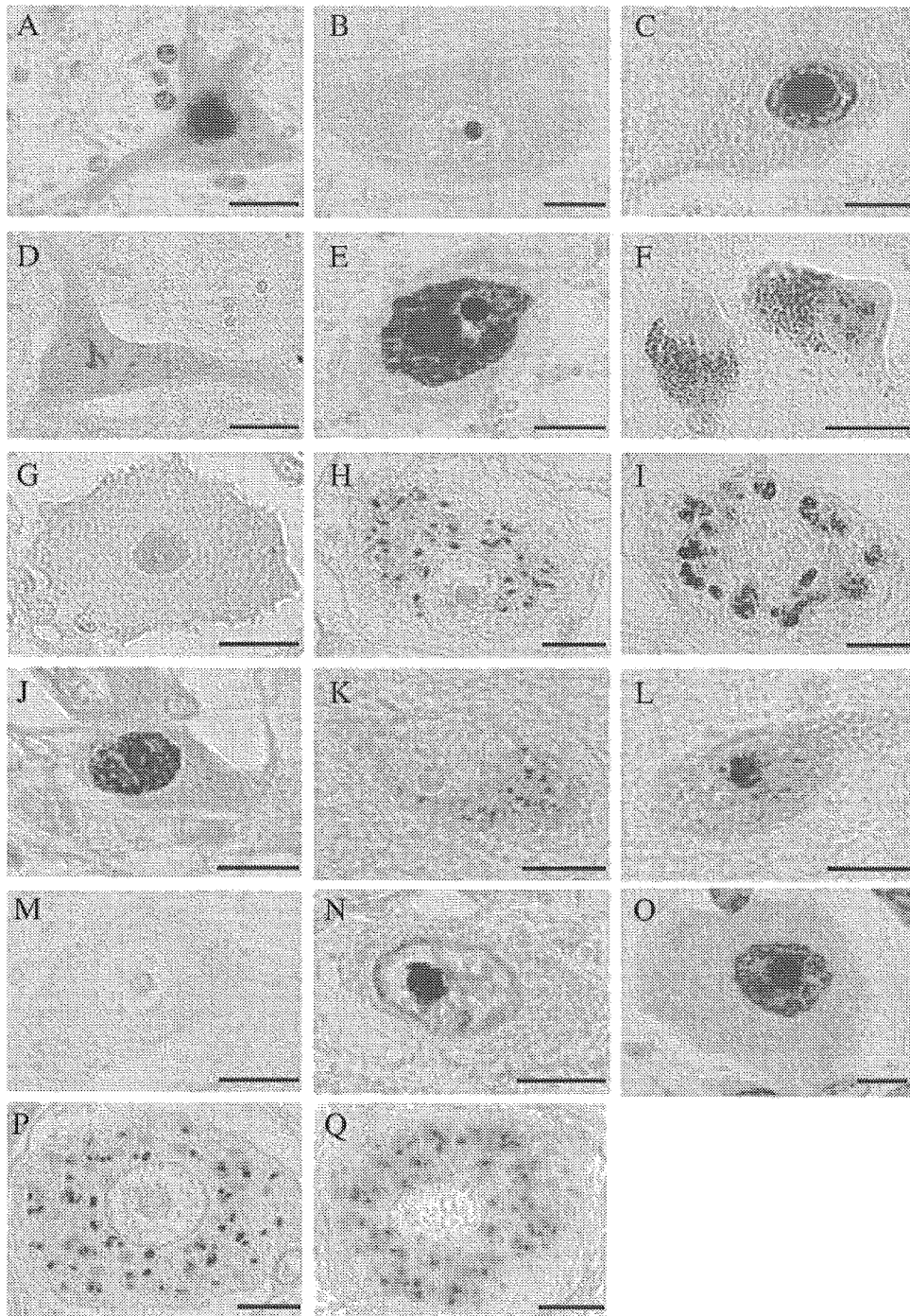


Fig. 1 Immunohistochemical analysis in the neural tissues from SBMA patients and control cases. In the CNS of SBMA patients, intense diffuse nuclear staining is present in neuronal nuclei of various regions using 1C2 antibody (A, E, F, G, J and L). Diffuse immunostaining of mutant androgen receptor (AR) is present in a web-like pattern in nuclei of anterior horn neurons (A). Diffuse nuclear staining is also observed in posterior horn neurons (E), substantia nigra (F), spinal dorsal root ganglia (G), paravertebral sympathetic ganglia (J) and hypothalamus (L). Some nuclei appear packed with mutant AR. Small or large nuclear inclusions are also stained intensely using 1C2 antibody in anterior horn neurons (B–D), posterior horn neurons (E), the substantia nigra (F) and the hypothalamus (L). Most of the dark brown pigment seen in the neuronal cytoplasm in the substantia nigra (F) is neuromelanin. In addition to nuclear inclusions, occasional neurons exhibit granular structures immunoreactive for 1C2 in the cytoplasm, such as in the anterior horn (D) and hypothalamus (K). In spinal dorsal root ganglia, small or large cytoplasmic inclusions are frequent (H and I). There is no immunoreactivity for 1C2 in the spinal anterior horn cell from the control case (M). Immunopositive nuclear inclusions and diffuse nuclear staining are also present using H280 antibody in the spinal anterior horn cell (N) and spinal dorsal root ganglia (O). Spinal dorsal root ganglia neurons exhibit granular structures immunoreactive for anti-Golgi 58K protein antibody in the cytoplasm in SBMA (P) and a control case (Q). Scale bars = 20 μ m for A, D, F, G, M and O; and 10 μ m for B, C, E, H, I, J, K, L, N, P and Q.

nucleus, posterior horn and intermediolateral nucleus of the spinal cord, dorsal root ganglia and sympathetic ganglia (Fig. 1, Table 2). Cytoplasmic inclusions were prominent in the dorsal root ganglia neurons, and some neurons in the mammillary body, hypothalamus and facial motor nucleus and anterior and posterior horns of the spinal cord showed a slight degree of cytoplasmic accumulation (Table 2). We detected both nuclear and cytoplasmic accumulations in some

Table 2 Immunohistochemical distribution of mutant AR in the neural tissues of patients with SBMA

Region	Nuclear accumulation		Cytoplasmic accumulation
	Diffuse nuclear accumulation	NI	
Cerebrum			
Cerebral cortex	—	—	—
Striatum	+	+	—
Caudate nucleus	+	—	—
Mammillary body	—	—	+
Thalamic nuclei	+	+	—
Hypothalamus	+ to ++	+	+
Midbrain			
Superior colliculus	—	—	—
Periaqueductal grey	+	+	—
Oculomotor nucleus	—	—	—
Reticular formation of midbrain	+	+	—
Red nucleus	+	+	—
Substantia nigra	+	+	—
Pons			
Locus coeruleus	+	+	—
Trigeminal motor nucleus	+ to ++	+	—
Reticular formation of pons	+	+	—
Facial motor nucleus	+ to +++	+	+
Nucleus raphe pontis	+	+	—
Pontine nuclei	+	+	—
Medulla			
Cuneate nucleus	+	+	—
Hypoglossal nucleus	+ to ++	+	—
Nucleus ambiguus	+ to ++	+	—
Gracile nucleus	+	+	—
Supraspinal nucleus	+ to ++	+	—
Accessory nucleus	+	+	—
Cerebellum			
Purkinje cell	—	—	—
Granule cell	—	—	—
Cerebellar dentate nucleus	+	+	—
Spinal cord			
Anterior horn motor neurons	+ to +++	+ to ++	+
Intermediate zone	+	+	—
Clarke's nucleus	+	—	—
Posterior horn neurons	+ to ++	— to +	+
Intermediolateral nucleus	+	+	—
Dorsal root ganglia	+	+	++ to +++
Sympathetic ganglia	+	+	—

Frequency of neurons expressing polyglutamine immunoreactivity: —, 0%; +, 0–4%; ++, 4–8%; +++, 8%.

neurons (Fig. 1D). No significant difference in staining pattern was evident between regions previously reported to be affected and unaffected. Diffuse nuclear staining was seen more frequently than NIs in most regions (Table 2). Relative numbers of stained neurons varied between patients, but no staining was detected in cerebral cortex, hippocampus or cerebellar cortex. In contrast to neurons, NIs and diffuse nuclear staining were very rare in glial cells. NIs stained strongly with anti-ubiquitin antibody, while cytoplasmic accumulations did not (data not shown). Anti-human AR antibodies also recognized NIs (Fig. 1N), and occasionally stained diffuse nuclear accumulations (Fig. 1O). However, cytoplasmic accumulations were not seen with anti-AR antibodies.

Electron microscopic immunohistochemistry for 1C2 demonstrated granular dense aggregates without a limiting membrane corresponding to NIs and cytoplasmic accumulations, whereas amorphous aggregates corresponded to diffuse nuclear staining in neurons (Fig. 2). No filamentous structures such as those reported in Huntington's disease, DRPLA and Machado–Joseph disease were seen. Neural tissues from five control cases were also examined in the same manner as that for SBMA cases; in these, NIs, diffuse nuclear staining and cytoplasmic accumulations were not seen, indicating that the immunohistochemical procedure with the highly diluted condition of 1C2 applied in this study did not recognize the TATA-binding protein, a transcription factor containing a stretch of polyQ residues (Trottier *et al.*, 1995), as previously demonstrated (Yamada *et al.*, 2001a, 2002a).

Although we did not quantitatively examine neuronal populations in this study, the motor neurons in the spinal cord and brainstem showed the most conspicuous depletion, as expected. Neurons in the posterior horn of the spinal cord, where diffuse nuclear accumulations and NIs were present in relatively high frequency, also appeared to be depleted to some extent. Quantitative assessment of neuronal cell populations in regions newly showing mutant AR accumulation will be needed.

Co-localization of cytoplasmic organelles with mutant AR

We performed immunofluorescence with double staining using primary antibodies to recognize specifically various cytoplasmic cell organelles together with 1C2 in the dorsal root ganglia, where cytoplasmic mutant AR accumulation was most prominent (Table 2). TGN46 and 1C2 were co-localized (Fig. 3), indicating that mutant AR exists in the Golgi apparatus. Spinal dorsal root ganglia neurons exhibit some granular structures immunoreactive for another Golgi apparatus marker anti-Golgi 58K protein antibody in the cytoplasm (Fig. 1P and Q). Other organelle markers, including antibodies for lysosomes, endoplasmic reticulum and mitochondria, did not show co-localization with 1C2 (Fig. 3), indicating that expanded polyQ sequences were not detected in these organelles.

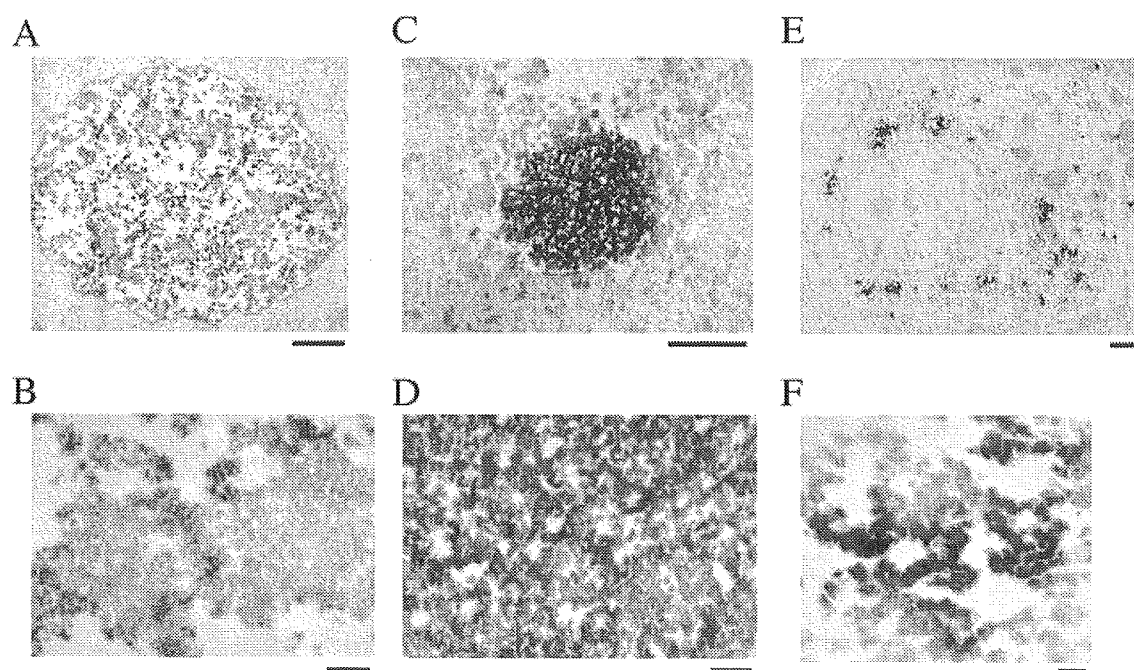


Fig. 2 Electron microscopic immunohistochemical study of nuclear inclusions in motor neurons, and of diffuse nuclear staining and cytoplasmic inclusions in sensory neurons. Electron microscopic immunohistochemistry using 1C2 demonstrated amorphous aggregates corresponding to diffuse nuclear staining in the spinal dorsal root ganglia (A and B), and granular dense aggregates without fibrous configurations corresponding to nuclear and cytoplasmic inclusions in the spinal anterior neuron (C and D) and spinal dorsal root ganglia (E and F). Scale bars = 2 μ m for A, C and E; and 200 nm for B, D and F.

Correlation of diffuse nuclear accumulation and NIs with degree of CAG repeat expansion

We examined the correlation of diffuse nuclear accumulation and NIs with the degree of CAG repeat expansion in anterior and posterior horn spinal cord neurons. Averaged frequencies of diffuse nuclear accumulations and NIs in cervical, thoracic and lumbar spinal segments were evaluated for correlation with numbers of CAG repeats in the *AR* gene. The frequency of diffuse nuclear accumulation in anterior and posterior horn neurons correlated well with the degree of CAG repeat expansion (Fig. 4; $r = 0.78$, $P < 0.05$ and $r = 0.69$, $P < 0.05$, respectively). However, the frequency of NIs in motor neurons and posterior horn neurons did not show a significant correlation with number of CAG repeats (Fig. 4; $r = 0.05$, $P = \text{NS}$ and $r = -0.14$, $P = \text{NS}$, respectively). These observations strongly suggest that diffuse nuclear accumulation of the mutant *AR* protein is more important pathogenetically than NIs.

Immunohistochemical localization of mutant AR in non-neural tissues

As in neural tissues, diffuse nuclear accumulations, NIs or cytoplasmic accumulations of mutant *AR* were observed in certain visceral organs and skin (Fig. 5, Table 3). Diffuse nuclear accumulations and NIs were detected in the liver, proximal tubules of the kidney, testis, prostate gland, and scrotal and other skin (Fig. 5, Table 3). Cytoplasmic

accumulations were detected in the liver, pancreatic islets of Langerhans, testis and prostate gland (Fig. 5, Table 3). Nuclear labelling and cytoplasmic accumulation both were absent in the pituitary gland, heart, lung, intestine, spleen, thyroid, adrenal gland and skeletal muscles. Pancreatic islet cells showed exclusively cytoplasmic accumulations without detectable nuclear accumulations, suggesting that the impaired glucose tolerance frequently observed in our patients (Table 1) could be attributed to cytoplasmic mutant *AR* accumulation. Ubiquitin staining detected only NIs and, as observed in neural tissues, anti-human *AR* antibodies occasionally showed diffuse nuclear accumulation without cytoplasmic staining (Fig. 5J). The five control cases did not show any 1C2 immunoreactivity in viscera or skin.

Discussion

The present study clearly demonstrated that diffuse nuclear accumulation of mutant *AR*, detected by the antibody 1C2 which specifically recognizes the expanded polyQ tract, occurred more frequently than NIs in neural and non-neural tissues in SBMA patients. In neural tissues, diffuse nuclear mutant *AR* accumulation occurred in the basal ganglia, thalamus, hypothalamus, various midbrain, pontine and medullary nuclei, posterior horn, intermediolateral and Clarke's nuclei of the spinal cord and in sensory and sympathetic ganglion neurons, as well as brainstem and spinal cord motor neurons. NIs detected by 1C2 were similar

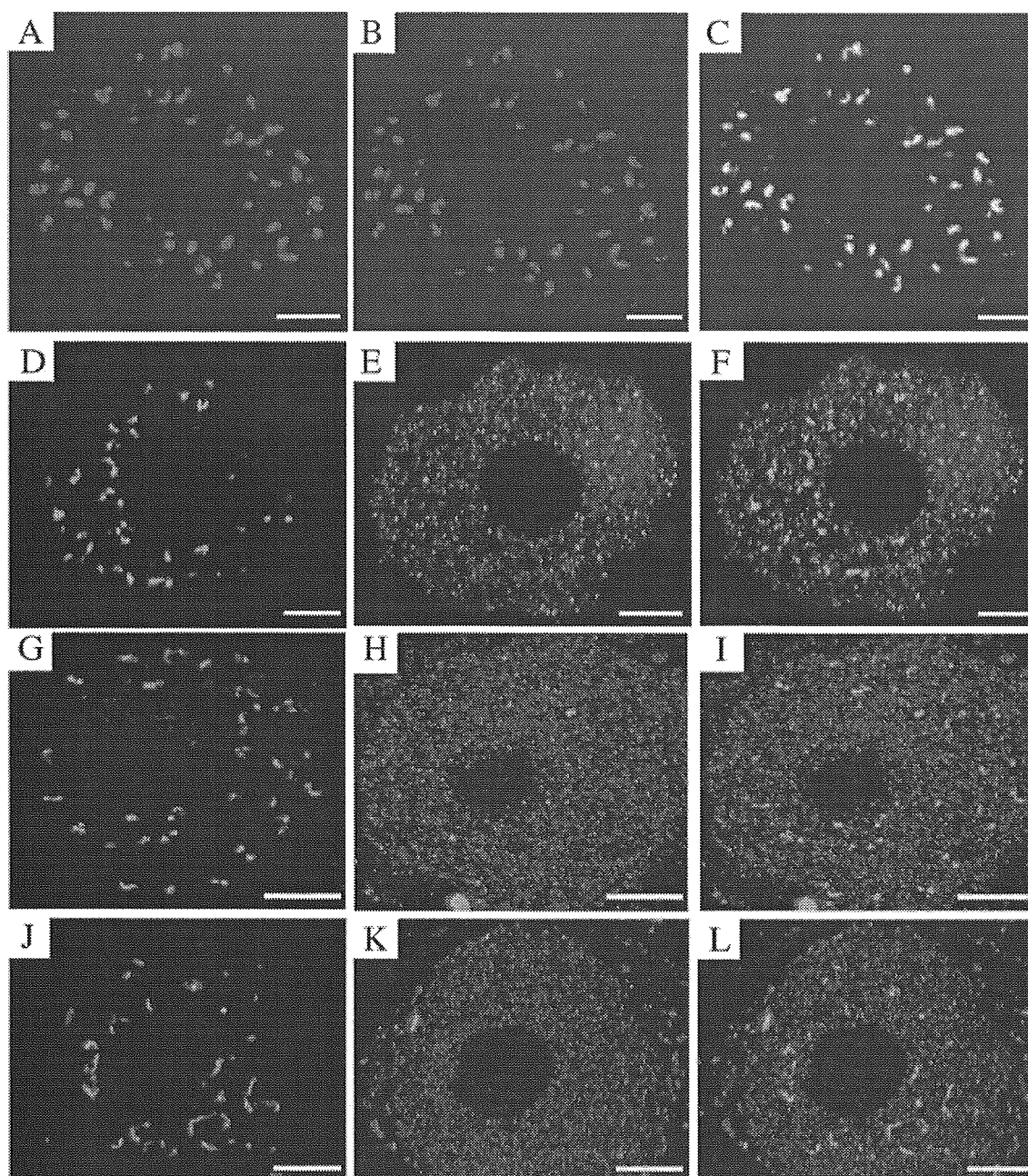


Fig. 3 Co-localization of organelles with mutant AR accumulation. Double immunofluorescence staining with antibodies against TGN46 and expanded polyQ reveals that TGN46 and mutant androgen receptor (AR) are co-localized, as shown for (A) expanded polyQ (green), (B) TGN46 (red) and (C) superimposition of the two signals (yellow) in neurons of the spinal dorsal root ganglia in SBMA, suggesting that mutant AR exists in the Golgi apparatus. Cytoplasmic co-localization of cathepsin B (E), GRP78 (H) and mitochondria (K) with mutant AR (D, G and J) is not observed in dorsal root ganglia (shown in F, I and L), suggesting that the endoplasmic reticulum, lysosomes and mitochondria are unassociated with mutant AR. Scale bars = 10 μ m for A–L.

in distribution to diffuse nuclear accumulation, but the frequency of NIs in each tissue was far less than for diffuse nuclear accumulation. We previously demonstrated that diffuse nuclear mutant AR protein accumulation was more extensive than NIs in male SBMA transgenic mice (Katsuno *et al.*, 2002, 2003). Furthermore, expression and severity of motor dysfunction, and abatement of abnormalities when the mice were castrated or given leuproterin, paralleled the extent

of diffuse nuclear mutant AR accumulation rather than that of NIs (Katsuno *et al.*, 2002, 2003). Accordingly, neuronal dysfunction appeared to be closely related to diffuse nuclear mutant AR accumulation. The key observation in the present study was a significant close correlation between frequency of diffuse nuclear mutant AR accumulation and length of CAG repeat expansion, while a similar correlation was not observed between frequency of NIs and CAG repeats. Diffuse nuclear

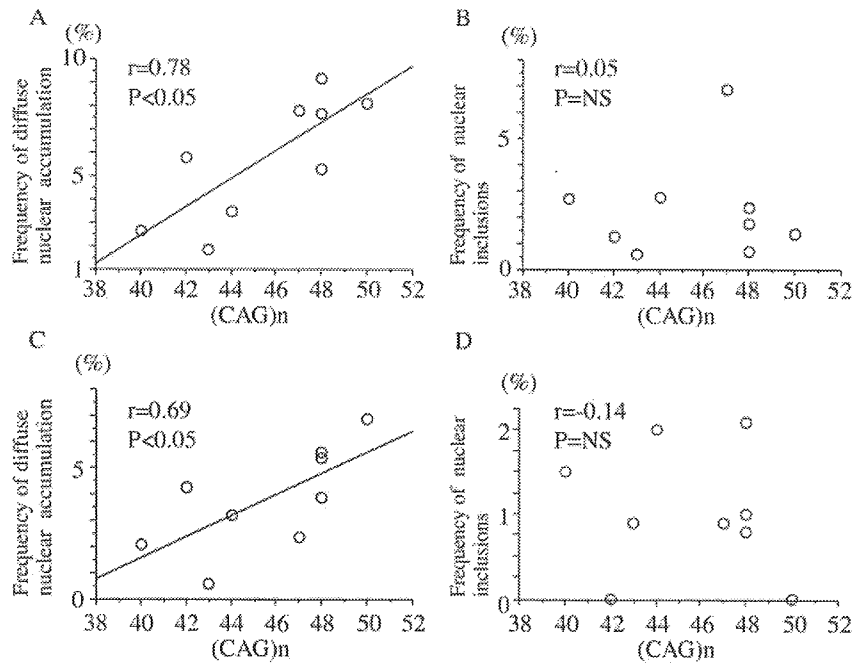


Fig. 4 The relationship of the number of CAG repeats to the frequency of diffuse nuclear accumulation and nuclear inclusions. Diffuse nuclear staining correlates significantly with the number of CAG repeats in anterior horn neurons (A) and posterior horn neurons (C). On the other hand, nuclear inclusions do not correlate significantly with the number of CAG repeats in the anterior horn neurons (B) or posterior horn neurons (D).

mutant protein accumulation also has been demonstrated in the neural tissues affected by DRPLA (Yamada *et al.*, 2001a, 2002a), Huntington's disease (Sapp *et al.*, 1997) and Machado–Joseph disease (Yamada *et al.*, 2001b) as well as corresponding transgenic mouse models (Schilling *et al.*, 1999; Yvert *et al.*, 2000; Lin *et al.*, 2001); and, here too, diffuse nuclear mutant protein accumulation was more widespread and extensive than NIs in DRPLA patients. These observations are in good agreement with our present observation in SBMA patients; together, they suggest that diffuse nuclear accumulation of mutant proteins with an expanded polyQ tract is an early event prior to NI formation that is closely related to manifestation of neuronal dysfunction (Yamada *et al.*, 2001a; Garden *et al.*, 2002; Katsuno *et al.*, 2002, 2003; Watase *et al.*, 2002; Yoo *et al.*, 2003). However, the molecular pathogenetic process by which diffuse nuclear mutant AR accumulation induces neuronal dysfunction still is unclear. Although considerable controversy recently surrounds the importance of NIs in the pathophysiology in polyQ diseases (Simeoni *et al.*, 2000; Walcott and Merry, 2002b; Bates, 2003; Michalik and Van Broeckhoven, 2003; Ross *et al.*, 2003), our data showed that diffuse mutant AR accumulation in nuclei could have potent cytotoxic effects inducing neuronal dysfunction through an active epitope of the expanded polyQ tract.

Anti-AR antibodies showed the ability to detect NIs, and some of them (H280, N-20 and Ab-1) occasionally stained diffuse nuclear accumulations. Diffuse nuclear accumulation had the appearance of amorphous aggregates of mutant AR as observed by electron microscopic immunohistochemistry

using 1C2 (Fig. 2) (Katsuno *et al.*, 2002). These observations suggest that the polyQ tract epitope can be detected by 1C2, while other AR protein epitopes may be protected by structural features of the aggregate state of the mutant AR. This view is supported by observations made by small-angle X-ray scattering and infrared spectroscopy carried out with myoglobin protein containing an inserted highly expanded polyQ tract, localizing the polyQ tract to the surface of aggregates, while other epitopes were sequestered within aggregates (Tanaka *et al.*, 2001, 2003). These observations suggest that 1C2 can detect the amorphous aggregate state of mutant AR protein, making 1C2 a more sensitive histological and pathophysiological marker than anti-AR protein antibodies. On the other hand, cytoplasmic accumulations were not seen with anti-AR antibodies. This cytoplasmic mutant AR was not ubiquitinated, in contrast to nuclear accumulated mutant AR, particularly the heavily ubiquitinated NIs, suggesting that protein modification varies between the nucleus and cytoplasm. Different protein modification might mask other AR protein epitopes directly or through structural alterations of the aggregate state of the mutant AR in the cytoplasm.

Another important observation in our study was the occurrence of cytoplasmic mutant AR accumulation in neural and non-neural tissues. In neural tissues, cytoplasmic accumulation was restricted to certain neuronal populations such as dorsal root ganglia neurons, mammillary body, hypothalamus, facial motor nucleus, and anterior and posterior horn neurons. In non-neural tissues, cytoplasmic accumulation also occurred in certain organs. Cytoplasmic mutant AR accumulation co-localized with a Golgi apparatus marker.

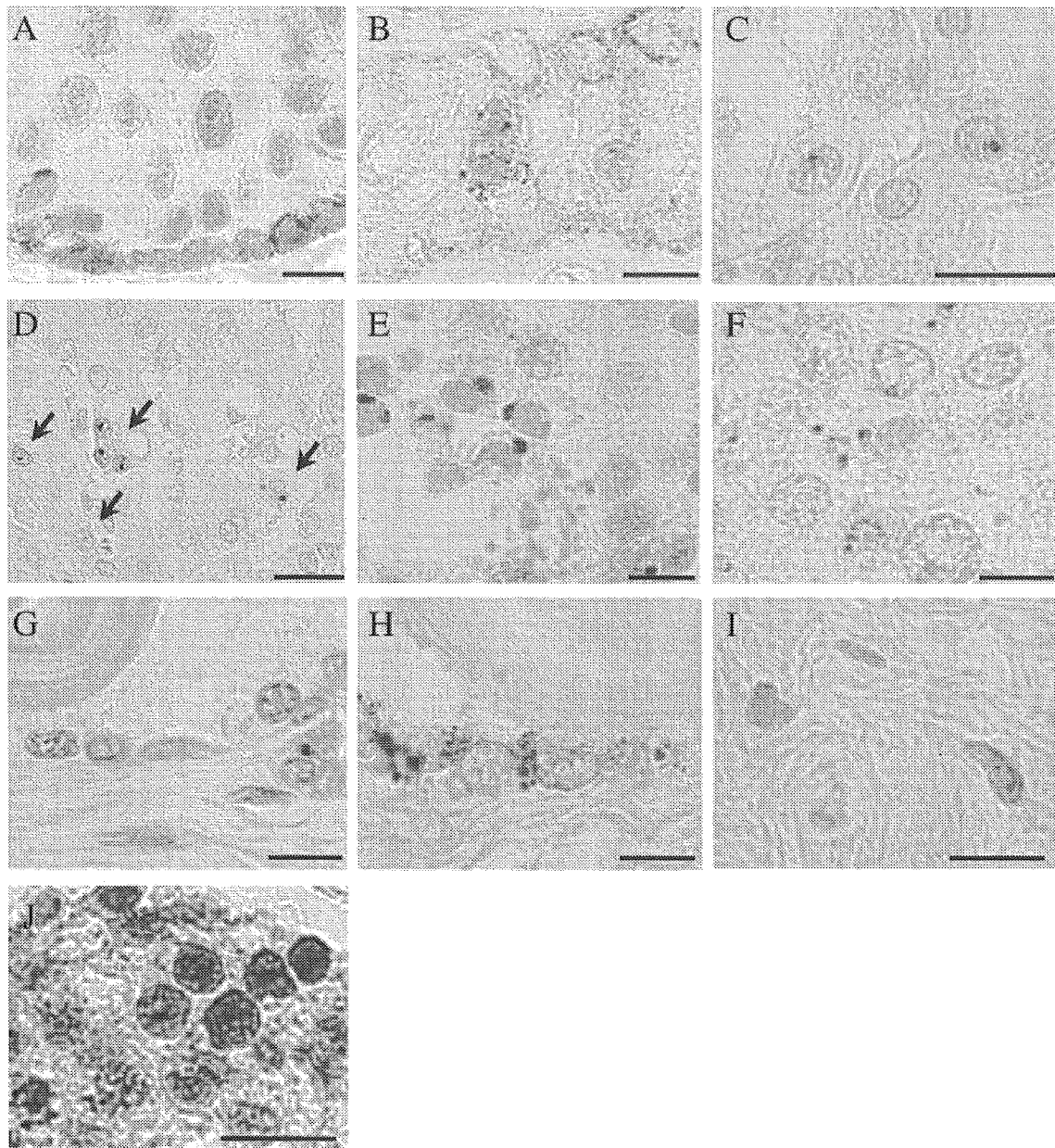


Fig. 5. Immunohistochemical study of the non-neural tissues in SBMA patients using 1C2 antibody. In the non-neural tissues, diffuse nuclear staining and nuclear inclusions are detectable in scrotal skin (A), other skin (B), proximal tubules of the kidney (C), hepatocytes (D), Sertoli cells of the testis (E), and glandular epithelium (G) and fibroblasts of the interstitial connective tissue (I) of the prostate gland. Moreover, cytoplasmic inclusions can be detected in hepatocytes (D), spermatocytes in the testis (E), pancreatic islets of Langerhans (F) and glandular epithelium of the prostate gland (H). Diffuse nuclear staining is also present using H280 antibody in Sertoli cells of the testis (J). Scale bars = 10 μm for A–C and E–I; and 20 μm for D and J.

Co-localization of a polyQ-expanded mutant protein with the Golgi apparatus has also been reported for ataxin-2 (Huynh *et al.*, 2003), although the significance of this localization remains unclear. Expression of polyQ-expanded mutant ataxin-2 disrupted the normal morphology of the Golgi complex and increased cell death (Huynh *et al.*, 2003). On the other hand, the lysosomal occurrence of other mutant proteins with an expanded polyQ tract has been reported in DRPLA (Yamada *et al.*, 2002b) and Huntington's disease (Sapp *et al.*, 1997). The lysosomal localization of polyQ-expanded mutant

proteins suggests a lysosomal autophagic degradation process acting independently of the ubiquitin–proteasome pathway in the polyQ diseases (Sapp *et al.*, 1997). Additionally, the reason why neural tissues develop more nuclear than cytoplasmic accumulation while most involved visceral organs show equal or predominantly cytoplasmic accumulation is unknown. Differences in the predominant degradation pathway dealing with the mutant AR could influence the intracellular site of accumulation and eventual cell toxicity. One important question is whether cytoplasmic mutant AR

Table 3 Immunohistochemical distribution of mutant AR in the non-neural tissues of patients with SBMA

Region	Nuclear accumulation		Cytoplasmic accumulation
	Diffuse nuclear accumulation	NI	
Pituitary gland	—	—	—
Heart	—	—	—
Lung	—	—	—
Liver	+	+	+
Kidney	+ to ++	+	—
Pancreas	—	—	+ to ++
Intestine	—	—	—
Spleen	—	—	—
Thyroid	—	—	—
Adrenal gland	—	—	—
Testis	+ to ++	+	+ to ++
Prostate gland	+ to +++	+ to ++	+ to +++
Skeletal muscle	—	—	—
Scrotal skin	+ to +++	+	—
Skin	+ to ++	+	—

Frequency of cells expressing polyglutamine immunoreactivity: —, 0%; +, 0–3%; ++, 3–6%; +++, 6%.

accumulation exerts cytotoxicity in neural and non-neural tissues. Cytoplasmic mutant AR accumulation (Taylor *et al.*, 2003) as well as other mutant protein accumulations (Kegel *et al.*, 2000; Ravikumar *et al.*, 2002; Huynh *et al.*, 2003) involving an expanded polyQ tract in Golgi apparatus and lysosomes indeed has been found to induce cytotoxicity. Accumulation of mutant protein with expanded polyQ in the Golgi apparatus or lysosomes increases death of cultured cells through activation of apoptosis-related effectors such as caspase-3 (Ishisaka *et al.*, 1998; Kegel *et al.*, 2000; Huynh *et al.*, 2003). One should note that histologically or immunohistochemically evident mutant protein accumulation is not necessarily cytotoxic, while microaggregates at the molecular level that are histologically undetectable can also exert cytotoxicity. Indeed, excessive accumulation of mutant AR in aggresomes was found to protect cells from a cytotoxic form of mutant AR (Taylor *et al.*, 2003). However, our present study strongly suggests that these cytoplasmic mutant AR accumulations may be related to mutant AR-mediated cytotoxicity and eventual symptom manifestation. For instance, the pancreas showed only cytoplasmic mutant AR accumulation without obvious nuclear accumulation. Elevated serum glucose and impaired glucose tolerance were present in most of our patients, suggesting islet cell dysfunction in the pancreas. The frequency of cytoplasmic accumulations of mutant AR in pancreatic islet cells did not show a significant correlation with fasting blood glucose levels in the examined SBMA patients (data not shown), while certain symptoms and signs of SBMA apparently can be induced by cytoplasmic accumulation of mutant AR protein. Although further study of the significance of cytoplasmic mutant AR accumulation is needed, nuclear accumulation of the mutant AR protein

appears to cause motor neuron dysfunction while cytoplasmic accumulation may underlie some visceral and possibly some neuronal dysfunction in SBMA. The pathological process is likely to differ between tissues, being more prominent in motor neuron nuclei, but mainly cytoplasmic in certain neuronal populations and visceral organs. We also need to clarify further which degradation process affecting mutant AR is most active in a given tissue, e.g. lysosomal in certain viscera versus via ubiquitination pathway in most neural tissues.

An important question here is why diffuse nuclear and possibly cytoplasmic accumulation of the mutant AR in the neuronal tissues beyond the major affected spinal and brainstem motor neurons has no apparent symptomatic involvement. First, the causative lesions for sensory impairment and essential-type tremor in SBMA patients have not yet been clearly substantiated. The novel lesion distribution of SBMA neurons shown in the present study, such as the posterior horn of the spinal cord, dorsal root ganglia, thalamus and cerebellum, might provide some explanations for these clinicopathological problems that have not been resolved. Since the cerebellothalamocortical pathway seems to be responsible for essential-type tremor (Pinto *et al.*, 2003), these lesions might contribute to mostly subclinical but definite sensory impairment and essential-type tremor in SBMA. Secondly, the occurrence of neuronal nuclear and cytoplasmic abnormalities in both clinically affected and non-affected neural regions in SBMA suggests that this alteration does not always induce neuronal cell dysfunction or death. The selective neuronal loss and dysfunction in neural lesions that are characteristic of SBMA might depend on additional factors that are specific to neurons in these systems. Recent studies have demonstrated that CREB-binding protein (CBP) is sequestered in AR-positive NIs, resulting in a decrease in CBP-dependent transcription (McC Campbell *et al.*, 2000), and further histone acetylation is reduced in affected cells (McC Campbell *et al.*, 2001; Steffan *et al.*, 2001; Minamiyama *et al.*, 2004). These reports suggest that CBP-dependent transcriptional dysregulations may cause symptomatic neuronal dysfunction. Since CBP-dependent transcriptional control differs among neurons, this difference may show the lack of their symptomatic involvement in certain polyQ-containing neurons. Alternatively, the population of neurons with nuclear accumulation of mutant AR in the regions beyond the commonly affected lesions may not be simply enough to manifest the responsible symptoms. A precise neuronal cell count assay combined with assessment of nuclear mutant AR accumulation will be needed to clarify these clinicopathological problems.

Clearly, motor neuron impairment with nuclear accumulation of mutant AR is the major problem in SBMA. Thus, for a therapeutic strategy against motor neuron dysfunction in SBMA, nuclear accumulation of mutant AR should be the main target, as we demonstrated in transgenic mice treated with leuproterin. Cytoplasmic accumulation of mutant AR, on the other hand, should be considered a therapeutic target with respect to certain symptoms in SBMA patients.

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