al,[3] our painful form of patients without obvious ataxia may be atypical, but we believe that this form should be considered for inclusion as paraneoplastic neuropathy.

Previously described painful neuropathies, such as familial amyloid polyneuropathy, the painful version of diabetic neuropathy, Sjögren syndrome–associated neuropathy, alcoholic polyneuropathy, and Fabry disease, are characterized by predominant small-fiber axonal degeneration with relative preservation of large myelinated fibers.[33,35-38] These pathologic findings are very similar to the findings in our painful cases, and suggest that painful symptoms in paraneoplastic neuropathy may be related to the impairment of small myelinated and unmyelinated fibers.

In the sensory ataxic form of paraneoplastic neuropathy, the loss of large sensory neurons in the DRG associated with lymphocytic infiltration, as well as the loss of large sensory axons in the central and peripheral rami, has been demonstrated in autopsied patients.[2,5,10,11] Therefore, the responsible primary lesion of paraneoplastic neuropathy may be a reduction of large DRG neurons due to some immune-inflammatory-mediated mechanism. Although further study is required, we postulate that the lesion responsible for a painful version of paraneoplastic neuropathy is also a loss of the small DRG neuron, which eventually leads to small fiber loss in peripheral nerves. The predominant neurologic symptoms in the upper limbs in painful patients also support this hypothesis.

In the painful cases, the main complaints involved severe painful sensations, with mechanical hyperalgesia being characteristic. However, other sensory modalities, including deep sensation or kinesthetic sensation, were also affected in these painful cases. In addition, painful sensation or painful dysesthesia was also present to some extent in the ataxic form patients. These observations suggest that these two forms of paraneoplastic neuropathy cover a continuous spectrum of pathophysiologic events with either the predominant impairment of small sensory neuro-axons or large neuro-axons as the extremes of the spectrum. Our findings of no obvious differences in age at onset, gender distribution, initial progression rate, distribution pattern of sensory signs, frequency of associated autonomic signs, frequency and nature of onconeural antibodies, and the spectrum and nature of the associated cancers among these two versions of neuropathic forms support the view that a common pathologic background is present in these two neuropathies.

Another interesting observation in this study was that the CMAPs and MCV were impaired in paraneoplastic neuropathy. This result suggests that motor nerves are also involved in paraneoplastic neuropathy, although muscle weakness and atrophy are not present or only mildly affected clinically. Some extent of motor impairment has been reported to associate with paraneoplastic neuropathy, since the original report by Denny-Brown.[1] A mild impairment of motor conduction has been suggested to be rather common.[39,40] These observations are fairly consistent with our present findings, but an additional important finding in our series is that the reduction of CMAPs was more prominent in the painful form than the ataxic form (table 3). The mechanism responsible for this observation is unknown; however, we speculate that motor axons or motor neurons are more severely impaired in the painful form of paraneoplastic neuropathy compared to the ataxic form.

The underlying mechanism for the pathogenesis in paraneoplastic neuropathy remains unknown. However, inflammatory cell infiltration in the DRG,[2,5,8,10] the peripheral nerve endoneurium, and epineural vessels,[16] as well as the occurrence of microvasculitis in nerve and muscles,[5,41,42] strongly suggests an immune-mediated mechanism. On the other hand, cytokines such as TNF-alpha, IL-1 beta, and IL-6 are known to be closely related to mechanical hyperalgesia in animal models.[43,44] Indeed, in patients with painful neuropathy or complex regional pain syndrome, TNF alpha is reported to correlate with the presence of mechanical hyperalgesia.[45,46] Together, these observations suggest that an immune-mediated pathogenic mechanism for paraneoplastic neuropathy, which may involve pain-related cytokines, may be a cause of painful symptoms in some patients with this disorder.

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SHORT COMMUNICATION

Myotonic dystrophy type 2 in Japan: ancestral origin distinct from Caucasian families

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Abstract Myotonic dystrophy type 2 (DM2) is caused by expansion of a tetranucleotide CCTG repeat in intron 1 of the *ZNF9* gene on chromosome 3q21. All studied DM2 mutations have been reported in Caucasians and share an identical haplotype, suggesting a common founder. We identified a Japanese patient with DM2 and showed that the affected haplotype is distinct from the previously identified DM2 haplotype shared among Caucasians. These data strongly suggest that DM2 expansion mutations originate from separate founders in Europe and Japan and are more widely distributed than previously recognized.

Keywords Myotonic dystrophy type 2 · CCTG tetranucleotide repeat expansion · Founder haplotype

Saito and Amakusa have contributed equally to the work.

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Introduction

Myotonic dystrophy type 2 (DM2) is an autosomal dominant, myotonic multisystemic disorder caused by the expansion of a tetranucleotide CCTG repeat in intron 1 of the zinc finger protein 9 (*ZNF9*) gene on chromosome 3q21 [1]. The size of expanded alleles is extremely variable, ranging from 75 to 11,000 repeats, with a very large mean of 5,000 CCTG repeats. Because of this unprecedented size and somatic heterogeneity, molecular diagnosis of DM2 is complicated. DM2 is also clinically variable, described as proximal myotonic myopathy [2], proximal myotonic dystrophy [3], or "myotonic dystrophy with no CTG expansion" [4]. Further studies suggested that all genetically confirmed DM2 patients arose from a single ancestral origin [5, 6]. No DM2 mutation, to date, has been identified in sub-Saharan or East-Asian populations [7]. Herein, we report the first Japanese family with a DM2 mutation.

Case report

A 59-year-old Japanese woman was admitted to our hospital for weakness of all four limbs that had progressed

slowly for more than 12 years. There was no complaint of muscle pain or stiffness. At age 47, she developed type 2 diabetes mellitus. At age 52, she had a right posterior subcapsular cataract extracted. There was no known consanguinity or genetic admixture with other ethnicities in her family. Her father and mother had no history of muscle weakness before having died at ages 67 and 72 years, respectively. There was a history of undiagnosed muscle disease in her brother and sister, but they were unavailable for examination. Her two children were asymptomatic.

Neurologically, this patient had normal language, speech, and cognition on routine clinical evaluation. She showed mild facial weakness and temporal wasting as well as weakness and atrophy of sternocleidomastoid muscles. Motor examination revealed predominantly proximal muscle weakness and atrophy in all limbs. Grip myotonia was present, but percussion myotonia was not elicitable. Tendon reflexes were present, but hypoactive and sensation was intact. Serum creatine kinase was 99 IU/l (normal range, 45–163 IU/l), and serum IgG level was slightly decreased to 828 mg/dl (normal range, 870–1,700 mg/dl). Electrocardiogram revealed complete right bundle branch block, and Holter monitoring detected premature ventricular contractions. Electromyography showed small motor unit potentials with early recruitment and myotonic discharges in all muscles examined. Nerve conduction studies were normal. Muscle computed tomography revealed diffuse muscle atrophy in the trunk and proximally in all limbs, whereas forearm and distal leg muscles were well preserved (Supplementary Figs. 2 and 3). T2-weighted brain magnetic resonance imaging demonstrated diffuse periventricular white matter hyperintensities without significant cerebral atrophy. No CTG expansion in the *DMPK* gene associated with myotonic dystrophy type 1 (DM1) [8] was detected.

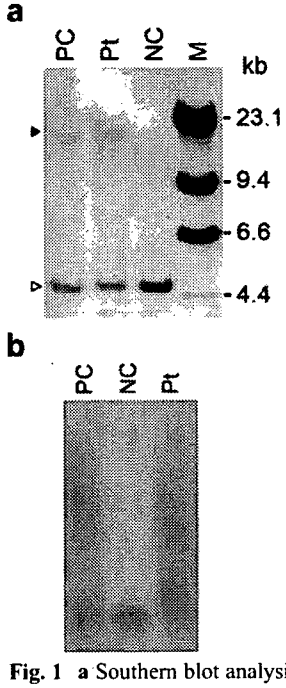
Materials and methods

Mutation analysis

Blood samples were obtained from the patient and her family members with informed consent approved by the institutional review boards of the National Dohoku Hospital and the Nagoya University Graduate School of Medicine for human research. High molecular weight genomic DNA was extracted by a standard procedure. Polymerase chain reaction (PCR) products across the DM2 repeat (marker CL3N58) in the first intron of the *ZNF9* [1] were analyzed by capillary electrophoresis using an automated DNA sequencer (ABI 310A Genetic Analyzer, Applied Biosystems). For detection of the DM2 CCTG expansion, Southern blot analysis and the repeat-primed PCR assay using an oligonucleotide primed within the DM2 CCTG repeat were performed as described elsewhere [1, 9, 10].

Haplotype analysis

To investigate the ancestral origin of Japanese DM2, we performed a haplotype analysis of our patient's family. We genotyped available family members (the patient, her spouse, and two children) for the previously described microsatellite markers: CL3N83; CL3N95; CL3N96; CL3N59; C3N116; CL3N117; CL3N118; CL3N119 [6] and a single nucleotide polymorphism (SNP): rs1871922, which is identical to TSC873597 in the report by Bachinski et al. [5]. We also analyzed three unrelated Caucasian DM2 DNA samples sharing the identical core haplotype as controls [6]. PCR products for all microsatellite markers were analyzed, and the SNP genotyping was performed by PCR amplification followed by restriction enzyme digestion (*Hae*III). The data were compared to the consensus Caucasian haplotypes reported in the previous report [6].



c

Location (kb)	Marker	Our pedigree	Caucasian haplotype B	Caucasian haplotype A	Caucasian haplotype C
273	CL3N83	191	189	195	185
241	CL3N95	224	222	222	212
240	CL3N96	159	159	159	157
119	CL3N59	129	149	149	141
0	DM2 repeat	exp.	exp.	exp.	exp.
11	rs1871922	A	C	C	C
74	CL3N116	222	222	222	222
103	CL3N117	239	235	235	235
127	CL3N118	227	221	221	221
216	CL3N119	220	234	230	230

Fig. 1 **a** Southern blot analysis of myotonic dystrophy type 2 (DM2). A *closed arrowhead* points to expanded alleles in DM2. *M* λDNA/*Hind* III marker, *NC* normal control, *Pt* our patient showing an 18.1-kb expanded allele as well as a normal allele, *PC* a Caucasian positive control with a DM2 expansion (17.5 kb). **b** Repeat-primed PCR analysis. Expanded CCTG repeats are detected as a continuous characteristic ladder in the patient (*Pt*) and positive control (*PC*) lanes, the size of which exceeds the range in the normal control (*NC*). **c** Comparison of DM2-affected haplotypes between Japanese and Caucasian DM2 families. Genotypes shared among Caucasian haplotypes are *shaded*. The distance of each marker from the DM2 CCTG repeat expansion (*exp*) is denoted on the *left*

Results

Mutation analysis

PCR amplification of the DM2 repeat detected a single normal allele at 228 bp. To determine if this patient also had a disease allele too large to be amplified by PCR, Southern analysis was performed and showed an expanded DM2-mutant allele of 18.1 kb, corresponding to approximately 3,400 CCTG repeats, as well as a normal allele of 4.5 kb (Fig. 1a). The repeat-primed PCR assay also showed a smear PCR product, confirming the presence of DM2 CCTG expansion (Fig. 1b).

Haplotype analysis

As shown in Fig. 1c, we found that this patient has an expansion-associated haplotype distinct from that commonly found in Caucasian DM2 patients [5, 6], indicating a different ancestral origin. Although a short common haplotype, less than 130 kb, between CL3N59 and rs1871922, is still possible, a telomeric recombination between the mutation and the SNP (11 kb telomeric of the mutation) is unlikely.

Discussion

Consistent with the typical DM2 phenotype [7, 10, 11], our patient clinically showed a combination of adult-onset proximal muscle weakness and myotonia. To our knowledge, this is the first DM2 patient identified from an East-Asian population [7]. Although DM2 mutations were reported in non-European populations including Morocco, Algeria, Lebanon, Afghanistan, and Sri Lanka [7, 11], all reported DM2 patients were considered to originate from a single common founder because they shared an identical haplotype [5, 6]. Our data have implications for molecular genetic diagnostics and counseling of East-Asian patients with the DM clinical phenotype and their families, as well as providing insight into the evolution of this complex disease. Physicians and genetic counselors should be aware that DM2 exists in non-Caucasian populations. Further epidemiologic studies, especially collection of additional non-Caucasian DM2 patients, will be of interest. It will also be of value to determine whether DM2 patients of different ethnic backgrounds originated from separate founders and whether they have unique clinical features and differences in genetic instability.

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CAG repeat size correlates to electrophysiological motor and sensory phenotypes in SBMA

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Spinal and bulbar muscular atrophy (SBMA) is an adult-onset, lower motor neuron disease caused by an aberrant elongation of a CAG repeat in the androgen receptor (AR) gene. The main symptoms are weakness and atrophy of bulbar, facial and limb muscles, but sensory disturbances are frequently found in SBMA patients. Motor symptoms have been attributed to the accumulation of mutant AR in the nucleus of lower motor neurons, which is more profound in patients with a longer CAG repeat. We examined nerve conduction properties including F-waves in a total of 106 patients with genetically confirmed SBMA (mean age at data collection = 53.8 years; range = 31–75 years) and 85 control subjects. Motor conduction velocities (MCV), compound muscle action potentials (CMAP), sensory conduction velocities (SCV) and sensory nerve action potentials (SNAP) were significantly decreased in all nerves examined in the SBMA patients compared with that in the normal controls, indicating that axonal degeneration is the primary process in both motor and sensory nerves. More profound abnormalities were observed in the nerves of the upper limbs than in those of the lower limbs. F-waves in the median nerve were absent in 30 of 106 cases (28.3%), but no cases of absent F-waves were observed in the tibial nerve. From an analysis of the relationship between CMAPs and SNAPs, patients were identified with different electrophysiological phenotypes: motor-dominant, sensory-dominant and non-dominant phenotypes. The CAG repeat size and the age at onset were significantly different among the patients with motor- and sensory-dominant phenotypes, indicating that a longer CAG repeat is more closely linked to the motor-dominant phenotype and a shorter CAG repeat is more closely linked to the sensory-dominant phenotype. Furthermore, when we classified the patients by CAG repeat size, CMAP values showed a tendency to be decreased in patients with a longer CAG repeat ($\geq$47), while SNAPs were significantly decreased in patients with a shorter CAG repeat (<47). In addition, we found that the frequency of aggregation in the sensory neuron cytoplasm tended to inversely correlate with the CAG repeat size in the autopsy study, supporting the view that the CAG repeat size differentially correlates with motor- and sensory-dominant phenotypes. In conclusion, our results suggest that there are unequivocal electrophysiological phenotypes influenced by CAG repeat size in SBMA.

Keywords: CAG repeat; spinal and bulbar muscular atrophy; electrophysiological phenotypes; motor-dominant; sensory-dominant

Abbreviations: CMAP = compound muscle action potential; MCV = motor conduction velocity; SBMA = spinal and bulbar muscular atrophy; SCV = sensory conduction velocity; SNAP = sensory nerve action potential

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Introduction

Spinal and bulbar muscular atrophy (SBMA) is a hereditary lower motor neuron disease affecting adult males (Kennedy *et al.*, 1968; Sobue *et al.*, 1989, 1993; Fischbeck *et al.*, 1997). The cause of SBMA is an aberrant elongation of a CAG repeat in the androgen receptor (AR) gene. Normally, 9–36 CAGs are observed in the AR gene in normal subjects, but 38–62 CAGs are observed in SBMA patients (La Spada *et al.*, 1991; Tanaka *et al.*, 1996; Andrew *et al.*, 1997). A similar gene mutation has been detected in Huntington's

disease (HD), dentatorubral-pallidoluysian atrophy (DRPLA) and several types of spinocerebellar ataxia (Gatchel *et al.*, 2005). Since CAG is translated to glutamine, these disorders, including SBMA, are called polyglutamine diseases. In SBMA patients, there is an inverse correlation between the number of CAGs and the age at onset (Doyu *et al.*, 1992; Atsuta *et al.*, 2006). The histopathological hallmarks of this disease are an extensive loss of lower motor neurons in the spinal cord and brain stem, together with degeneration of the dorsal root ganglions (DRG) (Sobue *et al.*, 1989; Adachi *et al.*, 2005). Intranuclear accumulations of mutant AR protein in the residual motor neurons are another hallmark (Li *et al.*, 1998; Adachi *et al.*, 2005). The molecular basis for motor neuron degeneration is thought to be testosterone-dependent nuclear accumulation of the mutant AR, and androgen deprivation rescues neuronal dysfunction in animal models of SBMA (Katsuno *et al.*, 2002, 2003; Takeyama *et al.*, 2002; Chevalier-Larsen *et al.*, 2004). Androgen deprivation with a luteinizing hormone-releasing hormone (LHRH) analog also suppresses nuclear accumulation of mutant AR in the scrotal skin of SBMA patients (Banno *et al.*, 2006). Other candidates for potent therapeutics such as 17-allylamino-17-demethoxygeldanamycin (17-AAG) or geranylgeranylacetone (GGA), enhancers of molecular chaperone expression and function, and a histone deacetylase (HDAC) inhibitor have also emerged from studies of animal models of SBMA (Minamiyama *et al.*, 2004; Katsuno *et al.*, 2005; Waza *et al.*, 2005).

The main symptoms of SBMA are weakness and atrophy of the bulbar, facial and limb muscles (Katsuno *et al.*, 2006). The onset of weakness is usually between 30 and 60 years of age. Postural tremor of the fingers is often observed prior to weakness. The symptoms are slowly progressive in SBMA, and the susceptibility for aspiration pneumonia increases as bulbar paralysis develops (Atsuta *et al.*, 2006). The most common cause of death is pneumonia. Many patients also have hypertension, hyperlipidemia, liver dysfunction and glucose intolerance. Serum creatine kinase is increased in the majority of patients.

In addition to motor symptoms, sensory impairment such as vibratory sensory disorder is often observed, and electrophysiological involvement has also been described in sensory nerves of SBMA patients (Harding *et al.*, 1982; Olney *et al.*, 1991; Li *et al.*, 1995; Guidetti *et al.*, 1996; Polo *et al.*, 1996; Ferrante *et al.*, 1997; Antonini *et al.*, 2000; Sperfeld *et al.*, 2002). In addition, sensory nerve axon loss, particularly of the central and peripheral rami of primary sensory neurons, has been documented to be profound (Harding *et al.*, 1982; Sobue *et al.*, 1989; Li *et al.*, 1995). Spinal dorsal column involvement and loss of axons in the sural nerve are common pathological features (Sobue *et al.*, 1989; Li *et al.*, 1995), and abnormalities in sensory nerve conduction and sensory evoked potentials are well known features of SBMA (Kachi *et al.*, 1992). Since the sensory symptoms are not generally severe in most patients, sensory nerve involvement has not been given much attention, particularly when compared to motor symptoms. However, the involvement of primary sensory neurons is one of the major phenotypic manifestations in SBMA (Sobue *et al.*, 1989).

The age at onset and the severity of motor symptoms are variable among SBMA patients (Kennedy *et al.*, 1968; Sperfeld *et al.*, 2002). One of the major factors determining clinical features is the CAG repeat size in the AR gene (Doyu *et al.*, 1992; Atsuta *et al.*, 2006). However, the age at onset and severity are also variable even among the patients with the same CAG repeat size (Doyu *et al.*, 1992; Atsuta *et al.*, 2006), indicating that some unknown genetic or environmental factors may influence the development of clinical heterogeneity (Atsuta *et al.*, 2006). In sensory impairments, there is also a variable degree of severity. Some patients show profound sensory symptoms and sensory nerve electrophysiological abnormalities, while other patients appear almost normal (Olney *et al.*, 1991; Li *et al.*, 1995; Guidetti *et al.*, 1996; Antonini *et al.*, 2000). In contrast to motor symptoms, the age at onset for sensory symptoms is rather difficult to determine, and the role of CAG repeat size in the severity of symptoms and the onset of sensory symptoms is unknown.

In order to clarify motor and sensory nerve involvement in SBMA, we examined nerve conduction properties including F-waves in 106 patients with genetically confirmed SBMA and 85 control subjects. We further analysed the influence of the CAG repeat size within the AR gene on the electrophysiological motor- and sensory-dominancy, as well as the histopathological background underlying the phenotypic diversity in nerve conduction of SBMA patients.

Subjects and Methods

Patients

A total of 106 male patients with the diagnosis of SBMA confirmed by genetic analysis and 85 male normal control subjects were included in this study. The data of SBMA patients were collected between May 2003 and May 2007. We analysed various electrophysiological examinations, motor function, sensory disturbance, disease duration and CAG repeat size in the AR gene in these patients. We defined the onset of disease as when the muscular weakness began, but not when tremor of the fingers appeared. As a functional assessment, we applied the Limb Norris score, Norris Bulbar score and ALS functional rating scale-revised (ALSFRS-R), which are aimed at motor function evaluations of patients with amyotrophic lateral sclerosis (ALS) (Norris *et al.*, 1974; The ALS CNTF Treatment Study (ACTS) Phase I-II Study Group, 1996).

All studies conformed to the ethics guideline for human genome/gene analysis research and the ethics guideline for epidemiological studies endorsed by the Japanese government. The ethics committee of Nagoya University Graduate School of Medicine approved the study, and all SBMA patients and normal subjects gave their written informed consent to the investigation.

Electrophysiological assessments

Motor and sensory nerve conduction studies were performed in the median, ulnar, tibial and sural nerves in 106 patients during their initial clinical assessment at Nagoya University Hospital using a standard method with surface electrodes for stimulation and recording as described previously (Sobue *et al.*, 1989; Kimura, 2001*a*, *b*; Koike *et al.*, 2003; Mori *et al.*, 2005). Motor conduction was investigated in the median, ulnar and tibial nerves, recording from the abductor pollicis brevis, abductor digiti minimi and abductor hallucis brevis, respectively. The following nerve segments were used for calculating motor conduction velocities (MCV): wrist to elbow for the median nerve, wrist to distally at the elbow for the ulnar nerve, and ankle to popliteal fossa for the tibial nerve. Sensory conduction was investigated in the median, ulnar and sural nerves, using antidromic recording from ring electrodes at the second and fifth digit for the median and ulnar nerves, respectively, and bar electrodes at the ankle for the sural nerve. Sensory conduction velocities (SCV) were calculated for the distal segment. Amplitudes of compound muscle action potentials (CMAP) and those of sensory nerve action potentials (SNAP) were measured from the baseline to the first negative peak. Control values were obtained in 56–85 age-matched normal volunteers (31–75 years) (Koike *et al.*, 2001; Mori *et al.*, 2005).

F-waves were also examined in the median and tibial nerves at the same time as the nerve conduction studies using a standard method as described previously (Kimura, 2001*c*). Sixteen consecutive supramaximal stimuli with frequency of 1 Hz were delivered to the median and tibial nerves, while recording from the same muscles as the normal nerve conduction studies. The following variables were estimated: occurrence, minimum latency and maximum F-wave conduction velocity (FWCV). FWCV was calculated using the formula $2D/(F-M-1)$, where D is the surface distance measured from the stimulus point to the C7 spinous process in the median nerves or to the T12 spinous process in the tibial nerves, F is the latency of the F-wave and M is the latency of the CMAP. Control values were obtained in 28–47 age-matched normal volunteers (31–75 years). All nerve conduction studies and F-wave studies were carried out on the right side of the body.

We defined the nerve conduction, CMAPs and SNAPs as abnormal, when these values were less than the mean −2 SD of normal controls on the examined nerves. We also expressed the CMAP and SNAP values as the percentage of the mean values of normal controls, when we need the standardized expression of the degree of CMAP and SNAP involvement as compared to normal controls.

Standard needle electromyography (EMG) was performed using concentric needle electrodes in 93 SBMA patients, with the muscles at rest and during weak and maximal efforts (Sobue *et al.*, 1993; Kimura, 2001*d*; Sone *et al.*, 2005).

Genetic analysis

Genomic DNA was extracted from peripheral blood of SBMA patients using conventional techniques (Tanaka *et al.*, 1996). PCR amplification of the CAG repeat in the AR gene was performed using a fluorescein-labelled forward primer (5′-TCC AGAATCTGTTCCAGAGCGTGC-3′) and a non-labelled reverse primer (5′-TGGCCTCGCTCAGGATGTCTTTAAG-3′). Detailed PCR conditions were described previously (Tanaka *et al.*, 1996, 1999). Aliquots of PCR products were combined with loading dye and separated by electrophoresis with an autoread sequencer SQ-5500 (Hitachi Electronics Engineering, Tokyo, Japan). The size of the CAG repeat was analysed on Fraglys software version 2.2 (Hitachi Electronics Engineering) by comparison to co-electrophoresed PCR standards with known repeat sizes. The CAG repeat size of the PCR standard was determined by direct sequence as described previously (Doyu *et al.*, 1992).

Immunohistochemistry for mutant AR in the sensory and motor neurons

For immunohistochemistry of primary sensory and spinal motor neurons, autopsy specimens of lumbar DRG and spinal cord from five genetically diagnosed SBMA patients (70.4 ± 11.0 years old) were used. The lumbar DRG and spinal cord were excised at autopsy and immediately fixed in 10% buffered formalin solution. The collection of tissues and their use for this study were approved by the Ethics Committee of Nagoya University Graduate School of Medicine. Lumbar DRG and spinal cord sections of 6 μm were deparaffinized, treated with 98% formic acid at room temperature for 3 min and with microwave oven heating for 10 min in 10 mM citrate buffer at pH 6.0, and incubated with an anti-polyglutamine antibody (1C2, 1:20 000; Chemicon, Temecula, CA). Subsequent staining procedures are performed using the Envision+ kit (Dako, Glostrup, Denmark).

For quantification of primary sensory neurons in which mutant AR accumulates, we prepared at least 100 transverse sections from the lumbar DRG, and performed 1C2 immunohistochemistry as described above. The frequency of 1C2-positive and -negative cells within the DRG was assessed by counting all the neurons with 1C2-positive cytoplasmic inclusions against total neuronal cells with obvious nuclei on every 10th section under the light microscope (BX51N-34, Olympas, Tokyo, Japan). The results were expressed as frequency of 1C2-positive cells in the 10 sections of the DRG. As for quantification of spinal motor neurons, the detailed procedure has been described previously (Adachi *et al.*, 2005). We have also examined five control autopsied specimens from patients died from non-neurological diseases, and found that there were no 1C2-positive cytoplasmic or nuclear staining.

Data analysis

Quantitative data was presented as means ± SD. Statistical comparisons were performed using the Student's *t*-test. Correlations among the parameters were analysed using Pearson's correlation coefficient. P values less than 0.05 and correlation coefficients (r) greater than 0.4 were considered to indicate significance. Calculations were performed using the statistical software package SPSS 14.0J (SPSS Japan Inc., Tokyo, Japan).

Results

Clinical and genetic backgrounds of SBMA patients

The clinical background of the SBMA patients is described in Table 1. All of the patients examined were of Japanese nationality. The duration from onset assessed from the first notice of motor impairment (Atsuta *et al.*, 2006) ranged from 1 to 32 years. There was no significant difference between the median CAG repeat size in the present study

Table 1 Clinical and genetic features of SBMA patients

Clinical and genetic features	Mean ± SD	Range	*n*
Age at examination (years)	53.8 ± 10.0	31–75	106
Duration from onset (years)	10.1 ± 6.8	1–32	106
Age at onset (years)	43.7 ± 10.4	25–68	106
CAG repeat size in AR gene (number)	47.8 ± 3.1	41–57	97[a]
Limb Norris score (normal score = 63)	53.9 ± 7.3	34–63	99
Norris Bulbar score (normal score = 39)	33.0 ± 4.3	20–39	99
ALSFRS-R (normal score = 48)	41.1 ± 4.3	22–48	99

[a]The abnormal elongation of the CAG repeat was confirmed by gene analysis using agarose gel electrophoresis without determining the repeat number in the remaining nine patients. AR = androgen receptor; ALSFRS-R = ALS functional rating scale-revised

and those reported previously in SBMA patients (La Spada *et al.*, 1991; Tanaka *et al.*, 1996; Andrew *et al.*, 1997).

All patients were ambulatory with or without aid, and none were bed-ridden. The mean Limb Norris score, Norris Bulbar score and ALSFRS-R also suggested that the ADL of patients in this study was not severely impaired. Vibratory sensation disturbance was detected in 78.2% of the SBMA patients. Touch and pain sensation abnormalities were found in 10.9 and 9.1% of the patients, respectively. Joint position sensation was intact in all of the patients examined.

In EMG, all the examined patients showed high amplitude potentials, reduced interference and polyphasic potentials, suggesting neurogenic changes in SBMA.

Nerve conduction and F-wave studies indicate CMAP and SNAP reduction as a profound feature of SBMA

MCV, CMAP, SCV and SNAP were significantly decreased in all the nerves examined in the SBMA patients when compared with those of the normal controls (Table 2). Sensory nerve activity could not be evoked in some cases, whereas activity in the motor nerves was elicited in all patients examined. The most profound finding in the nerve conduction studies was the reduction in the amplitude of the evoked potentials in both motor and sensory nerves. The mean values of CMAPs were reduced to 47–76%, and SNAPs were reduced to 31–47% of the normal mean values. The decrease in conduction velocity was relatively mild, but definitely present in both motor and sensory nerves. The conduction velocity was reduced to 94–96% in MCV and 87–91% in SCV of the normal mean values. The F-wave latencies were also mildly, but significantly prolonged in the median and tibial nerves of SBMA patients. The mean occurrence rate of F-waves in the median nerve was significantly less in SBMA patients, and they were absent in 30 cases (28.3%) (Table 2).

When we compared the CMAP and MCV values of the individual patients in the median, ulnar and tibial nerves, MCV was decreased only in the patients with a severely decreased CMAP (Supplementary Fig. 1). In addition, SCV reduction was observed only in the patients with severely decreased SNAP (Supplementary Fig. 1). These observations strongly suggest that the most profound impairment of the SBMA patients is a reduction of the amplitude of evoked potentials, possibly due to axonal loss (Sobue *et al.*, 1989; Li *et al.*, 1995).

As for the spatial distribution of electrophysiological involvements, the frequency of abnormal values of CMAP was most remarkable in the median nerve followed by the ulnar and tibial nerves (Table 3). The decrease in SNAP was also remarkable in the median and ulnar nerves when compared with those in the sural nerve (Table 3). The absence of F-waves was more frequent in the median nerve than in the tibial nerve (Table 3). These findings indicate that more significant abnormalities in nerve conduction and F-waves are observed in the nerves of the upper limbs than in those of the lower limbs.

Electrophysiologically defined motor and sensory phenotypes

When we analysed the relationship between the degree of motor and sensory nerve involvement by assessing the number of nerves showing abnormally reduced amplitudes (less than control mean − 2 SD) in the sensory (median, ulnar and sural nerves) and motor (median, ulnar and tibial nerves) nerves, we found that the patients could be distinguished by either a motor-dominant, sensory-dominant or non-dominant phenotype (Fig. 1A). It should be noted that there were patients showing only abnormally reduced SNAPs, while the CMAPs were well preserved (Fig. 1A). Alternatively, patients demonstrating CMAPs abnormalities with well preserved SNAPs were also seen (Fig. 1A).

When we analysed the relationship between CMAPs and SNAPs on a standardized scale of percentage of the mean values of normal controls in the median and ulnar nerves (Fig. 1B and C), we found that there were patients with different electrophysiological phenotypes. Some patients showed well preserved CMAPs, being 50% or more of the mean value in the controls, while showing profoundly reduced SNAPs of less than 50% of the mean value in the controls. In contrast, other patients showed well-preserved SNAPs and significantly reduced CMAPs (Fig. 1B and C). Finally, some patients showed a similar involvement of CMAPs and SNAPs. These observations suggest that a subset of SBMA patients shows predominantly motor impairments, while another subset shows predominantly sensory impairments.

The CAG repeat size correlates to electrophysiologically defined motor and sensory phenotypes

Since the CAG repeat size is a key factor dictating clinical presentation in polyglutamine diseases (Zoghbi *et al.*, 2000),

Table 2 Nerve conduction studies and F-wave examinations

	SBMA		Normal		*P*
	(Mean ± SD)	*n*	(Mean ± SD)	*n*	
Median nerve					
MCV (m/s)	54.3 ± 6.5	106	57.9 ± 3.6	79	<0.001
Distal latency (m/s)	4.3 ± 1.0	106	3.4 ± 0.4	79	<0.001
CMAP (mV)	5.1 ± 2.9	106	10.8 ± 3.3	79	<0.001
SCV (m/s)	52.3 ± 6.1	103	57.4 ± 4.4	85	<0.001
SNAP (μV)	7.0 ± 5.2	103	20.0 ± 7.9	85	<0.001
Not evoked	Three cases (2.8%)		None		
F-wave minimum latency (ms)	28.2 ± 3.0	76	22.3 ± 1.9	46	<0.001
FWCV maximum (m/s)	58.7 ± 10.5	74	66.4 ± 8.6	41	<0.001
F-wave occurrence (%)	24.5 ± 22.5	106	67.6 ± 20.3	47	<0.001
Absent	30 cases (28.3%)		None		
Ulnar nerve					
MCV (m/s)	55.9 ± 5.2	106	58.2 ± 4.7	71	0.003
Distal latency (ms)	3.2 ± 0.6	106	2.7 ± 0.3	71	<0.001
CMAP (mV)	5.1 ± 2.4	106	8.4 ± 2.4	71	<0.001
SCV (m/s)	48.1 ± 7.5	102	55.0 ± 3.8	74	<0.001
SNAP (μV)	5.6 ± 4.6	102	18.3 ± 7.4	74	<0.001
Not evoked	Four cases (3.8%)		None		
Tibial nerve					
MCV (m/s)	44.5 ± 3.8	106	47.2 ± 3.7	56	<0.001
Distal latency (ms)	5.0 ± 1.0	106	4.5 ± 0.8	56	0.003
CMAP (mV)	7.8 ± 3.7	106	10.3 ± 3.4	56	<0.001
F-wave minimum latency (ms)	48.3 ± 4.1	106	41.4 ± 3.0	31	<0.001
FWCV maximum (ms)	43.9 ± 5.6	105	47.4 ± 3.3	28	<0.001
F-wave occurrence (%)	94.3 ± 11.6	106	96.3 ± 12.5	31	NS
Absent	None		None		
Sural nerve					
SCV (m/s)	44.1 ± 5.7	94	50.8 ± 5.1	68	<0.001
SNAP (μV)	5.1 ± 3.5	94	10.8 ± 4.6	68	<0.001
Not evoked	12 cases (11.3%)		None		

MCV = motor nerve conduction velocity; CMAP = compound muscle action potential; SCV = sensory nerve conduction velocity; SNAP = sensory nerve action potential; FWCV = F-wave conduction velocity; NS = not significant.

Table 3 Frequency of patients with abnormal values in nerve conduction studies and F-wave examinations

	Number of patients with abnormal values[a]	*n*	Frequency (%)
Median nerve			
MCV	20	106	18.9
CMAP	43	106	40.6
SCV	23	106	21.7
SNAP	45	106	42.5
FWCV maximum	38	104	36.5
F-wave occurrence	91	106	85.8
Ulnar nerve			
MCV	5	106	4.7
CMAP	24	106	22.6
SCV	40	106	37.7
SNAP	49	106	46.2
Tibial nerve			
MCV	6	106	5.7
CMAP	8	106	7.5
FWCV maximum	17	105	16.2
F-wave occurrence	7	106	6.6
Sural nerve			
SCV	36	106	34.0
SNAP	26	106	24.5

MCV = motor nerve conduction velocity; CMAP = compound muscle action potential; SCV = sensory nerve conduction velocity; SNAP = sensory nerve action potential; FWCV = F-wave conduction velocity.

[a]We defined the abnormal values as those values that were either less than the mean − 2 SD of normal controls on the examined nerves or not evoked.

we compared the phenotypes based on present symptoms and the electrophysiological phenotypes between patients with a CAG repeat size <47 and those with 47 or more CAGs, according to the previous report on clinical features of SBMA (Atsuta *et al.*, 2006) (Table 4). The age at onset and the age at examination were higher in patients with a shorter CAG repeat than in those with a longer repeat ($P < 0.001$). Disease duration and functional scale, including the Limb Norris score, Norris Bulbar score and ALSFRS-R, were similar between these groups. The CMAP values in the median, ulnar and tibial nerves were not significantly different, but showed a tendency to be decreased in the patients with a longer CAG repeat in all three nerves (Table 4). SNAPs in the median, ulnar and sural nerves were all significantly decreased in the patients with a shorter CAG repeat (Table 4). These observations suggest that a shorter CAG repeat is linked to a more significant SNAP decrease, while a longer CAG repeat is linked to a more profound CMAP decrease.

Furthermore, considering the possibility that action potentials are influenced by the age at examination, we compared the CMAPs and SNAPs in the patient subsets with a longer CAG repeat and those with a shorter CAG repeat between different age groups (Fig. 2). Patients <49 years old showed a significant difference in CMAPs and SNAPs ($P = 0.041–0.002$). The patients <49 years old and with a longer CAG repeat showed a more significant decrease in CMAPs, while those with a shorter CAG repeat showed a more significant decrease in SNAPs.

We selected patients with the sensory-dominant phenotype and those with the motor-dominant phenotype to further analyse the implication of CAG repeat size on the age at onset and electrophysiological phenotypes of SBMA.

A

Number of nerves with abnormally reduced SNAPs	Number of nerves with abnormally reduced CMAPs: 0	1	2	3
0	22	16	4	1
1	11	6	3	2
2	12	9	2	2
3	10	2	3	1

Motor-dominant
Sensory-dominant
Non-dominant

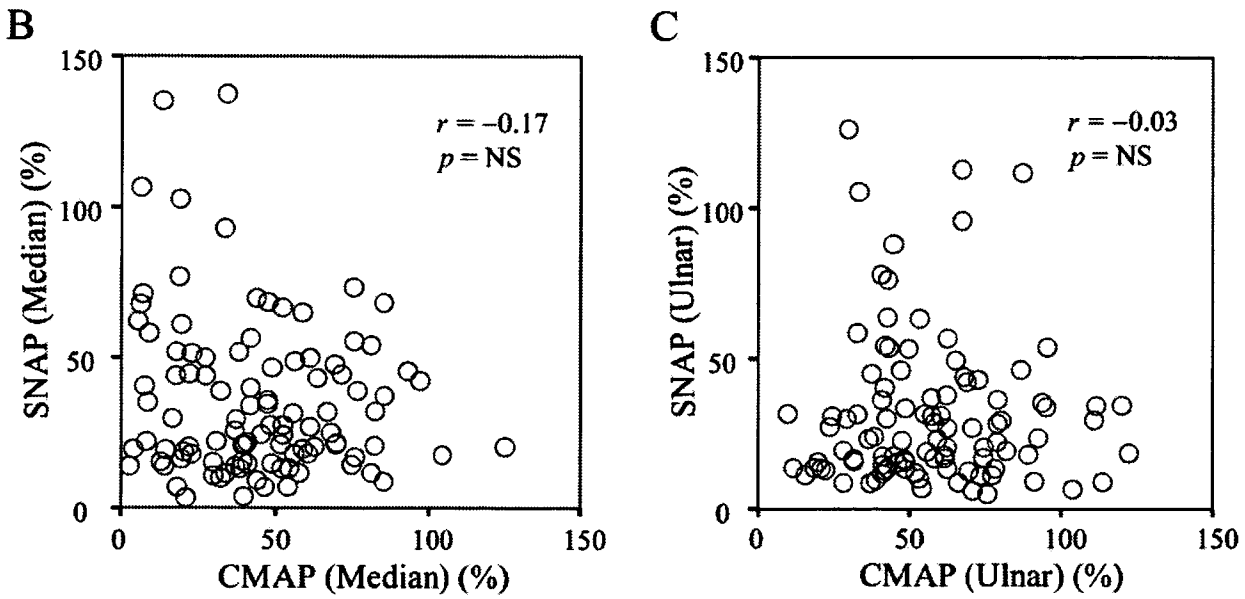

Fig. 1 Electrophysiological discrepancies in motor and sensory nerve involvement in SBMA patients. (**A**) The cross tabulation of the number of motor and sensory nerves showing an abnormally decreased action potential. The vertical stripe area corresponds to the motor-dominant phenotype and the gray area denotes the sensory-dominant phenotype. The white area represents the non-dominant phenotype. (**B** and **C**). Relation between CMAP and SNAP in the median and ulnar nerves on a standardized scale of percentage to normal control mean values. Some patients have only decreases of CMAP with preserved SNAP, while other patients show declines of SNAP with conserved CMAP.

As shown in Fig. 1A, the sensory-dominant phenotype was determined if patients show a reduced SNAP (less than control mean −2 SD) in at least one nerve without any decrease in CMAPs, whereas the motor-dominant phenotype denotes patients showing a reduced CMAP (less than control mean −2 SD) in at least one nerve without any decrease in SNAPs. We examined the relationship between CAG repeat number and the age at onset in these patients ($n=54$) (Fig. 3A). We found that the mean CAG repeat number and the age at onset were significantly different between patients with motor- and sensory-dominant phenotypes ($P<0.001$, Fig. 3A), indicating that a longer CAG repeat is more closely linked to the motor-dominant phenotype, and a shorter CAG repeat is more closely linked to sensory-dominant phenotype. Similar findings were observed when we classified patients based on abnormally reduced action potentials (less than control mean −2 SD) in the median nerve or the ulnar nerve (Fig. 3B and C).

Table 4 Clinical and electrophysiological features in terms of CAG repeat size in AR gene

	CAG repeat <47		CAG repeat ⩾47		P
	(Mean ± SD)	n	(Mean ± SD)	n	
Age at examination	58.9 ± 10.2	32	51.7 ± 8.9	65	0.001
Duration from onset	9.6 ± 7.4	32	10.7 ± 6.6	65	NS
Age at onset	49.3 ± 11.5	32	41.0 ± 8.9	65	0.002
Limb Norris score	54.2 ± 8.3	28	53.9 ± 7.0	63	NS
Norris Bulbar score	32.4 ± 5.1	28	33.4 ± 3.9	63	NS
ALSFRS-R	41.1 ± 4.1	28	41.2 ± 4.5	63	NS
CMAP (mV)					
Median	5.7 ± 2.4	32	4.8 ± 3.1	65	NS
Ulnar	5.6 ± 2.2	32	4.9 ± 2.4	65	NS
Tibial	8.7 ± 4.9	32	7.4 ± 3.1	65	NS
SNAP (μV)					
Median	4.8 ± 3.3	29	7.7 ± 5.6	65	0.011
Ulnar	4.1 ± 2.6	29	6.2 ± 5.0	64	0.037
Sural	3.8 ± 2.6	26	5.4 ± 3.4	59	0.022

AR = androgen receptor; ALSFRS-R = ALS functional rating scale-revised; CAMP = compound muscle action potential; SNAP = sensory nerve action potential; NS = not significant.

The CAG repeat size correlates directly with the frequency of nuclear accumulation in the motor neurons and inversely with that of cytoplasmic aggregation in the DRG

In order to investigate the relationship between CAG repeat size and the degree of motor and sensory nerve involvement, we performed immunohistochemistry using anti-polyglutamine antibody (1C2) on autopsied spinal cord and DRG specimens from SBMA patients, and quantified the primary sensory neurons in which mutant AR accumulated. In primary sensory neurons within the DRG, mutant AR was detected immunochistochemically as punctuate aggregates in the cytoplasm (Fig. 4A). On the other hand, diffuse nuclear accumulation of mutant AR was detected in motor neurons of the spinal anterior horn (Fig. 4B). The size of CAG repeat in the AR gene tended to be inversely correlated with the number of primary sensory neurons bearing cytoplasmic aggregates (Fig. 4C). This result is in contrast with the previously reported correlation between the frequency of mutant AR accumulation in spinal motor neuron and the CAG repeat size (Adachi *et al.*, 2005) (Fig. 4D).

Discussion

The present study demonstrated extensive abnormalities in both motor and sensory nerve conduction in SBMA patients, reflecting principal pathological lesions in the lower motor neurons and in the DRG. Previous studies on nerve conduction in SBMA patients showed a characteristic decrease in SNAP compared with normal controls, whereas SCV and MCV were variably reported as either normal or decreased, and CMAP decreased to variable extents (Harding *et al.*, 1982; Olney *et al.*, 1991; Li *et al.*, 1995; Guidetti *et al.*, 1996; Polo *et al.*, 1996; Ferrante *et al.*, 1997; Antonini *et al.*, 2000; Sperfeld *et al.*, 2002). In the present study, the reductions in both CMAP and SNAP were remarkable, in agreement with previous reports. This suggests that axonal degeneration is the principal peripheral nerve damage in SBMA patients. In addition, MCVs and SCVs were significantly decreased in the SBMA patients, and distal latencies were also significantly increased.

Several reports have examined the F-wave in SBMA patients. Those studies showed that the latency is almost normal or slightly extended (Olney *et al.*, 1991; Guidetti *et al.*, 1996). In the present study, the minimum F-wave latency was significantly longer and the maximum FWCV was significantly decreased in SBMA patients compared to that in normal controls. The occurrence of F-waves in SBMA patients was significantly less in the upper limb, but not in the lower limb compared with that of controls.

As for the spatial distribution of involvement, we demonstrated that nerves of the upper limbs are more severely disturbed than those of the lower limbs in SBMA patients. These observations suggest that nerve involvement does not reflect a length-dependent process of primary neuropathy, but a neuronopathy process, which is consistent with our results from histopathological studies (Sobue *et al.*, 1989; Li *et al.*, 1995).

The most striking observations in the present study are that motor and sensory nerves are differentially affected in SBMA patients, that electrophysiologically defined motor-dominant and sensory-dominant phenotypes are present, especially in young patients, and that the CAG repeat size in the AR gene is a factor determining these electrophysiologically defined motor and sensory phenotypes. Previous studies have reported that the number of CAGs determine not only the age at onset, but also the clinical phenotype in polyglutamine diseases (Ikeutchi *et al.*, 1995; Johansson *et al.*, 1998; Mahant *et al.*, 2003). For example, DRPLA patients with a longer CAG repeat (earlier age at onset) showed a progressive myoclonus epilepsy phenotype, whereas patients with a shorter CAG repeat (later age at onset) showed a non-progressive myoclonus epilepsy phenotype, but high frequencies of choreoathetosis and psychiatric symptoms (Ikeutchi *et al.*, 1995). Moreover, in spinocerebellar ataxia type-7 (SCA7) patients with ⩾59 CAGs, visual impairment was the most common initial symptom observed, while ataxia predominated in patients with <59 CAGs (Johansson *et al.*, 1998). Additionally, in HD patients, younger age at onset was associated with less chorea and more dystonia (Mahant *et al.*, 2003). In SBMA, only the relationship between CAG repeat and the age at onset or the severity of motor

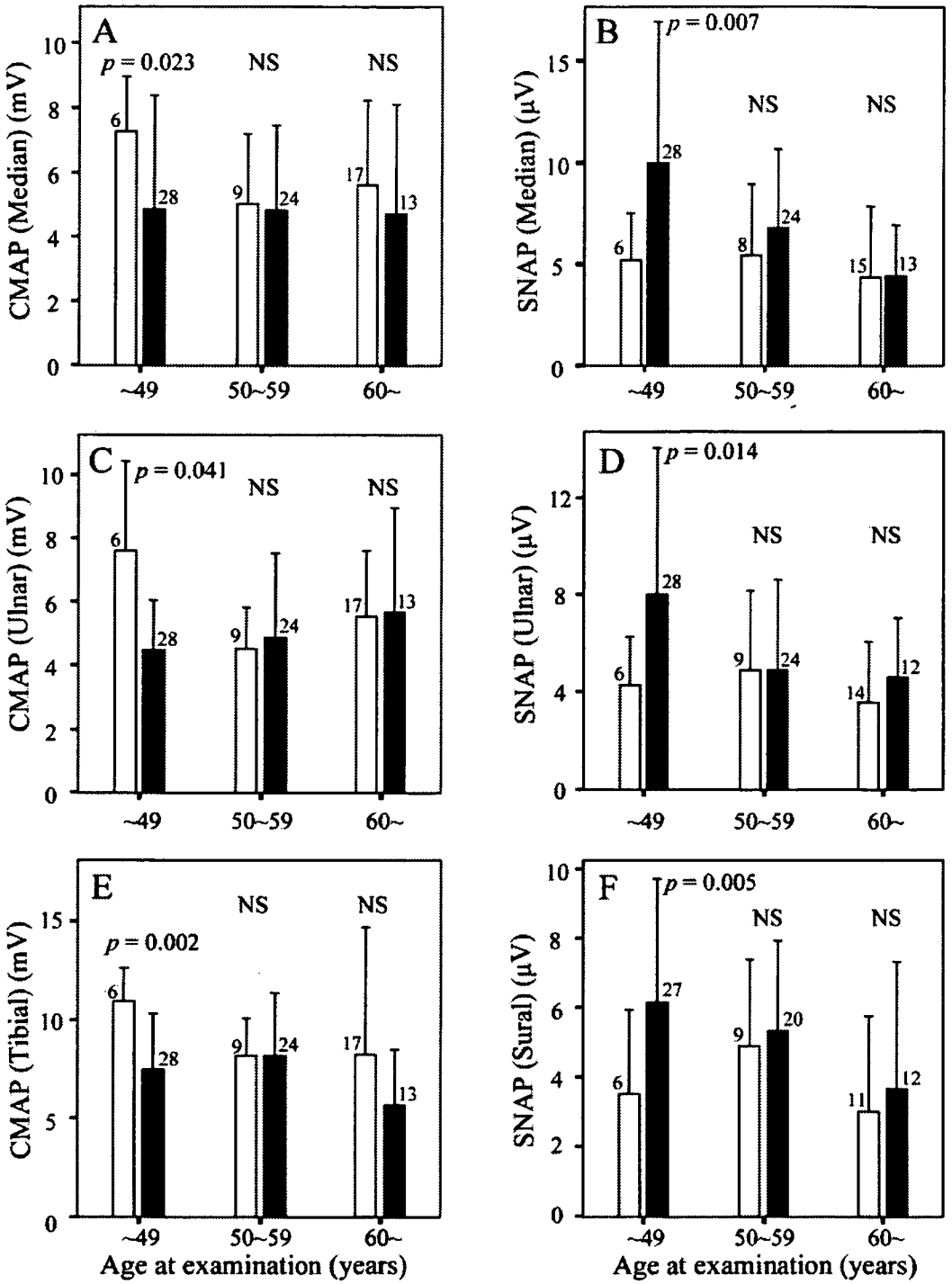

Fig. 2 (A–F) Age- and CAG-dependent changes in motor and sensory amplitudes in SBMA. CMAPs and SNAPs in the median (A and B), ulnar (C and D), tibial (E) and sural (F) nerves in different age groups are shown. The white columns are the mean values of the patients with a shorter CAG repeat (<47), while the black columns are the mean values of the patients with a longer CAG repeat (≥47). The error bars are SD. The number of patients examined is shown above each column. The young patients with a longer CAG repeat showed significantly low values of CMAPs compared to those with a shorter CAG repeat. Conversely, young patients with a shorter CAG repeat showed significantly lower values of SNAPs than those with a longer CAG repeat. Patients more than 49 years old did not show a significant difference between shorter and longer CAG repeat.

function has been reported (Doyu *et al.*, 1992; Atsuta *et al.*, 2006), but a CAG size-dependent clinical phenotype has not been described. This may be because the expansion of CAG repeat in the AR gene is shorter than that in the causative genes for DRPLA, SCA7 or HD. Alternatively, as compared to outstanding motor dysfunction, the clinical manifestations of sensory nerve impairment are less severe in SBMA patients, which may result in overlooking the motor and

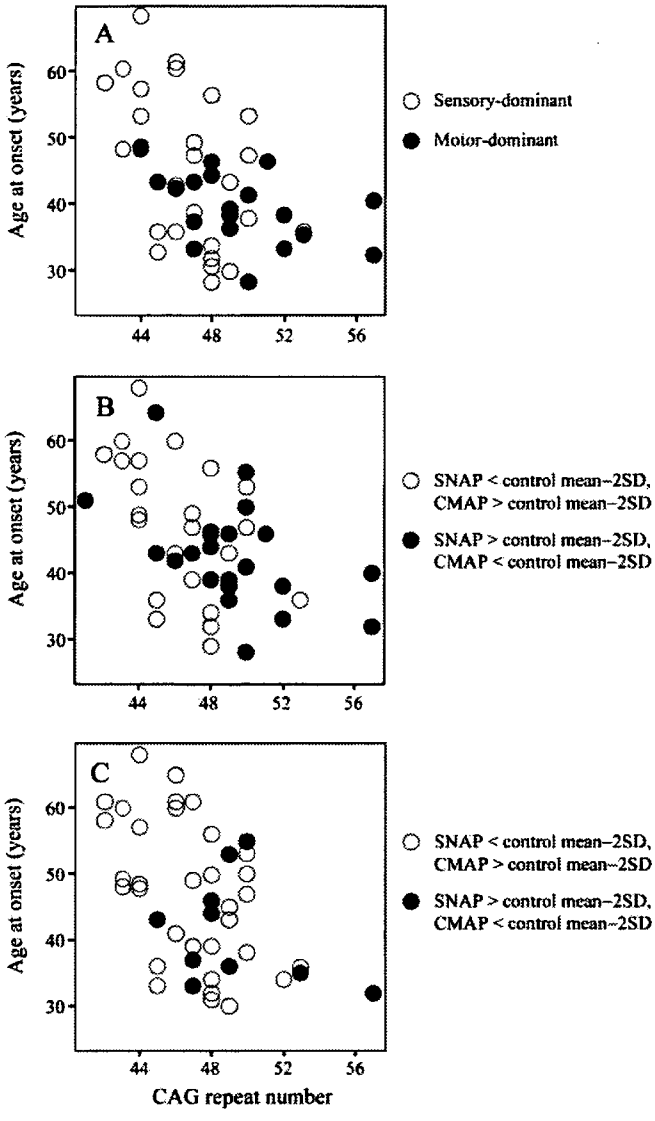

Fig. 3 CAG repeat size determines the age at onset in SBMA. (**A**) Relation between the CAG repeat size and the age at onset according to the phenotypes determined by CMAPs and SNAPs. A longer CAG repeat was closely linked to the motor-dominant phenotype, and a shorter CAG repeat was closely linked to the sensory-dominant phenotype. Motor- and sensory-phenotypes were determined as shown in Fig. 1A. (**B**) Relation between the CAG repeat size and the age at onset according to the phenotype determined by using CMAPs and SNAPs in the median nerve. (**C**) Relation between the CAG repeat size and the age at onset according to the phenotype determined by using CMAPs and SNAPs in the ulnar nerve.

sensory discrepancy. Our present findings in SBMA patients strongly suggest that the phenotypic diversity determined by CAG repeat size is a common feature shared by various polyglutamine diseases.

Although the pathological mechanism by which CAG repeat size influences clinical phenotype is unknown, a common molecular basis appears to underlie the heterogeneity of clinical presentations in polyglutamine diseases. The polyglutamine tract encoded by an expanded CAG repeat forms a β-sheet structure, leading to conformational changes and the eventual accumulation of causative proteins (Perutz *et al.*, 2002; Sakahira *et al.*, 2002). Since the propensity of aggregation is dependent on CAG repeat size, the different length of polyglutamine tract may result in a CAG repeat size-dependent pathology.

The observations that a longer CAG repeat results in the motor-dominant phenotype, while a shorter CAG leads to the sensory-dominant presentation, are further reinforced by results of previous studies on the cell-specific histopathological changes in SBMA. A diffuse loss and atrophy of anterior horn cells accompanied by a mild gliosis is characteristic of SBMA (Kennedy *et al.*, 1968; Sobue *et al.*, 1989), suggesting that the pathology of spinal motor neurons is neuronopathy. On the other hand, no substantial neuronal loss in the DRG despite severe axonal loss in the central and peripheral rami suggests that the pathology of sensory neurons is distally accentuated axonopathy, although the primary pathological process may be present in the perikarya of sensory neurons (Sobue *et al.*, 1989; Li *et al.*, 1995). Moreover, the accumulation of mutant AR, a pivotal feature of SBMA pathology, is also different in motor and sensory neurons (Adachi *et al.*, 2005). Mutant AR accumulates diffusely in the nucleus of spinal motor neurons, but cytoplasmic aggregation is predominant in sensory neurons within the DRG (Adachi *et al.*, 2005). The extent of diffuse nuclear accumulation of mutant AR in motor neurons is closely related to CAG repeat size, providing a molecular basis for the present observations that patients with a longer CAG repeat show a greater decrease in CMAPs. On the other hand, the results of anti-polyglutamine immunohistochemistry in this study indicate that cytoplasmic aggregation of mutant AR is more frequent in the patients with a shorter CAG repeat. Taken together, the differential accumulation pattern of mutant AR between motor and sensory neurons, and their differential correlation to CAG repeat size may be the pathophysiological background for the development of motor- and sensory-dominant phenotypes.

In conclusion, the results of the present study are unequivocal electrophysiological phenotypes, motor-dominant, sensory-dominant and non-dominant, especially in young patients of SBMA. These features are dependent on the CAG repeat size within the AR gene, with a longer CAG repeat size is more closely related to the motor-dominant phenotype and a shorter CAG repeat size related to the sensory-dominant phenotype. Our observations shed light on new roles of CAG repeat size in the clinical presentation of SBMA.

Supplementary materials

Supplementary materials are available at *Brain* online.

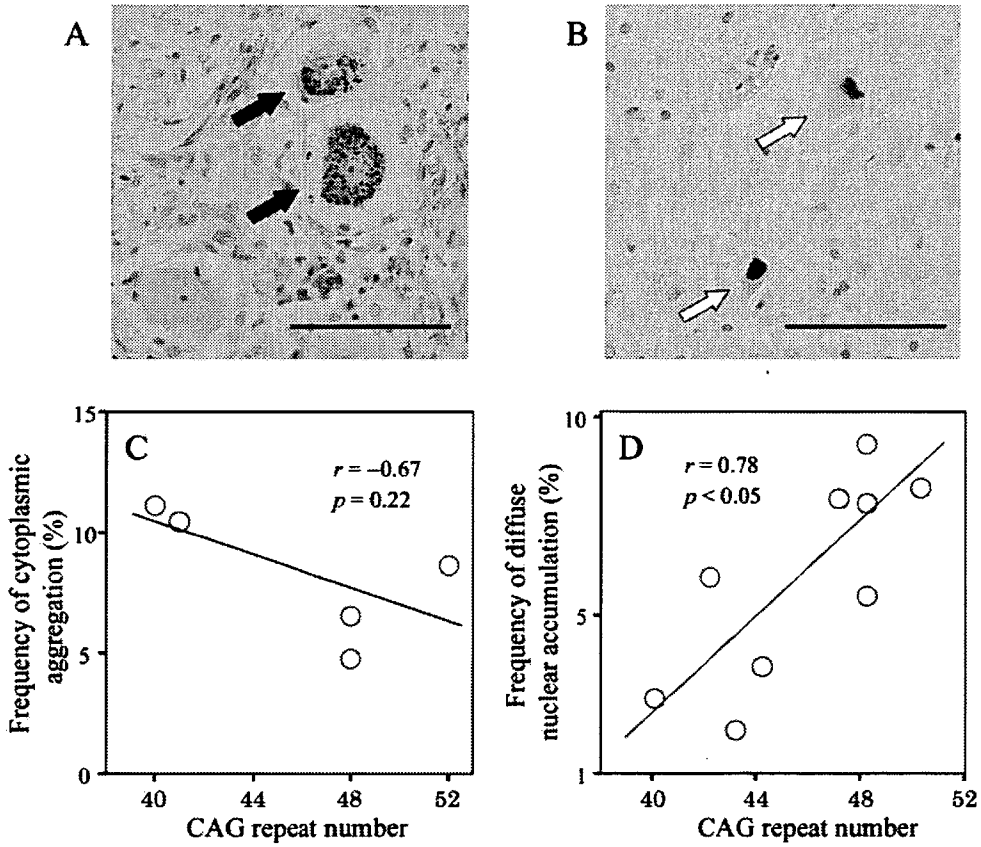

Fig. 4 Immunohistochemical analyses of mutant androgen receptor (AR) accumulation in the dorsal root ganglion (DRG) and that in the spinal anterior horn of SBMA patients. (**A**) Aggregates of mutant AR in the cytoplasm of DRG neurons (black arrows). Scale bar = 100 μm. (**B**) Mutant AR accumulates in the motor neuron nuclei (white arrows). Scale bar = 100 μm. (**C**) Relation between the CAG repeat size and cytoplasmic aggregations in the primary sensory neuron. Cytoplasmic aggregation tended to be more frequent in the patients with a shorter CAG repeat. (**D**) Relation between the CAG repeat size and diffuse nuclear accumulation of mutant AR in the spinal motor neuron. Panel D is reconstructed from the previous report (Adachi *et al.*, 2005).

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Viral vector-medicated expression of human collage Q in cultured cells

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Abstract

Congenital myasthenic syndromes are caused by mutations in molecules expressed at the neuromuscular junction. Collagen Q (ColQ) makes a triple helical structure and anchors the catalytic subunit of acetylcholinesterase (AChE) to the synaptic basal lamina in the form of asymmetric AChE. Mutations in the collagen Q gene (*COLQ*) cause endplate AChE deficiency. As an initial step to develop a novel therapeutic strategy for endplate acetylcholinesterase deficiency, we expressed AChE species in cultured cells delivered by retrovirus and adeno-associated virus (AAV).

The retroviral vectors carried human *ACHE* and *COLQ* either in a single construct (EF1α-*ACHE*-IRES-*COLQ*) or in two separate constructs (EF1α-*ACHE* and EF1α-*COLQ*). We produced high-titer retroviruses using the PLAT-E retrovirus packaging cells. We also confirmed expression of asymmetric AChE in the PLAT-E cells. We infected NIH3T3 and confirmed expression of the transgenes by RT-PCR. The AAV vector carried human *COLQ*-IRES-*EGFP* downstream of the CMV promoter (pAAV-CMV-*COLQ*-IRES-*EGFP*). We produced recombinant AAV using HEK293 cells carrying pDF6 encoding the AAV6 capsid gene. We infected AAVHT1080 cells and confirmed expression of *COLQ* by RT-PCR and EGFP by flow cytometry. We are currently working on achieving further higher expression levels of transgenes in cultured cells to make the current strategy applicable to an animal model.

Keywords: collagen Q; acetylcholinesterase; retrovirus; adeno-associated virus

Introduction

There has been no rational therapy for endplate acetylcholinesterase deficiency caused by a congenital defect of collagen Q (ColQ). Unlike other molecules defective in congenital myasthenic syndromes, ColQ is an extracellular matrix protein [1]. Asymmetric acetylcholinesterase (AChE) harboring the ColQ is excreted from muscle cells and is precisely anchored to the synaptic basal lamina [2].

We tried to exploit these features of the asymmetric acetylcholinesterase to develop a novel therapeutic strategy for endplate acetylcholinesterase deficiency. As an initial step of this strategy, we here report retrovirus vector-mediated expression of collagen-tailed AChE, as well as adeno-associated virus (AAV)-mediated expression of ColQ.

Materials and methods

Retrovirus vectors

We inserted an EF1α promoter into the pSIREN-RetroQ retroviral vector (Clonetech). We then inserted either human *ACHE* to make pSIREN-EF1α-*ACHE*, human *COLQ* to make pSIREN-EF1α-*COLQ*, or human *ACHE*, IRES, and human *COLQ* to make pSIREN-EF1α-*ACHE*-IRES-*COLQ*. We employed PLAT-E packaging cells (a kind gift of Dr. Toshio Kitamura, Univ. of Tokyo) to produce recombinant retrovirus particles. PLAT-E cells were transfected with retroviral vectors using FuGENE6 (Roche). Sedimentation analysis was performed in a 5-20% sucrose density gradient to separate AChE