

Table 6. Selected Genes Characterized by SOM (select each 15)

GeneBank	Gene Name	Fold Change (ALS/control)
SOM1/6: genes downregulated in ALS motor neurons		
NM_002695	Polymerase (RNA) II (DNA directed) polypeptide E (25kD)	0.20
M24857	Retinoic acid receptor gamma 1 (RAR- γ 1)	0.20
NM_002375	Microtubule-associated protein 4	0.20
NM_001651	Aquaporin 5	0.21
NM_003178	Synapsin II	0.22
NM_004624	Vasoactive intestinal peptide receptor 1	0.23
NM_001740	Calbindin 2, (29kD, calretinin)	0.24
M73812	G1/S-specific cyclin E (CCNE)	0.25
NM_003206	Transcription factor 21	0.25
NM_004082	Dynalectin 1 (p150)	0.30
U05012	TRK-C; NT-3 growth factor receptor precursor	0.31
NM_003632	Contactin associated protein 1	0.32
NM_005910	Microtubule-associated protein tau	0.49
NM_002373	Microtubule-associated protein 1A	0.51
NM_002442	Musashi (Drosophila) homolog 1	0.52
SOM24/25: genes upregulated in ALS motor neurons		
M60718	Hepatocyte growth factor (HGF)	3.42
L20814	Glutamate receptor subunit 2 (GLUR-2)	3.34
K02268	β -neoendorphin-dynorphin precursor	3.13
L19063	Glial cell line-derived neurotrophic factor (GDNF)	3.08
NM_005543	Insulin-like 3	2.79
J04088	DNA topoisomerase II alpha (TOP2A)	2.58
M22489	Bone morphogenetic protein 2A (BMP2A)	2.57
U51004	Hint protein; protein kinase C inhibitor	2.26
M87507	Caspase 1, interleukin-1 β convertase precursor	2.21
NM_006196	Poly(rC)-binding protein 1	2.16
L29511	Growth factor receptor-bound protein 2	2.04
U78798	TRAF6	1.98
NM_001229	Caspase 9, apoptosis-related cysteine protease	1.89
U84388	Caspase and rip adaptor with death domain (CRADD)	1.83
U13737	Caspase-3	1.77

Gene expression levels are expressed as means of fold-change, which is calculated by dividing the signals of each ALS sample by those of control samples, in the five patients with ALS. Genes listed in Tables 2 and 3 are excluded. SOM = self-organizing map.

KIAA0231 was one of the mostly overexpressed genes in ALS motor neurons, but the function of this gene product is not known.

In the greatly downregulated genes of interest related to transcription, EGR3, whose expression was remarkably reduced in motor neurons of ALS, is a zinc-finger immediate-early transcription factor that is important for neurotrophin-3 (NT-3) regulation. It is known that EGR3 knockout mice develop gait ataxia, scoliosis, resting tremors, and ptosis due to the degeneration of muscle spindles, through disruption of NT-3 regulation.³¹ In ALS motor neurons, TrkC receptor for NT-3 was underexpressed, whereas NT-3 expression was not changed. The finding about TrkC-null mutant and NT-3-null mutant mice show that NT-3-TrkC signaling is required to maintain Ia afferent central synapses of DRG neurons.³² The marked downregulation of EGR3 in spinal motor neurons may disrupt sensory-motor connections by decreasing NT-3-TrkC signaling, resulting in motor neuron degeneration.

For neurotrophic support for ALS motor neurons, this study showed the overexpression of CNTF, GDNF, and HGF involved in the functional category of cell signaling, suggesting that these neurotrophic factors would be secondarily and compensatorily upregulated after motor neuron degeneration. Indeed, GDNF expression has been reported to increase in the spinal cords and decrease in the muscles of sporadic ALS patients.³³ In contrast with these neurotrophic factors, midkine was one of the significantly downregulated neurotrophic factors. Because midkine plays important roles in promotion of neuronal survival as well as modulation of neuromuscular junctions,³⁴ its underexpression may be related to motor neuron degeneration. In addition, the gene expression of vascular endothelial growth factor, which has been identified as a critical factor for motor neuron degeneration,³⁵⁻³⁷ did not change significantly in gene expression in this study. SOD1 gene expression was not altered in spinal motor neurons and ventral horns. Moreover, the gene expres-

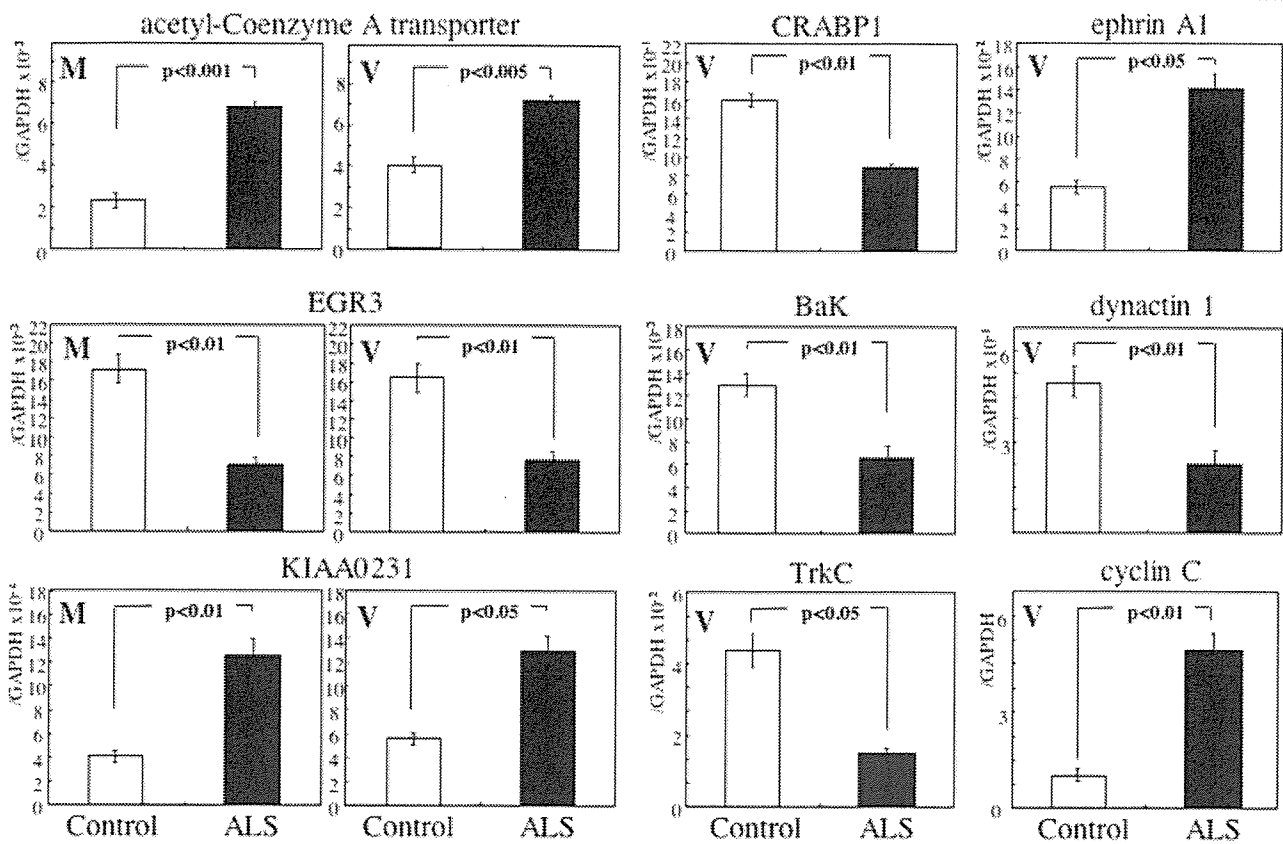


Fig 3. Quantitative real-time reverse transcription polymerase chain reaction verification of the selected genes. Expression levels of acetyl-coenzyme A transporter, EGR3, KIAA0231, CRABP1, BaK, TrkC, ephrin A1, cyclin C, dynactin 1, and GAPDH were measured using spinal motor neurons (M) and ventral horns (V), and those of the target genes were normalized against the GAPDH level. The relative expression levels are expressed as mean \pm standard error for 13 amyotrophic lateral sclerosis (ALS) cases and 11 controls. Gene names (Genbank accession number), primers and probe sequences (forward primer, reverse primer, and TaqMan probe; 5' to 3' sequence) were as follows [acetyl-CoA transporter: D88152] (GGGTTACTTTTGGGCAATG, AACGATTCCTCTGGGTTGAG, FAM-TTGGCCCTTGAATCTGCCGATAMRA); [BaK: NM_001188] (CTGGAAGATCAGCACCTTAAG, CCCCTCTCCTAGTAGGTCCTG, FAM-TGCTCCCAITTCCTCCCTCCG-TAMRA); [CRABP1: NM_004378] (TGCAGGAGTTTAGCCACTTG, CTCACGGGTCCAGTAGGTTT, FAM-TGAGAACAAGATCCACTGCACCCCA-TAMRA); [cyclin C: M74091] (TGAGCAGTGAAGAATTTCG, ACCCTGCTCCTTCACTGT, FAM-TGCCAAAACAAAACCCACTCCA-TAMRA); [dynactin 1: NM_004082] (ATGTGAATCGGGAACCTGACA, GGGCCCTTAGTCTCAGCAAAC, FAM-TG-GAGAGGCAACAGCAGCCAC-TAMRA); [EGR3: NM_004430] (CTTCCCCATGATTCCTGACT, TTGAATGCCTTGATG-GTCTC, FAM-TTCCAGGGCATGGACCCCAT-TAMRA); [ephrin A1: M57730] (GGCAAGGAGTTCAAAGAAGG, TCACCTTCAACCTCAAGCAG, FAM-CCATCCACCAGCATGAAGACCG-TAMRA); [GAPDH: NM_002046] (TCAAGAAG-GTGGTGAAGCAG, GGTGTGCTGTGTAAGTCAG, FAM-CCTCAAGGGCATCCTGGGCT-TAMRA); [KIAA0231: D86984] (CAACGGTCTTCCAGACAATG, GAGGTGACCAGCTGTGAGA, FAM-TCCCAGAGGTGAAGCTGCCCTC-TAMRA); and [TrkC: U05012] (TGAGAACCCCAAGTACTTCC, TCAGCACGATGTCTCTCCTC, FAM-CTGCCACAAGCCGGACACGT-TAMRA).

sion level of GluR2 was upregulated, as shown by its classification in SOM25, but the expression of its editing enzyme (adenosine deaminase, RNA-specific, 2; ADAR2) was not altered in this study, although the editing efficiency of GluR2 mRNA has been demonstrated to be low in spinal motor neurons of ALS.³⁸

Genes subject to transcriptional regulation constitute a crucial part of the whole human genome as demonstrated by human genome projects.³⁹ In addition to the gene expression of EGR3, the gene expression of

retinoic acid receptor α and γ together with cellular retinoic acid-binding protein 1 (CRABP1), and Musashi 1, all of which are known to be inducers of neuronal differentiation,^{40,41} was downregulated in spinal motor neurons of ALS. The dysregulation of retinoic acid receptor and retinol binding protein has been reported in the postmortem spinal cords of ALS and SOD1 mutant mice.^{17,26} These interesting results imply the potential involvement of the differentiation signals in maintaining motor neuron integrity, which

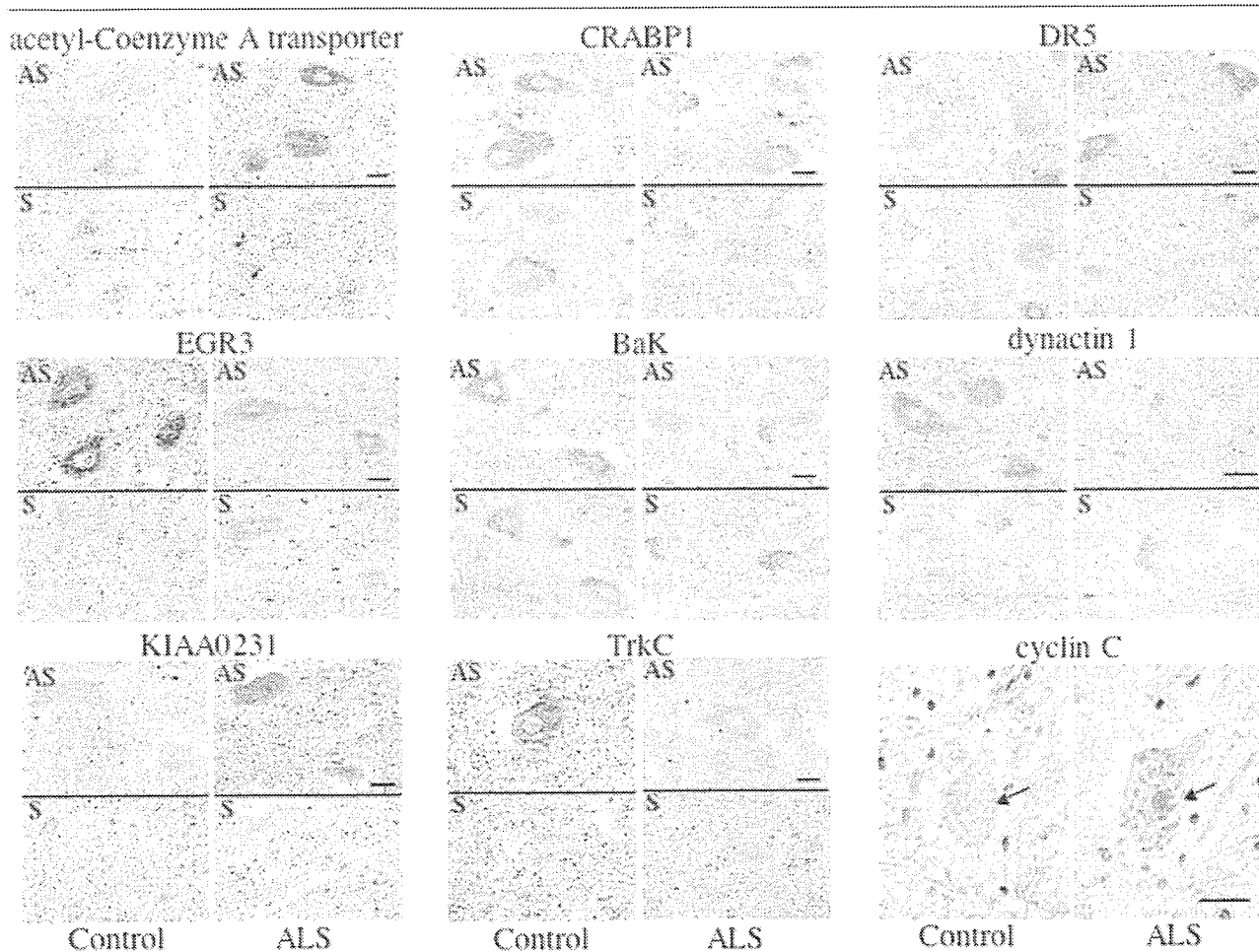


Fig 4. In situ hybridization and immunohistochemistry of the selected genes. Representative in situ hybridization is shown for acetyl-Coenzyme A transporter, EGR3, KIAA0231, CRABP1, Bak, TrkC, DR5, and dynactin 1. The antisense probe (AS) detects positive signals for the expression of each gene in spinal motor neurons for ALS and/or controls, but the sense probe (S) does not. Lipofuscin granules are seen as yellowish granules. Immunohistochemistry was performed for cyclin C, and nuclear staining was prominent in ALS motor neurons. Arrows denote the nuclei. Bars = 25 μ m.

might be impaired in the neurodegenerative process of ALS. Among the genes related to transcriptional regulation, the number of significantly downregulated genes was twofold greater than that of the upregulated ones in ALS motor neurons. These downregulated transcription-related genes were not restricted to genes regulating neuronal differentiation as neuron-specific cellular properties, but also included genes such as RNA polymerase and transcription factors, which regulate general cellular functions. These observations suggest that downregulation of transcriptional activity may be the reflection of motor neuron dysfunction because of a wide range of impairments of cellular maintenance systems.

Interestingly, the expression of dynactin 1, which recently has been identified as a causative gene for human motor neuron disease,⁴² was reduced in ALS motor neurons. Some other motor proteins including the kinesin family responsible for anterograde axonal trans-

port and dyneins for retrograde axonal transport were not changed significantly, but the expression levels of MAPs 1A, 4, and tau were decreased, shown by their classification in SOM1 and SOM6. The impairment of axonal transport is thought to be an early event of motor neuron degeneration, and especially the protein levels of MAPs 1A and tau have been reported to decrease early before the onset of symptoms in mutant SOD1 transgenic mice.⁴³⁻⁴⁵ The upregulation of ubiquitin-specific protease 11 (USP11), listed in Tables 2 and 3, also may be related to microtubule abnormality because the RanGTP-associated protein RanBPM, which is required for correct nucleation of microtubules, is the enzymatic substrate for USP11.⁴⁶ The present results imply that retrograde axonal transport, especially that associated with dynactin, might be affected even at the terminal stage of ALS and be crucial for motor neurons, although cytoskeletal proteins are the major functional group in the downregulated genes.

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.

This work was supported by a Center of Excellence grant from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and grants from the Ministry of Health, Labor and Welfare of Japan.

References

1. Ince PG, Lowe J, Shaw PJ. Amyotrophic lateral sclerosis: current issues in classification, pathogenesis and molecular pathology. *Neuropathol Appl Neurobiol* 1998;24:104-117.
2. Bunina TL. On intracellular inclusions in familial amyotrophic lateral sclerosis. *Korsakov J Neuropath Psychiat* 1962;62:1293-1296.
3. Okamoto K, Hirai S, Amari M, et al. Bunina bodies in amyotrophic lateral sclerosis immunostained with rabbit anti-cystatin C serum. *Neurosci Lett* 1993;162:125-128.
4. Cleveland DW, Rothstein JD. From Charcot to Lou Gehrig: deciphering selective motor neuron death in ALS. *Nat Rev Neurosci* 2001;2:806-819.
5. Bergeron C. Oxidative stress: its role in the pathogenesis of amyotrophic lateral sclerosis. *J Neurol Sci* 1995;129(suppl):81-84.
6. Sathasivam S, Ince PG, Shaw PJ. Apoptosis in amyotrophic lateral sclerosis: a review of the evidence. *Neuropathol Appl Neurobiol* 2001;27:257-274.
7. Hand CK, Rouleau GA. Familial amyotrophic lateral sclerosis. *Muscle Nerve* 2002;25:135-159.
8. Ishigaki S, Liang Y, Yamamoto M, et al. X-Linked inhibitor of apoptosis protein is involved in mutant SOD1-mediated neuronal degeneration. *J Neurochem* 2002;82:576-584.

9. Kawahara Y, Ito K, Sun H, et al. Glutamate receptors: RNA editing and death of motor neurons. *Nature* 2004;427:801.
10. Niwa J, Ishigaki S, Hishikawa N, et al. Dornin ubiquitylates mutant SOD1 and prevents mutant SOD1-mediated neurotoxicity. *J Biol Chem* 2002;277:36793–36798.
11. Hishikawa N, Niwa J, Doyu M, et al. Dornin localizes to the ubiquitylated inclusions in Parkinson's disease, dementia with Lewy bodies, multiple system atrophy, and amyotrophic lateral sclerosis. *Am J Pathol* 2003;163:609–619.
12. Luo J, Isaacs WB, Trent JM, Duggan DJ. Looking beyond morphology: cancer gene expression profiling using DNA microarrays. *Cancer Invest* 2003;21:937–949.
13. Alizadeh AA, Ross DT, Perou CM, van de Rijn M. Towards a novel classification of human malignancies based on gene expression patterns. *J Pathol* 2001;195:41–52.
14. Luo L, Salunga RC, Guo H, et al. Gene expression profiles of laser-captured adjacent neuronal subtypes. *Nat Med* 1999;5:117–122.
15. Ando Y, Liang Y, Ishigaki S, et al. Caspase-1 and -3 mRNAs are differentially upregulated in motor neurons and glial cells in mutant SOD1 transgenic mouse spinal cord: a study using laser microdissection and real-time RT-PCR. *Neurochem Res* 2003;28:839–846.
16. Ginsberg SD, Hemby SE, Lee VM, et al. Expression profile of transcripts in Alzheimer's disease tangle-bearing CA1 neurons. *Ann Neurol* 2000;48:77–87.
17. Yoshihara T, Ishigaki S, Yamamoto M, et al. Differential expression of inflammation- and apoptosis-related genes in spinal cords of a mutant SOD1 transgenic mouse model of familial amyotrophic lateral sclerosis. *J Neurochem* 2002;80:158–167.
18. Kamme F, Salunga R, Yu J, et al. Single-cell microarray analysis in hippocampus CA1: demonstration and validation of cellular heterogeneity. *J Neurosci* 2003;23:3607–3615.
19. Terao S, Sobue G, Hashizume Y, et al. Disease-specific patterns of neuronal loss in the spinal ventral horn in amyotrophic lateral sclerosis, multiple system atrophy and X-linked recessive bulbospinal neuronopathy, with special reference to the loss of small neurons in the intermediate zone. *J Neurol* 1994;241:196–203.
20. Sobue G, Hashizume Y, Yasuda T, et al. Phosphorylated high molecular weight neurofilament protein in lower motor neurons in amyotrophic lateral sclerosis and other neurodegenerative diseases involving ventral horn cells. *Acta Neuropathol (Berl)* 1990;79:402–408.
21. Bohm M, Wieland I, Schutze K, Rubben H. Microbeam MOMENT: non-contact laser microdissection of membrane-mounted native tissue. *Am J Pathol* 1997;151:63–67.
22. Watanabe H, Tanaka F, Doyu M, et al. Differential somatic CAG repeat instability in variable brain cell lineage in dentatorubral pallidoluysian atrophy (DRPLA): a laser-captured microdissection (LCM)-based analysis. *Hum Genet* 2000;107:452–457.
23. Schutze K, Lahr G. Identification of expressed genes by laser-mediated manipulation of single cells. *Nat Biotechnol* 1998;16:737–742.
24. Wang J, Delabie J, Aasheim H, et al. Clustering of the SOM easily reveals distinct gene expression patterns: results of a re-analysis of lymphoma study. *BMC Bioinformatics* 2002;3:36–44.
25. Ross ME, Zhou X, Song G, et al. Classification of pediatric acute lymphoblastic leukemia by gene expression profiling. *Blood* 2003;102:2951–2959.
26. Malaspina A, Kaushik N, de Belleroche J. Differential expression of 14 genes in amyotrophic lateral sclerosis spinal cord detected using gridded cDNA arrays. *J Neurochem* 2001;77:132–145.
27. Dangond F, Hwang D, Camelo S, et al. The molecular signature of late-stage human ALS revealed by expression profiling of post-mortem spinal cord gray matter. *Physiol Genomics* 2004;16:229–239.
28. Kanamori A, Nakayama J, Fukuda MN, et al. Expression cloning and characterization of a cDNA encoding a novel membrane protein required for the formation of O-acetylated ganglioside: a putative acetyl-CoA transporter. *Proc Natl Acad Sci USA* 1997;94:2897–2902.
29. Malisan F, Franchi L, Tomassini B, et al. Acetylation suppresses the proapoptotic activity of GD3 ganglioside. *J Exp Med* 2002;196:1535–1541.
30. Rapport MM, Donnenfeld H, Brunner W, et al. Ganglioside patterns in amyotrophic lateral sclerosis brain regions. *Ann Neurol* 1985;18:60–67.
31. Chen HH, Tourtellotte WG, Frank E. Muscle spindle-derived neurotrophin 3 regulates synaptic connectivity between muscle sensory and motor neurons. *J Neurosci* 2002;22:3512–3519.
32. McEers SC, Frank E. Formation of specific monosynaptic connections between muscle spindle afferents and motoneurons in the mouse. *J Neurosci* 1997;17:3138–3135.
33. Yamamoto M, Sobue G, Yamamoto K, et al. Expression of glial cell line-derived neurotrophic factor mRNA in the spinal cord and muscle in amyotrophic lateral sclerosis. *Neurosci Lett* 1996;204:117–120.
34. Zhou H, Muramatsu T, Halfter W, et al. A role of midline in the development of the neuromuscular junction. *Mol Cell Neurosci* 1997;10:56–70.
35. Oosthuysen B, Moons L, Storkebaum E, et al. Deletion of the hypoxia-response element in the vascular endothelial growth factor promoter causes motor neuron degeneration. *Nat Genet* 2001;28:131–138.
36. Lambrechts D, Storkebaum E, Morimoto M, et al. VEGF is a modifier of amyotrophic lateral sclerosis in mice and humans and protects motoneurons against ischemic death. *Nat Genet* 2003;34:383–394.
37. Gros-Louis F, Laurent S, Lopes AA, et al. Absence of mutations in the hypoxia response element of VEGF in ALS. *Muscle Nerve* 2003;28:774–775.
38. Kawahara Y, Ito K, Sun H, et al. Low editing efficiency of GluR2 mRNA is associated with a low relative abundance of ADAR2 mRNA in white matter of normal human brain. *Eur J Neurosci* 2003;18:23–33.
39. Venter JC, Adams MD, Myers EW, et al. The sequence of the human genome. *Science* 2001;291:1304–1351.
40. Colbert MC, Rubin WW, Linney E, LaMantia AS. Retinoid signaling and the generation of cellular diversity in the embryonic mouse spinal cord. *Dev Dyn* 1995;204:1–12.
41. Sakakibara S, Okano H. Expression of neural RNA-binding proteins in the postnatal CNS: implications of their roles in neuronal and glial cell development. *J Neurosci* 1997;17:8300–8312.
42. Puls I, Jonnakuty C, LaMonte BH, et al. Mutant dynactin in motor neuron disease. *Nat Genet* 2003;33:455–456.
43. Williamson TL, Cleveland DW. Slowing of axonal transport is a very early event in the toxicity of ALS-linked SOD1 mutants to motor neurons. *Nat Neurosci* 1999;2:50–56.
44. Zhang B, Tu P, Abtahian F, et al. Neurofilaments and orthograde transport are reduced in ventral root axons of transgenic mice that express human SOD1 with a G93A mutation. *J Cell Biol* 1997;139:1307–1315.
45. Farah CA, Nguyen MD, Julien JP, Lederc N. Altered levels and distribution of microtubule-associated protein before disease onset in a mouse model of amyotrophic lateral sclerosis. *J Neurochem* 2003;84:77–86.

46. Ideguchi H, Ueda A, Tanaka M, et al. Structural and functional characterization of the USP11 deubiquitinating enzyme, which interacts with the RanGTP-associated protein RanBPM. *Biochem J* 2002;367:87-95.
47. Hensley K, Floyd RA, Gordon B, et al. Temporal patterns of cytokines and apoptosis-related gene expression in spinal cords of the G93A-SOD1 mouse model of amyotrophic lateral sclerosis. *J Neurochem* 2002;82:365-374.
48. Hall ED, Oostveen JA, Gurney ME. Relationship of microglial and astrocytic activation to disease onset and progression in a transgenic model of familial ALS. *Glia* 1998;23:249-256.
49. Mu X, He J, Anderson DW, et al. Altered expression of bcl-2 and bax mRNA in amyotrophic lateral sclerosis spinal cord motor neurons. *Ann Neurol* 1996;40:379-386.
50. Ekegren T, Grundstrom E, Lindholm D, Aquilonius SM. Up-regulation of Bax protein and increased DNA degradation in ALS spinal cord motor neurons. *Acta Neurol Scand* 1999;100:317-321.
51. Ranganathan S, Bowser R. Alterations in G(1) to S phase cell-cycle regulators during amyotrophic lateral sclerosis. *Am J Pathol* 2003;162:823-835.
52. Malaspina A, de Belleruche J. Spinal cord molecular profiling provides a better understanding of amyotrophic lateral sclerosis pathogenesis. *Brain Res Brain Res Rev* 2004;45:213-229.
53. Sobue G, Sahashi K, Takahashi A, et al. Degenerating compartment and functioning compartment of motor neurons in ALS: possible process of motor neuron loss. *Neurology* 1983;33:654-657.

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

Hiroaki Adachi,¹ Masahisa Katsuno,¹ Makoto Minamiyama,¹ Masahiro Waza,¹ Chen Sang,¹ Yuji Nakagomi,² Yasushi Kobayashi,¹ Furniaki Tanaka,¹ Manabu Doyu,¹ Akira Inukai,¹ Mari Yoshida,³ Yoshio Hashizume³ and Gen Sobue¹

¹Department of Neurology, Nagoya University Graduate School of Medicine, Nagoya, ²Central Research Laboratories, School of Medicine, Aichi Medical University and ³Department of Neuropathology, Institute for Medical Sciences of Aging, Aichi Medical University of Aichi, Japan

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

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-pallidoluysian atrophy; CBP = CREB-binding protein

Received June 15, 2004. First revision August 23, 2004. Second revision November 26, 2004. Accepted November 30, 2004.
Advance Access publication January 19, 2005

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-pallidolusian 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 mm 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 nm 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

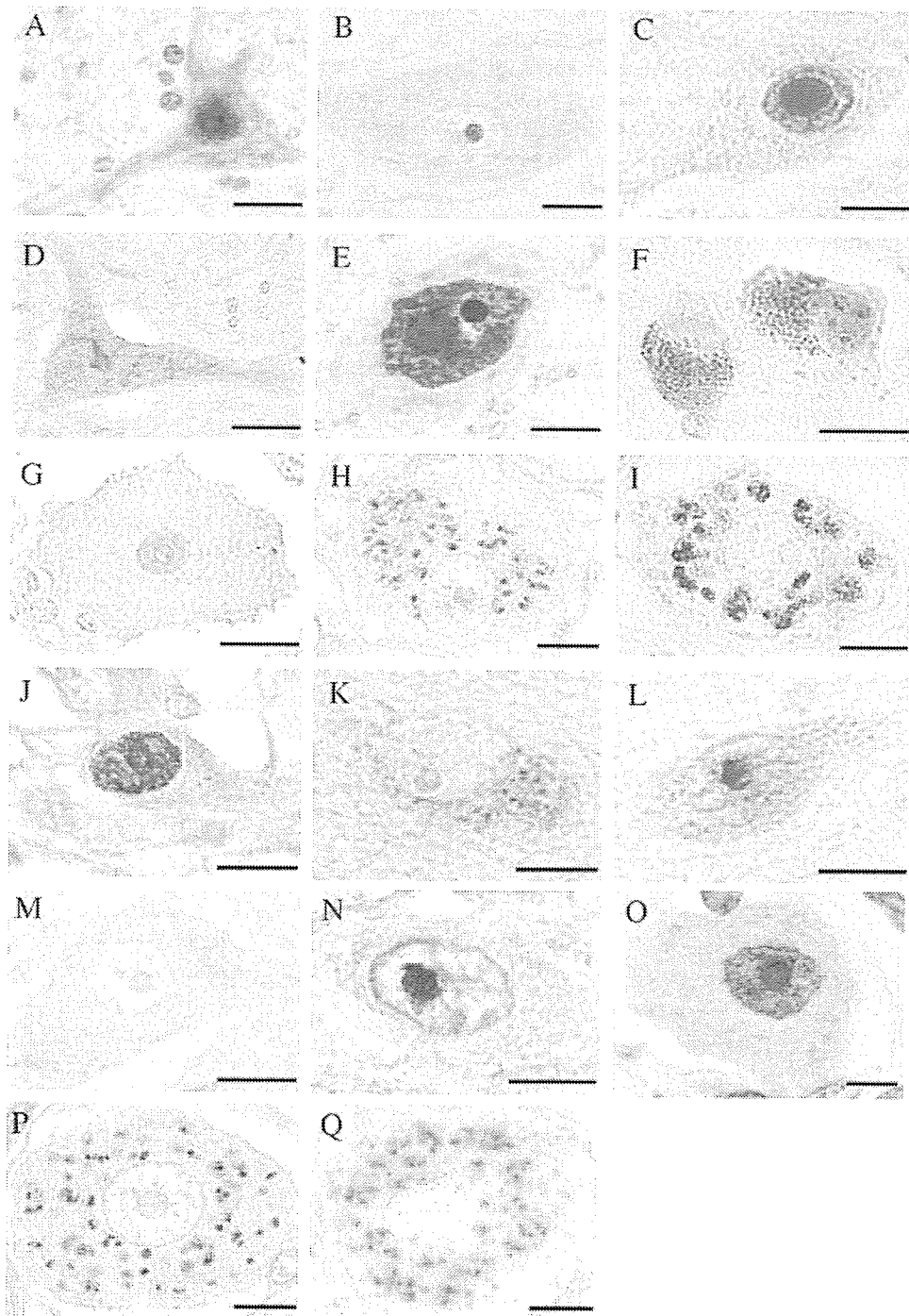


Fig. 1 Immunohistochemical analysis in the neural tissues from SBMA patients and control cases. In the CNS of SBMA patients, intense diffuse nuclear staining is present in neuronal nuclei of various regions using 1C2 antibody (A, E, F, G, J and L). Diffuse immunostaining of mutant androgen receptor (AR) 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 nm for A, D, F, G, M and O; and 10 nm 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

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.

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%.

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.

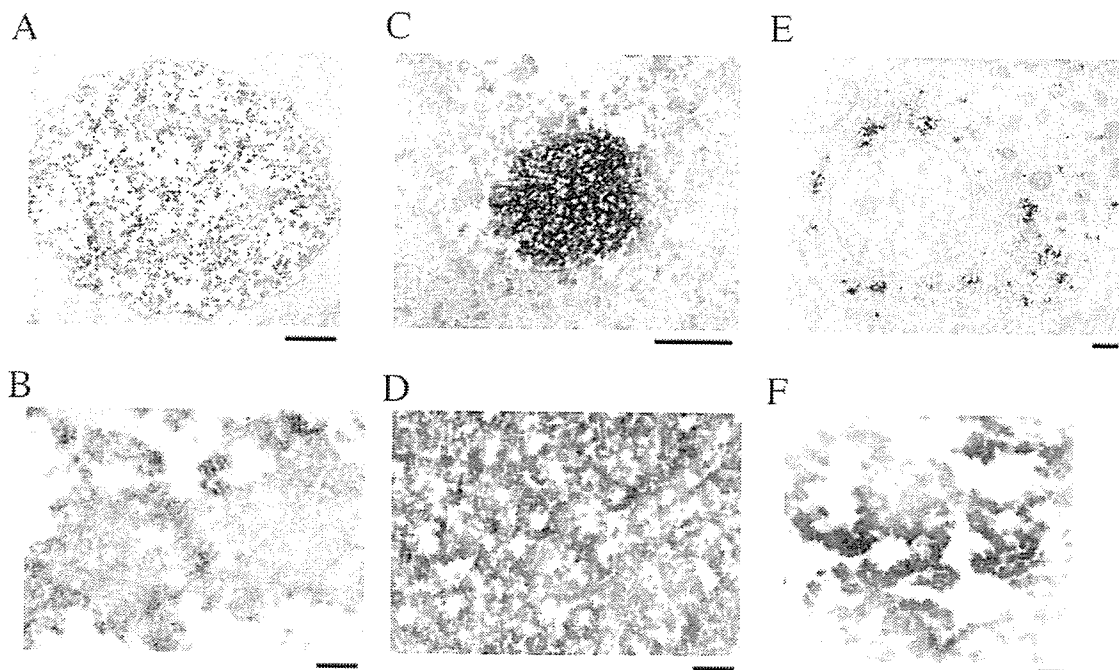


Fig. 2 Electron microscopic immunohistochemical study of nuclear inclusions in motor neurons, and of diffuse nuclear staining and cytoplasmic inclusions in sensory neurons. Electron microscopic immunohistochemistry using 1C2 demonstrated amorphous aggregates corresponding to diffuse nuclear staining 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 nm 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

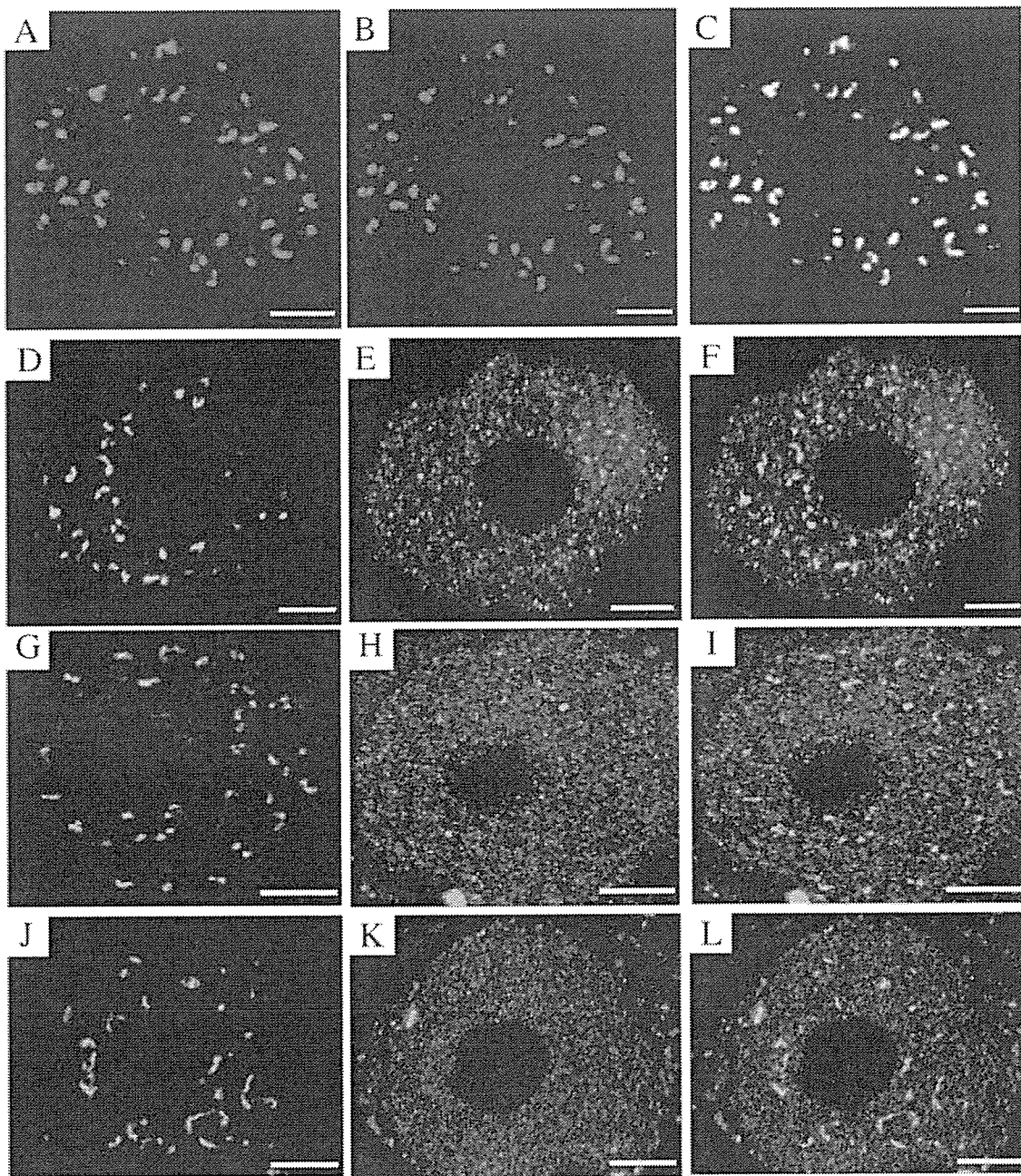


Fig. 3 Co-localization of organelles with mutant AR accumulation. Double immunofluorescence staining with antibodies against TGN46 and expanded polyQ reveals that TGN46 and mutant androgen receptor (AR) are co-localized, as shown for (A) expanded polyQ (green), (B) TGN46 (red) and (C) superimposition 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 nm 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

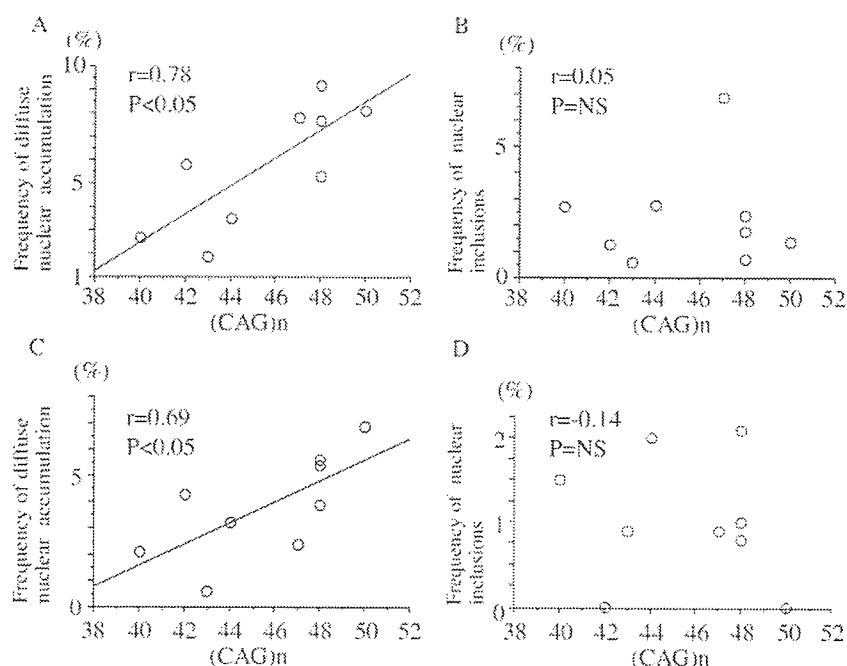


Fig. 4 The relationship of the number of CAG repeats to the frequency of diffuse nuclear accumulation and nuclear inclusions. Diffuse nuclear staining correlates significantly with the number of CAG repeats in anterior horn neurons (A) and posterior horn neurons (C). On the other hand, nuclear inclusions 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.

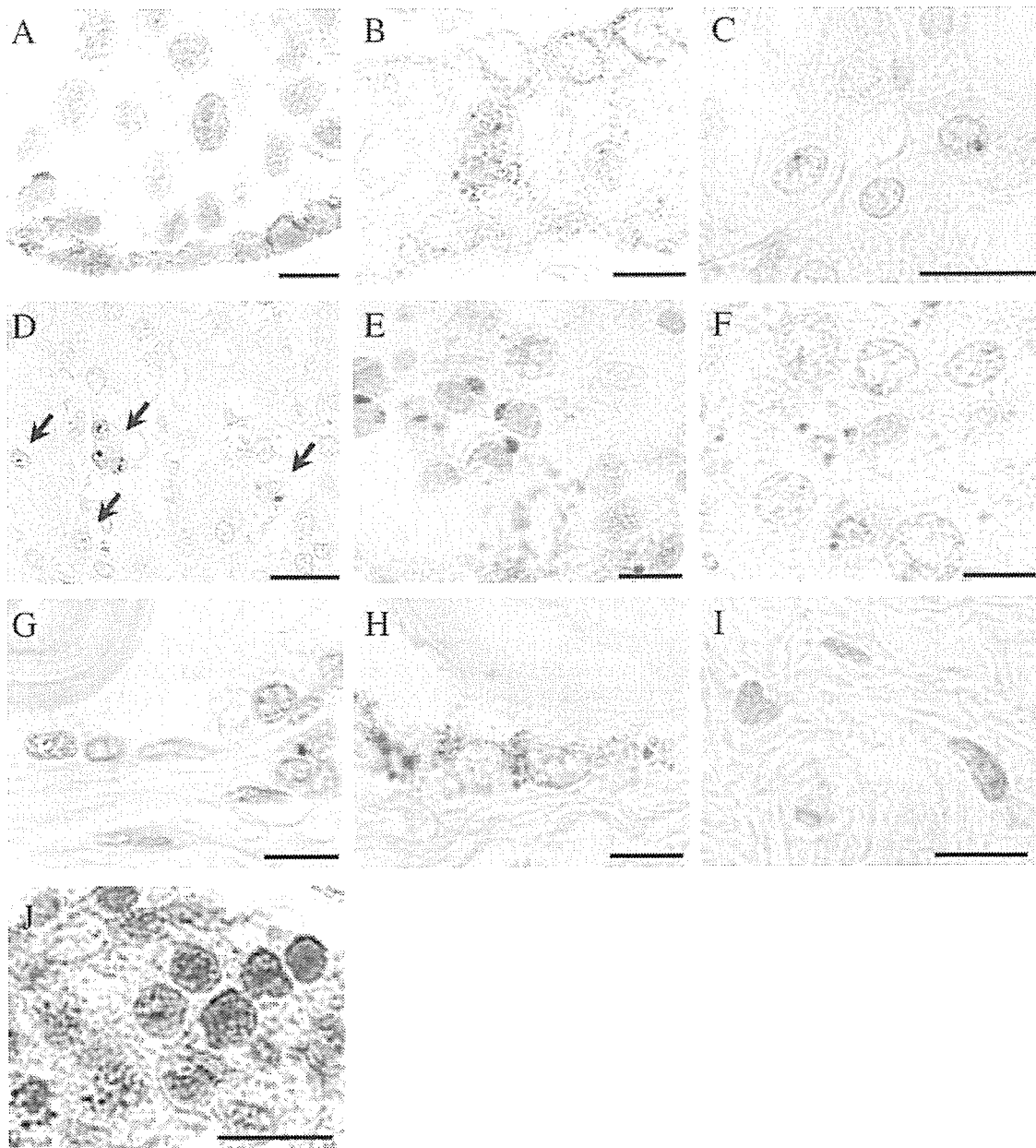


Fig. 5. Immunohistochemical study of the non-neural tissues in SBMA patients using 1C2 antibody. In the non-neural tissues, diffuse nuclear staining and nuclear inclusions are detectable in scrotal skin (A), other skin (B), proximal tubules of the kidney (C), hepatocytes (D), Sertoli cells of the testis (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 nm for A–C and E–I; and 20 nm 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 (McCampbell et al., 2000), and further histone acetylation is reduced in affected cells (McCampbell 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.

Acknowledgements

We wish to thank Dr Yasushi Iwasaki and Mrs Sugiko Yokoi for technical assistance. This work was supported by a Center of Excellence (COE) grant from the Ministry of Education, Culture, Sports, Science, and Technology of Japan, and grants from the Ministry of Health, Labor, and Welfare of Japan.

References

- Adachi H, Kume A, Li M, Nakagomi Y, Niwa H, Do J, et al. Transgenic mice with an expanded CAG repeat controlled by the human AR promoter show polyglutamine nuclear inclusions and neuronal dysfunction without neuronal cell death. *Hum Mol Genet* 2001; 10: 1039–48.
- Adachi H, Katsuno M, Minamiyama M, Sang C, Pagoulatos G, Angelidis C, et al. Heat shock protein 70 chaperone overexpression ameliorates phenotypes of the spinal and bulbar muscular atrophy transgenic mouse model by reducing nuclear-localized mutant androgen receptor protein. *J Neurosci* 2003; 23: 2203–11.
- Arbizu T, Santamaria J, Gomez JM, Quilez A, Serra JP. A family with adult spinal and bulbar muscular atrophy, X-linked inheritance and associated testicular failure. *J Neurol Sci* 1983; 59: 371–82.
- Bates G. Huntingtin aggregation and toxicity in Huntington's disease. *Lancet* 2003; 361: 1642–4.
- Chevalier-Larsen ES, O'Brien CJ, Wang H, Jenkins SC, Holder L, Lieberman AP, et al. Castration restores function and neurofilament alterations of aged symptomatic males in a transgenic mouse model of spinal and bulbar muscular atrophy. *J Neurosci* 2004; 24: 4778–86.
- Doyu M, Sobue G, Mukai E, Kachi T, Yasuda T, Mitsuma T, et al. Severity of X-linked recessive bulbospinal neuronopathy correlates with size of the tandem CAG repeat in androgen receptor gene. *Ann Neurol* 1992; 32: 707–10.
- Garden GA, Libby RT, Fu YH, Kinoshita Y, Huang J, Possin DE, et al. Polyglutamine-expanded ataxin-7 promotes non-cell-autonomous Purkinje cell degeneration and displays proteolytic cleavage in ataxic transgenic mice. *J Neurosci* 2002; 22: 4897–905.
- Huynh DP, Yang HT, Vakharina H, Nguyen D, Pulst SM. Expansion of the polyQ repeat in ataxin-2 alters its Golgi localization, disrupts the Golgi complex and causes cell death. *Hum Mol Genet* 2003; 12: 1485–96.
- Igarashi S, Tanno Y, Onodera O, Yamazaki M, Sato S, Ishikawa A, et al. Strong correlation between the number of CAG repeats in androgen receptor genes and the clinical onset of features of spinal and bulbar muscular atrophy. *Neurology* 1992; 42: 2300–2.
- Ishisaka R, Utsumi T, Yabuki M, Kanno T, Furuno T, Inoue M, et al. Activation of caspase-3-like protease by digitonin-treated lysosomes. *FEBS Lett* 1998; 435: 233–6.
- Katsuno M, Adachi H, Kume A, Li M, Nakagomi Y, Niwa H, et al. Testosterone reduction prevents phenotypic expression in a transgenic mouse model of spinal and bulbar muscular atrophy. *Neuron* 2002; 35: 843–54.
- Katsuno M, Adachi H, Doyu M, Minamiyama M, Sang C, Kobayashi Y, et al. Leuprorelin rescues polyglutamine-dependent phenotypes in a transgenic mouse model of spinal and bulbar muscular atrophy. *Nat Med* 2003; 9: 768–73.
- Kegel KB, Kim M, Sapp E, McIntyre C, Castano JG, Aronin N, et al. Huntingtin expression stimulates endosomal-lysosomal activity, endosome tubulation and autophagy. *J Neurosci* 2000; 20: 7268–78.
- Kennedy WR, Alter M, Sung JH. Progressive proximal spinal and bulbar muscular atrophy of late onset. A sex-linked recessive trait. *Neurology* 1968; 18: 671–80.
- La Spada AR, Wilson EM, Lubahn DB, Harding AE, Fischbeck KH. Androgen receptor gene mutations in X-linked spinal and bulbar muscular atrophy. *Nature* 1991; 352: 77–9.
- La Spada AR, Roling DB, Harding AE, Warner CL, Spiegel R, Hausmanowa-Petrusewicz I, et al. Meiotic stability and genotype-phenotype correlation of the trinucleotide repeat in X-linked spinal and bulbar muscular atrophy. *Nat Genet* 1992; 2: 301–4.
- Li M, Sobue G, Doyu M, Mukai E, Hashizume Y, Mitsuma T. Primary sensory neurons in X-linked recessive bulbospinal neuropathy: histopathology and androgen receptor gene expression. *Muscle Nerve* 1995; 18: 301–8.
- Li M, Miwa S, Kobayashi Y, Merry DE, Yamamoto M, Tanaka F, et al. Nuclear inclusions of the androgen receptor protein in spinal and bulbar muscular atrophy. *Ann Neurol* 1998a; 44: 249–54.
- Li M, Nakagomi Y, Kobayashi Y, Merry DE, Tanaka F, Doyu M, et al. Nonneural nuclear inclusions of androgen receptor protein in spinal and bulbar muscular atrophy. *Am J Pathol* 1998b; 153: 695–701.
- Lieberman AP, Harmison G, Strand AD, Olson JM, Fischbeck KH. Altered transcriptional regulation in cells expressing the expanded polyglutamine androgen receptor. *Hum Mol Genet* 2002; 11: 1967–76.
- Lin CH, Tallaksen-Greene S, Chien WM, Cearley JA, Jackson WS, Crouse AB, et al. Neurological abnormalities in a knock-in mouse model of Huntington's disease. *Hum Mol Genet* 2001; 10: 137–44.
- Mariotti C, Castellotti B, Pareyson D, Testa D, Eoli M, Antozzi C, et al. Phenotypic manifestations associated with CAG-repeat expansion in the androgen receptor gene in male patients and heterozygous females: a clinical and molecular study of 30 families. *Neuromuscul Disord* 2000; 10: 391–7.
- McCannell A, Taylor JP, Taye AA, Robitschek J, Li M, Walcott J, et al. CREB-binding protein sequestration by expanded polyglutamine. *Hum Mol Genet* 2000; 9: 2197–202.
- McCannell A, Taye AA, Whitty L, Penney E, Steffan JS, Fischbeck KH. Histone deacetylase inhibitors reduce polyglutamine toxicity. *Proc Natl Acad Sci USA* 2001; 98: 15179–84.
- Michalik A, Van Broeckhoven C. Pathogenesis of polyglutamine disorders: aggregation revisited. *Hum Mol Genet* 2003; 12: R173–86.
- Minamiyama M, Katsuno M, Adachi H, Waza M, Sang C, Kobayashi Y, et al. Sodium butyrate ameliorates phenotypic expression in a transgenic mouse model of spinal and bulbar muscular atrophy. *Hum Mol Genet* 2004; 13: 1183–92.
- Pinto AD, Lang AE, Chen R. The cerebellothalamocortical pathway in essential tremor. *Neurology* 2003; 60: 1985–7.
- Ravikumar B, Duden R, Rubinsztein DC. Aggregate-prone proteins with polyglutamine and polyalanine expansions are degraded by autophagy. *Hum Mol Genet* 2002; 11: 1107–17.
- Ross CA. Polyglutamine pathogenesis: emergence of unifying mechanisms for Huntington's disease and related disorders. *Neuron* 2002; 35: 819–22.
- Ross CA, Poirier MA, Wanker EE, Amzel M. Polyglutamine fibrillogenesis: the pathway unfolds. *Proc Natl Acad Sci USA* 2003; 100: 1–3.
- Sapp E, Schwarz C, Chase K, Bhide PG, Young AB, Penney J, et al. Huntingtin localization in brains of normal and Huntington's disease patients. *Ann Neurol* 1997; 42: 604–12.
- Schilling G, Wood JD, Duan K, Slunt HH, Gonzales V, Yamada M, et al. Nuclear accumulation of truncated atrophin-1 fragments in a transgenic mouse model of DRPLA. *Neuron* 1999; 24: 275–86.
- Schmidt BJ, Greenberg CR, Allingham-Hawkins DJ, Spriggs EL. Expression of X-linked bulbospinal muscular atrophy (Kennedy disease) in two homozygous women. *Neurology* 2002; 59: 770–2.
- Simeoni S, Mancini MA, Stenoi DL, Marcelli M, Weigel NL, Zanisi M, et al. Motoneuronal cell death is not correlated with aggregate formation of androgen receptors containing an elongated polyglutamine tract. *Hum Mol Genet* 2000; 9: 133–44.
- Sobue G, Hashizume Y, Mukai E, Hirayama M, Mitsuma T, Takahashi A. X-linked recessive bulbospinal neuronopathy. A clinicopathological study. *Brain* 1989; 12: 209–32.
- Sobue G, Doyu M, Kachi T, Yasuda T, Mukai E, Kumagai T, et al. Subclinical phenotypic expressions in heterozygous females of X-linked recessive bulbospinal neuronopathy. *J Neurol Sci* 1993; 117: 74–8.
- Sperfeld AD, Karitzky J, Brummer D, Schreiber H, Haussler J, Ludolph AC, et al. X-linked bulbospinal neuronopathy: Kennedy disease. *Arch Neurol* 2002; 59: 1921–6.
- Steffan JS, Bodai L, Pallos J, Poelman M, McCannell A, Apostol BL, et al. Histone deacetylase inhibitors arrest polyglutamine-dependent neurodegeneration in *Drosophila*. *Nature* 2001; 413: 739–43.

- Tanaka F, Doyu M, Ito Y, Matsumoto M, Mitsuma T, Abe K, et al. Founder effect in spinal and bulbar muscular atrophy (SBMA). *Hum Mol Genet* 1996; 5: 1253-7.
- Tanaka F, Reeves MF, Ito Y, Matsumoto M, Li M, Miwa S, et al. Tissue-specific somatic mosaicism in spinal and bulbar muscular atrophy (SBMA) is dependent on CAG repeat length and androgen receptor gene expression level. *Am J Hum Genet* 1999; 65: 966-73.
- Tanaka M, Morishima I, Akagi T, Hashikawa T, Nukina N. Intra- and intermolecular beta-plated sheet formation in glutamine-repeat inserted myoglobin as a model for polyglutamine diseases. *J Biol Chem* 2001; 276: 45470-5.
- Tanaka M, Machida Y, Nishikawa Y, Akagi T, Hashikawa T, Fujisawa T, et al. Expansion of polyglutamine induces the formation of quasi-aggregate in the early stage of protein fibrillization. *J Biol Chem* 2003; 278: 34717-24.
- Taylor JP, Tanaka F, Robitschek J, Sandoval CM, Taye A, Markovic-Plese S, et al. Aggresomes protect cells by enhancing the degradation of toxic polyglutamine-containing protein. *Hum Mol Genet* 2003; 12: 749-57.
- Terao S, Sobue G, Hashizume Y, Li M, Inagaki T, Mitsuma T. Age-related changes in human spinal ventral horn cells with special reference to the loss of small neurons in the intermediate zone: a quantitative analysis. *Acta Neuropathol (Berl)* 1996; 92: 109-14.
- Trottier Y, Lutz Y, Stevanin G, Imbert G, Devys D, Cancel G, et al. Polyglutamine expansion as a pathological epitope in Huntington's disease and four dominant cerebellar ataxias. *Nature* 1995; 378: 403-6.
- Walcott JL, Merry DE. Ligand promotes intranuclear inclusions in a novel cell model of spinal and bulbar muscular atrophy. *J Biol Chem* 2002a; 277: 50855-9.
- Walcott JL, Merry DE. Trinucleotide repeat disease. The androgen receptor in spinal and bulbar muscular atrophy. *Vitam Horm* 2002b; 65: 127-47.
- Watake K, Weeber EJ, Xu B, Antalffy B, Yuva-Paylor L, Hashimoto K, et al. A long CAG repeat in the mouse *Scal* locus replicates SCA1 features and reveals the impact of protein solubility on selective neurodegeneration. *Neuron* 2002; 34: 905-19.
- Yamada M, Wood JD, Shimohata T, Hayashi S, Tsuji S, Ross CA, et al. Widespread occurrence of intranuclear atrophin-1 accumulation in the central nervous system neurons of patients with dentatorubral-pallidoluysian atrophy. *Ann Neurol* 2001a; 49: 14-23.
- Yamada M, Hayashi S, Tsuji S, Takahashi H. Involvement of the cerebral cortex and autonomic ganglia in Machado-Joseph disease. *Acta Neuropathol (Berl)* 2001b; 101: 140-4.
- Yamada M, Sato T, Tsuji S, Takahashi H. Oligodendrocytic polyglutamine pathology in dentatorubral-pallidoluysian atrophy. *Ann Neurol* 2002a; 52: 670-4.
- Yamada M, Tsuji S, Takahashi H. Involvement of lysosomes in the pathogenesis of CAG repeat diseases. *Ann Neurol* 2002b; 52: 498-503.
- Yoo SY, Pennesi ME, Weeber EJ, Xu B, Atkinson R, Chen S, et al. SCA7 knock in mice model human SCA7 and reveal gradual accumulation of mutant ataxin-7 in neurons and abnormalities in short-term plasticity. *Neuron* 2003; 37: 383-401.
- Yvert G, Lindenberg KS, Picaut S, Landwehrmeyer GB, Sahel JA, Mandel JL. Expanded polyglutamines induce neurodegeneration and trans-neuronal alterations in cerebellum and retina of SCA7 transgenic mice. *Hum Mol Genet* 2000; 9: 2491-506.

The wide spectrum of clinical manifestations in Sjögren's syndrome-associated neuropathy

Keiko Mori,¹ Masahiro Iijima,¹ Haruki Koike,¹ Naoki Hattori,¹ Fumiaki Tanaka,¹ Hirohisa Watanabe,¹ Masahisa Katsuno,¹ Asako Fujita,² Ikuko Aiba,² Akihiko Ogata,³ Toyokazu Saito,⁴ Kunihiko Asakura,⁵ Mari Yoshida,⁶ Masaaki Hirayama¹ and Gen Sobue¹

¹Department of Neurology, Nagoya University Graduate School of Medicine, ²Department of Neurology, Higashi Nagoya Hospital, Nagoya, ³Department of Neurology, Hokkaido University School of Medicine, Sapporo, Japan, ⁴Rehabilitation Center, Kitasato University School of Medicine, Kanagawa, Japan, ⁵Department of Microbiology and Immunology, Kanazawa Medical College, Kanazawa, Japan and ⁶Department of Neuropathology, Institute for Medical Science of Aging, Aichi Medical University, Japan

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

We assessed the clinicopathological features of 92 patients with primary Sjögren's syndrome-associated neuropathy (76 women, 16 men, 54.7 years, age at onset). The majority of patients (93%) were diagnosed with Sjögren's **syndrome after neuropathic symptoms appeared. We classified these patients into seven forms of neuropathy:** sensory ataxic neuropathy (n=36), painful sensory neuropathy without sensory ataxia (n=18), multiple mononeuropathy (n=11), multiple cranial neuropathy (n=5), trigeminal neuropathy (n=15), autonomic neuropathy (n=3) and radiculoneuropathy (n=4), based on the predominant neuropathic symptoms. Acute or subacute onset was seen more frequently in multiple mononeuropathy and multiple cranial neuropathy, whereas chronic progression was predominant in other forms of neuropathy. Sensory symptoms without substantial motor involvement were seen predominantly in sensory ataxic, painful sensory, trigeminal and autonomic neuropathy, although the affected sensory modalities and distribution pattern varied. In contrast, motor weakness and muscle atrophy were observed in multiple mononeuropathy, multiple cranial neuropathy and radiculoneuropathy. Autonomic symptoms were often seen in all forms of neuropathy. Abnormal pupils and orthostatic hypotension were particularly frequent in sensory ataxic, painful, trigeminal and autonomic neuropathy. Unelicited somatosensory evoked potentials and spinal cord posterior column abnormalities in MRI were observed in sensory ataxic, painful and autonomic neuropathy. Sural nerve biopsy specimens (n=55) revealed variable **degrees of axon loss. Predominantly large fibre loss was observed in sensory ataxic neuropathy, whereas predominantly small fibre loss occurred in painful sensory neuropathy. Angiitis and perivascular cell invasion were seen most frequently in multiple mononeuropathy, followed by sensory ataxic neuropathy. The autopsy findings** of one patient with sensory ataxic neuropathy showed severe large sensory neuron loss paralleling to dorsal root and posterior column involvement of the spinal cord, and severe sympathetic neuron loss. Degrees of neuron loss in the dorsal and sympathetic ganglion corresponded to segmental distribution of sensory and sweating impairment. Multifocal T-cell invasion was seen in the dorsal root and sympathetic ganglion, perineurial space and vessel walls in the nerve trunks. Differential therapeutic responses for corticosteroids and IVIg were seen among the neuropathic forms. These clinicopathological observations suggest that sensory ataxic, painful and perhaps trigeminal neuropathy are related to ganglioneuronopathic process, whereas multiple mononeuropathy and multiple cranial neuropathy would be more closely associated with vasculitic process.

Keywords: angiitis; autonomic nerve dysfunction; ganglionopathy; neuropathy; Sjögren's syndrome

Abbreviations: CMAP = compound muscle action potential; DL = distal motor latency; MGB = meta-iodobenzylguanidine; MCV = motor nerve conduction velocity; NCS = nerve conduction studies; SNAP = sensory nerve action potential; SCV = sensory nerve conduction velocity; SEP = somatosensory evoked potential

Received April 19, 2005. Revised June 20, 2005. Accepted June 30, 2005. Advance Access publication July 27, 2005

Introduction

Primary Sjögren's syndrome is a systemic autoimmune disease characterized by xerophthalmia and xerostomia, and is associated with systemic visceral involvement, including pneumonitis, renal tubular acidosis, pancreatitis, myositis, and occasionally lymphocytic proliferation. A wide variety of neurological complications also are characteristic features of primary Sjögren's syndrome (Attwood et al., 1961; Alexander et al., 1982; Delalande et al., 2004). Peripheral neuropathy is a major neurological manifestation of Sjögren's syndrome and its aetiology has been considered to be vasculitis in the peripheral nerves, similar to that observed in other collagen diseases. In 1986 and 1990, it was demonstrated that dorsal root ganglionitis with degeneration of dorsal root ganglion neurons and mononuclear cell infiltration without vasculitis are associated with the sensory ataxic form of Sjögren's syndrome-associated neuropathy, suggesting that ganglion neurons themselves can be a target of Sjögren's syndrome (Malinow et al., 1986; Griffin et al., 1990).

Sjögren's syndrome-associated neuropathy has been shown to manifest as a variety of forms of neuropathy, including sensory ataxic neuropathy (Kennett et al., 1986; Griffin et al., 1990; Kaplan et al., 1990; Sobue et al., 1993), trigeminal neuropathy (Kaltreider et al., 1969), multiple mononeuropathy (Peyronnard et al., 1982; Molina et al., 1985), radiculoneuropathy (Gross et al., 1987; Grant et al., 1997), painful sensory neuropathy without sensory ataxia (Denislic et al., 1995; Mori et al., 2003), autonomic neuropathy with anhidrosis (Kumazawa et al., 1993; Goto et al., 2000) and multiple cranial neuropathy (Touze et al., 1999; Chu et al., 2000; Urban et al., 2001). While the wide spectrum of these neuropathies has been described in anecdotal reports or in studies of the systemic manifestations of Sjögren's syndrome (Mellgren et al., 1989; Gemignani et al., 1994; Mauch et al., 1994), the pathogenic mechanism responsible for most forms of Sjögren's syndrome-associated neuropathy remains unresolved. Furthermore, the spectrum of neuropathy and neuropathic symptoms of each form of neuropathy, particularly in the pathological and electrophysiological background, have not been well elucidated. In addition, since the prevalence of Sjögren's syndrome is growing in the elderly (Lafitte et al., 2001), Sjögren's syndrome-associated neuropathy also has become more prevalent. It is therefore necessary to re-evaluate the clinical spectrum, and pathological and electrophysiological features of Sjögren's syndrome-associated neuropathy.

In this study, we assessed the clinicopathological and electrophysiological features of a large number of patients associated with primary Sjögren's syndrome-associated neuropathy and determined the range of clinical manifestations of neuropathy.

Patients and methods

Patients

A total of 92 patients (76 women, 16 men; mean age, 59.7 years), all of Japanese descent, who fulfilled the diagnostic criteria for primary

Sjögren's syndrome and who had been referred to the Hospital of Nagoya University School of Medicine and its affiliated hospitals between 1985 and 2004 were the subjects of this study. The diagnosis of primary Sjögren's syndrome was established by the criteria proposed by the Diagnostic Committee of Health and Welfare of Japan (Fujibayashi et al., 1999) and by the American-European Community (Vitali et al., 2002). These criteria included symptoms of xerophthalmia and xerostomia, objective evidence of keratoconjunctivitis such as an abnormal Schirmer's test and an abnormal Rose Bengal score, evidence of chronic lymphocytic sialoadenitis on a minor salivary gland biopsy specimen, abnormal salivary gland scintigraphy or sialography, decreased salivary flow determined by a gum test and the presence of either anti-Sjögren's syndrome A or B (anti-SS-A or SS-B) autoantibodies (Fujibayashi et al., 1999; Vitali et al., 2002). Patients with other collagen diseases, such as systemic lupus erythematosus, rheumatoid arthritis, mixed connective tissue disease, progressive systemic sclerosis, polyarteritis nodosa, polymyositis and Churg–Strauss syndrome, diagnosed by the diagnostic criteria appropriate to each condition (Anonymous, 1980; Tan et al., 1982; Arnett et al., 1988; Masi et al., 1990) and designated as having secondary Sjögren's syndrome, were excluded from this study. Patients underwent neurological examinations, blood studies, CSF studies, nerve conduction studies (NCS), sural nerve biopsies, somatosensory evoked potentials (SEPs) and spinal MRI.

A patient with the sensory ataxic form of Sjögren's syndrome-associated neuropathy who died at 88 years of age underwent autopsy and histological examination.

Assessment of neurological symptoms, activities of daily living and autonomic nerve dysfunction

Neurological examinations for somatic motor and sensory symptoms were performed by at least one neurologist. Sensory examinations were performed for light touch, pinprick, vibratory sensation and joint position sense, as well as for the presence of sensory ataxia and pain or painful dysaesthesia. Muscle strength was assessed using the Medical Research Council scale. Cranial nerve function, Romberg's sign, walking pattern, deep tendon reflexes and pathological reflexes were also assessed. Autonomic symptoms were assessed as described elsewhere.

For the assessment of clinical disability on daily life the modified Rankin scale (van Swieten et al., 1988) was used. For the assessment of autonomic nerve dysfunction, we evaluated pupil abnormalities, including presence of Adie's pupils, anisocoria and elliptic pupils, urinary disturbances, diarrhoea and constipation, hypohidrosis and anhidrosis, orthostatic hypotension and ^{123}I -meta-iodobenzylguanidine (MIBG) cardiac accumulation. Autonomic symptoms generally were assessed by examining or interviewing patients or interviewing patients' family members, or reviewing the clinical records. Urinary symptoms were estimated by nocturnal or diurnal urinary frequency, a sensation of urgency, urinary incontinence, voiding difficulty and retention. Constipation was considered to be present if there were no stools for more than 3 days. Orthostatic hypotension was defined as a fall in systolic blood pressure of ≥ 30 mmHg upon standing from a recumbent position. For assessment of ^{123}I -MIBG cardiac accumulation, ^{123}I -MIBG 111 mBq (myo-MIBG- ^{123}I) for injection; Daiichi Radioisotope Laboratories Co.,