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Review

Clinical and Molecular Analysis of Neurodegenerative Diseases

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ABE, K. *Clinical and Molecular Analysis of Neurodegenerative Diseases.* Thoku J. Exp. Med., 1997, **181** (4), 389-409 —— Clinical and molecular analyses of neurodegenerative diseases such as Alzheimer's disease (AD), amyotrophic lateral sclerosis (ALS), and spinocerebellar ataxia type 1 (SCA1) were performed. In the present study, a Japanese family of AD with an Ala285Val substitution in exon 8 of the presenilin-1 (PS-1) gene was found. This family was characterized by relatively late onset (mean age at 50 years) in familial AD with PS-1 gene mutation and by absence of myoclonus, seizure or paratonia. Magnetic resonance image (MRI) study showed marked linear signal abnormalities in white matter of parieto-occtipital lobes, suggesting a presence of cortical amyloid angiopathy of the patient with PS-1 gene mutation. Clinical characteristics of familial amyotrophic lateral sclerosis (FALS) with four different missense point mutations in exons 2, 4, and 5 of the Cu/Zn superoxide dismutase (SOD) gene were reported. Although features of progressive neurogenic muscular atrophy was common in patients of these families, patients of each family showed characteristic clinical features. Although lower motor sign was evident in all cases, hyperreflexia varied from 0 to 100% among patients with the different mutations, and Babinski sign was not observed in any cases. Bulbar palsy was frequent with a mutation, but not present with another mutation. SOD activity of red blood cells was generally reduced with minor variations. CAG trinucleotide repeat expansion was analyzed in 25 families with hereditary ataxia of Menzel type in the northeast of Japan. Twenty of 38 patients in 12 families had expanded allele for spinocerebellar ataxia type 1 (SCA1). Study of the number of CAG repeats in various tissues showed no differences in the repeat length in lymphocytes, muscle or brain; sperm, however, showed an obvious expansion. This may be a clue to a possible mechanism for the molecular basis of paternal anticipation of the disease. These results suggest that clinical features of some familial cases of neurodegenerative diseases such as AD, ALS, and SCA1 are well correlated with their genetic mutations. ———— Alzheimer's disease; presenilin-1; Cu/Zn SOD gene; amyotrophic lateral sclerosis; spinocerebellar ataxia type 1 (SCA1)

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Abbreviations: AD, Alzheimer's disease; Aβ, amyloid β protein; ALS, amyotrophic lateral sclerosis; CSF, cerebrospinal fluid; FALS, familial amyotrophic lateral sclerosis; MRI, magnetic resonance image; NFTs, neurofibrillary tangles; PD, Parkinson's disease; PS-1, presenilin-1; SOD, superoxide dismutase.

Neurodegenerative diseases mainly include Alzheimer's disease (AD), amyotrophic lateral sclerosis (ALS), spinocerebellar ataxia (SCA), and Parkinson's disease (PD). These disorders are progressive, and most cases fall in severe clinical situations within several years of onset except cases with PD. No effective curative therapy has been established. Familial history has been known in 10-30% of AD, 5-10% of ALS, and 30-50% of SCA in Japan, but familial cases of PD is rare. Recent advancement of molecular genetic study revealed genetic abnormality of the familial cases of the above diseases. Discovery of such causative genes encouraged a potential development of the future curative therapy of those difficult disorders. Profound correlation of clinical and genetic abnormalities may suggest molecular mechanism of the disease. In the present study, clinical and molecular studies were performed in three cases of neurodegenerative diseases such as AD, ALS and SCA especially in SCA type 1 (SCA1). Thus, the works was divided into three subparts in the following.

A Japanese Family of Alzheimer's Disease with Presenilin-1 Mutation

Alzheimer's disease (AD) is a degenerative disorder of the central nervous system that causes progressive memory and cognitive decline during mid to late adult life. AD is accompanied by a wide range of neuropathologic features including extracellular amyloid plaques and intra-neural neurofibrillary tangles (NFTs). Although the etiology of the disease is complex, some cases of AD are inherited as an autosomal dominant trait. To date, at least four different genetic loci that confer inherited susceptibility to this disease have been identified. Mutations in the amyloid precursor protein gene on chromosome 21 cause early-onset (<65 years) autosomal dominant AD. For late-onset AD, the Apo E $\varepsilon 4$ allele elevates risk factor for AD. Recently, a novel gene, presenilin-1 (PS-1) on chromosome 14q was cloned and some different mutations have been found with early-onset familial AD (Sherrington et al. 1995). Although PS-1 mutations were identified early-onset familial AD families, no relationship between a mutation in the PS-1 gene and phenotype of each familial AD has been described.

Case reports

This family has lived in Japan, and no marital relation to Caucasian people is known (Fig. 1). There was no apparent history of dementia in the first generation as far as we investigated. The proband was 48 years-old woman who noticed memory disturbance at age 45. The memory disturbance was progressively developed accompanying a character change. The Japanese version of Alzheimer's disease assessment scale cognitive scores were 29.4 points. She has no history of epilepsy or involuntary movement. Hachinski's ischemic score was zero. She had no sign of involvement in cranial nerves, motor function, sensation, extra-pyramidal tract or autonomic system. There was no involuntary

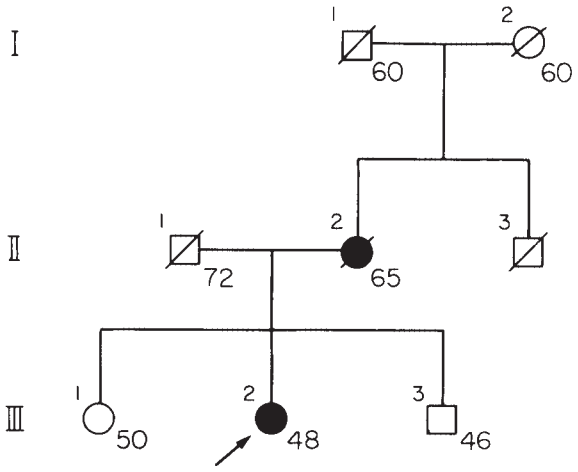

Fig. 1. Pedigree of familial Alzheimer's disease (AD). Male family members are represented by squares, female members by circles, and deceased family members by symbols with diagonals; members affected with familial AD are represented by solid symbols. Male member (II-3) died of the World War II. A case (patient 1) indicated by an arrow represents the proband of the present study.

movement. The reflexes in the upper and lower extremities were symmetrical, planter response flexor and abdominal reflexes were preserved. Results of laboratory test, including urinalysis, immunoelectrophoresis, vitamins, thyroid functions, antinuclear antibodies, chest x-rays, ECG were all normal. MRI study revealed atrophy of the cerebral cortex, especially of temporo-parietal lobes and linear high signal abnormalities in T2 weighted series in the deep white matter of parieto-occtipital lobes. Electroencephalogram demonstrated a rhythm slowing and θ activity without epileptic activity. Single-photon emission computerized tomography showed hypoperfusion in the parietal and occutipital areas. This patient, the mother of case 1, noted memory disturbance at age 55. The memory disturbance was progressively developed with a character change. She was diagnosed as AD by a neurologist. She has no history of epilepsy or involuntary movement. Results of laboratory test, including urinalysis, immunoelectrophoresis, vitamins, thyroid functions, antinuclear antibodies, chest x-rays, ECG were all normal. She died of bronchopneumonia at age 65. No postmortem examination was obtained.

DNA analysis

DNA was extracted from peripheral blood lymphocytes of the above family members as well as Japanese control subjects. Informed consent was obtained from each individual and the families. The PS-1 gene was examined in the proband as previous methods (Aoki et al. 1994b; Tsuda et al. 1995). A novel missense mutation (C to T) was identified in exon 8 of the PS-1 gene, which resulted in amino acid substitution of alanine^{285} by valine (Ala285Val) in the

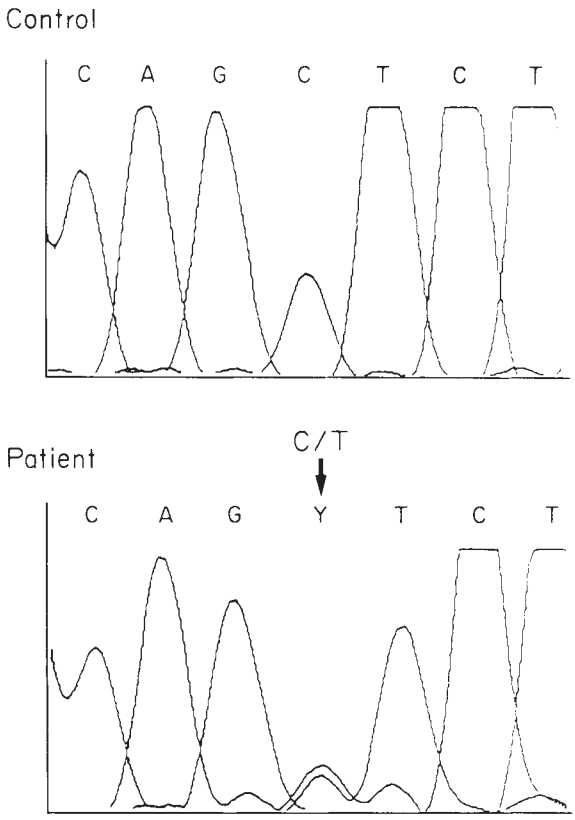

Fig. 2. Sequence analysis of a point mutation in the PS-1 gene. The upper panel shows the normal sequence whereas the lower panel shows equal C and T signals (arrow) at position 285, indicating that both nucleotides are present at this site in the patient.

patient (Fig. 2). To assess whether the possible presence of the mutation in the non-affected member of the family and the normal population, genomic DNA from 70 unrelated Japanese control subjects were investigated using allele specific oligonucleotide hybridization. No mutant allele, however, was present in these subjects (data not shown).

Cerebrospinal fluid analysis

Cerebrospinal fluid (CSF) was collected by lumber puncture from the case 1 and 20 Japanese control subjects. The cell number and protein concentration in CSF were normal in all subjects. Tau and amyloid β protein (Aβ) were measured in enzyme-linked immunosorbent assays as previously described (Suzuki et al. 1994; Mori et al. 1995). The amount of CSF tau in patient 1 was 119.2 pg/ml. The level was 5.8 times higher than the mean tau level in our control subjects (20.7 ± 16.0 pg/ml, mean $\pm$ S.D., $n=20$). The amount of Aβ1-40 and Aβ1-42(43) in CSF of case 1 was 1789.4 fmol/ml and 195.9 fmol/ml, respectively. These levels were not different compared with those of control levels (data not shown).

Discussion

Common feature of the phenotype of 14q linked familial AD in the families (FAD4, FAD2, A, B, L) included onset of dementia before age 50 and shorter duration of disease, early progressive aphasia, early appearance of myoclonus and generalized seizure, paratonia and cortical atrophy (Lampe et al. 1994). Neuropathological study revealed numerous and extensive senile plaque and NFTs, and prominent amyloid angiopathy. In contrast, in other families (SNW/FAD3, FAD1) also linked to 14q, affected individuals in some instance were noted to survive age 70 or beyond and the mean age at onset in these kindred was relatively higher. In these families, seizure and myoclonus or extrapyramidal signs were absent. Our Japanese family was characterized by relatively late onset (mean age at onset 50 years) in familial AD with PS-1 mutation and by absence of myoclonus, seizure or paratonia as compared to previous reports. It is interesting that Leu286Val mutation in the PS-1 gene was revealed to be associated with German FAD2 family (Sherrington et al. 1995) that was characterized by early-appearing myoclonus and generalized seizure, and extrapyramidal features. Thus, the clinical phenotype may be different among the families with the different mutations within the same hydrophilic loop of exon 8. Marked elevation of the amount of CSF tau in the genetically determined case supports its reliability for diagnostic test for AD (Mori et al. 1995) and suggests that the AD pathology may progress extensively in patient 1 brain, although clinical degree of her dementia is still mild.

The contribution of white matter changes to the clinical features of dementia syndrome in AD is still under debate (Scheltens et al. 1992; Brilliant et al. 1995). Although there has been few report about white matter changes in 14q linked familial AD, Scheltens et al. (1992) reported that there were no significant differences in any of the hyperintensity scores on MRI image between sporadic presenile onset AD and the age-matched control group. On the other hand, they reported that subcortical and deep white matter hyperintensity scores, especially in the frontal and parietal regions, were significantly higher in the senile onset AD group than control group and that these higher scores may reflect the presence of more severe cerebral amyloid angiopathy.

Neuropathological findings consistently reported in all 14q linked familial AD kindreds included numerous and extensive senile plaque and NFTs and prominent amyloid angiopathy. Immunohistochemical study of cerebral amyloid angiopathy revealed that the relative absence of Aβ immunoreactivity within the parenchymal white matter and that Aβ was not responsible for white matter changes. On the other hand, the recent report revealed that white matter hyperintensity on MRI, dominantly in deep white matter of parietal and occipital lobes, in hereditary cerebral hemorrhage with amyloidosis are probably caused by chronic ischemia due to stenosis of meningocortical arterioles (Bornebroek et al.

1996). Therefore, the white matter hyperintensity on MRI of the patient with Ala285Val mutation may reflect a presense of cortical amyloid angiopathy, although there was few report such as linear lesions as this case. Further studies should be done to clarify the exact nature of these linear lesions associated with PS-1 gene mutation.

Clinical Characteristics of Familial Amyotrophic Lateral Sclerosis with Cu/Zn Superoxide Dismutase Gene Mutations

Amyotrophic lateral sclerosis (ALS) is a degenerative disorder affecting predominantly motor neurons in the central nervous system. The clinical course of ALS is characterized by progressive muscle weakness and atrophy, leading to fatal paralysis and death (Horton et al. 1976; Boylan and Carnblath 1992; Shaw et al. 1992; Melki et al. 1994). There has been no effective treatment, and the duration is usually 2-5 years. About 5-10% of ALS is thought to be familial (FALS) with an autosomal dominant inheritance, or less often recessive, with age dependent penetrance (Mulder et al. 1986). Genetic linkage studies showed that a part of FALS is linked to chromosome 21q22.1 including the locus of Cu/Zn superoxide dismutase (SOD) gene (Siddique et al. 1991). Although FALS is thus genetically heterogeneous, missense point mutations and a small deletion have recently been identified in the Cu/Zn SOD gene in a part of patients with familial and sporadic ALS (Aoki et al. 1993; Deng et al. 1993; Rosen et al. 1993; Elshafey et al. 1994; Esteban et al. 1994; Jones et al. 1994; Nakano et al. 1994; Rainero et al. 1994;) as a putative cause of the disease. SOD has a role to remove superoxide radicals produced in normal cellular metabolism and under pathological conditions (Halliwell and Gutteridge 1985; Fridovich 1986; Mitchell et al. 1993). The activities of Cu/Zn SOD protein were reduced with the mutations than that of normal protein. However, an overexpression of mutated Cu/Zn SOD caused ALS-like symptom in transgenic mice (Gurney et al. 1994). Therefore, even though an association of the mutation of the Cu/Zn SOD gene with a part of FALS was found, the exact mechanism of the disease has not been fully understood.

Although 32 mutations in 24 amino acids of Cu/Zn SOD have been reported in Caucasian and Japanese families, relationships between each mutation of the gene and phenotypic characteristics of the family have not been fully described. We report here five FALS families associated with four different missense mutations of the Cu/Zn SOD gene (H46R, L84V, I104F, and V148I), and each family has unique clinical characteristics.

Clinical description of the family

Seven FALS families (family 1-7) were examined. All the families are Japanese, and have no marital relation to Caucasian or other races. The family

histories showed autosomal dominant inheritances, and penetrance was high in six families (family 1-6) and low in one (family-7).

In family-1, 13 individuals were affected by progressive muscle weakness and atrophy in four successive generations. The family history suggests an almost complete penetrance. Mean age at onset was 49.6 ± 10.9 ($n=10$, mean$\pm$S.D.) years, and mean duration after the onset was 15.8 ± 9.9 ($n=5$) years. The proband was a 76-year-old woman who first noted weakness of the left leg and difficulty in standing on her tiptoes at age 68. Her symptoms slowly developed. General physical examination was normal. On the neurological examination, distal lower limbs showed weakness and atrophy with fasciculations with only minimal change in the upper limbs. Deep tendon reflexes were generally decreased. No pathological reflexes were found. Cognitive function, cranial nerves, sensation and autonomic system were intact. Hemogram, brain computed tomogram, urinalysis, serum chemical studies including creatine kinase (CK), and CSF studies were normal. Serum antibody against human T-cell leukemia virus type-1 was negative. Biopsy of the gastrocnemius muscle showed a marked group atrophy of myofibers, while that of anterior tibial muscle revealed only a slight neurogenic change with type I fiber predominancy. The clinical features of rest of the patients were almost similar to the proband. All the patients had weakness of legs as the first symptom (especially in the left side), and bulbar palsy was seen only 30% of cases. This family is characterized by relatively benign course and uniform progression of the symptoms.

In family-2, 14 individuals were affected. The family history suggests an almost complete penetrance in four successive generations. The mean age at onset was 48.0 ± 9.5 ($n=14$) years with duration of 16.8 ± 6.8 ($n=9$) years. The proband was a 71-year-old woman who first noticed weakness of the left leg at age 65. The symptoms became gradually worsened. On the neurological examination, her leg showed weakness and atrophy especially in the distal parts with fasciculations. Deep tendon reflexes were generally decreased. Pathological reflexes were not found. Her hands were slightly involved, but cognitive function, cranial nerves, sensation or autonomic system was not involved. Serum CK level was normal. The clinical features of the other affected family members were almost the same as the proband, and similar to those of family-1.

In family-3, three successive generations, five individuals were affected. The family history suggests an almost complete penetrance. The mean age at onset was 53.8 ± 15.3 ($n=5$) years with duration of 1.6 ± 0.5 ($n=5$) years. The proband was a man who first noted a weakness and atrophy of left hand at age 38. The age at onset was quite different between generations or men and women. That of the first generation was 74 years, the second 52.3 ± 11.6 ($n=3$), and the third 38. That was 38 and 39 ($n=2$) in men, and 64.0 ± 8.7 ($n=3$) in women. However, with exception of the age at onset, the clinical progression was rather uniform in all patients of this family, the symptom characterized by beginning from the

hands, rapid progression, and early death usually within 1.6 years.

In family-4, five individuals were affected, and the genetic trait is considered to be autosomal dominant in three successive generations. The age at onset was variable in this family ranging from 6 to 55 years (33.0 ± 20.7, $n=4$) with duration also varying from 12 to 38 years (21.3 ± 11.8, $n=3$). Cases II-1 and -13 died before the mean age at onset (33.0) due to other diseases at 59 and 34 years old without developing ALS symptoms, suggesting that they were obligate gene carriers. The proband, first noted weakness in both hands at age 41, and followed by weakness in the lower limbs and bulbar symptoms 1.5 years after the onset. Deep tendon reflexes were generally increased without pathologic reflexes. The affected members have clinical varieties in the age at onset, first symptom, and duration of the disease. Bulbar palsy was observed in all the cases, but also took variable years to appear ranging from 1.5 to 14 years (7.8 ± 5.7, $n=4$).

In family-5, five individuals were affected with almost complete penetrance in three successive generations. The mean age at onset was 28.0 ± 3.8 ($n=4$) years with duration of 1.8 ± 0.5 ($n=3$) years. There was a significant difference in the sequential expression of symptoms among the affected family members. The first symptom of the proband appeared in legs, that was then followed by muscle atrophy of upper limbs and bulbar palsy 1 to 1.5 years after the onset. He died of respiratory failure at age 33 without developing dysarthria or dysphagia even after 2.5 years of the onset. On the other hand, his brother and mother first noted symptoms in tongue, neck and upper limbs, and the lower limbs were affected 1 year later. They died 1.3–1.7 years after the onset. The family is characterized by younger age at onset, rapid progression, and variable first symptoms. Serum CK was normal except in the proband (782 IU/liter).

DNA analysis of SOD and the protein activity

Examination of possible mutations in the Cu/Zn SOD gene in cases with FALS was performed according to a previous report (Levanon et al. 1985; Abe et al. 1996). In order to investigate how the Cu/Zn SOD mutations affect the function of the protein, the protein activity of erythrocyte lysate was measured in the patients and age-matched normal Japanese controls ($n=6$) by the nitrite method (Oyanagui 1984) according to our previous report (Aoki et al. 1993). In brief, heparinized blood was centrifuged and the plasma was removed. After a rinse with physiological saline, the cell pellet was diluted with water to obtain a lysis of erythrocytes. To remove the hemoglobin, ethanol and chloroform were added, and then the water/ethanol layer was separated to measure the Cu/Zn SOD activity. For the case with L84V mutation, the activity was measured with cultured fibroblasts because of the death of the patient, and was compared with that of normal controls. Data were expressed as % of control.

Four missense mutations of A to G in exon 2, T to G and A to T in exon 4, and G to A in exon 5 were identified in the Cu/Zn SOD cDNA in 5 out of 7

Japanese FALS families. Those mutations result in amino acid substitutions of histidine[46] by arginine (H46R), leucine[84] by valine (L84V), isoleucine[104] by phenylalanine (I104F), and valine[148] by isoleucine (V148I), respectively. No other abnormality was found in the cDNA sequences in the five families. No mutation was found in the rest of two families (family-6 and -7). Furthermore, single strand conformational polymorphism (SSCP) analysis of five exons showed normal pattern in the family-6 and -7 while that showed abnormal patterns in the five families (family 1-5).

In the genomic analysis for H46R, L84V, and V148I, a control study with plasmid clones confirmed the discriminative detection of mutant/wild type sequence by allele specific oligonucleotide (ASO) analysis. The mutation was heterozygously present only in the patients, but was absent in the non-affected members of the families. Fifty seven normal Japanese subjects and 27 sporadic ALS patients did not have such mutant alleles. In this analysis, an individual of family-2 (IV-2), who is healthy at this moment but much more under the mean age at onset of the family, showed an abnormal SSCP pattern, and carried the H46R mutation by direct sequence analysis. This person is suspected as a gene carrier. For an analysis of I104F, genomic polymerase chain reaction (PCR) products of exon 4 were digested by Sau3A1 because this point mutation results in loss of the normal restriction site for the enzyme. In all of 116 alleles of genomic DNA from 58 normal controls, the digestion of 233 bp PCR products produced two smaller fragments of 126 bp and 107 bp. On the other hand, genomic DNA of the patient showed fragments of undigested 233 bp and the two digested fragments.

Cu/Zn SOD activities in erythrocytes were lower in the affected members of the families with H46R (family-1, $75.9 \pm 11.0\%$, $n=4$; family-2, $71.4 \pm 14.6\%$, $n=4$), I104F (43.1%, $n=1$), and V148I (56.3 and 59.0%, $n=2$) than age-matched normal controls. The activity of cultured fibroblast with L84V mutation was 74.8% ($n=1$) of control.

Discussion

Four kinds of missense mutations were found in exons 2, 4, and 5 of the Cu/Zn SOD cDNA resulting in amino acid substitutions of H46R, L84V, I104F, anutation, that was observed in family-1 and -2, is located in the Cu binding site of Cu/Zn SOD protein. Patients of FALS with H46R mutation showed benign clinical course and uniform sequential expression of the symptoms. These clinical characteristics were reproducible among different families (family-1 and -2). The slow progression may be related with the mild decrease of the Cu/Zn SOD activity. It is intriguing that the reduction of the activity was only 24-29% of control in family-1 and -2 although H46R mutation locates just in the Cu binding site that is essential for the enzyme activity to react with superoxide. L84V mutation in a case of family-3 locates in the C-terminal of the IV active site loop

of the protein. The IV active site loop covers whole amino acid sequences derived from exon 3 and a part of exons 2 and 4 of the genome, and L84V is the only mutation in this longest active site loop. No mutation in exon 3 of the gene has been reported. Thus, L84V is in the unique position of the protein. It is interesting that affected members with this mutation suggested differences of age at onset between both sexes. Similar difference was not found in other FALS families with Cn/Zn SOD mutations (family-1, -2, -4, and -5). Although limited number of patients defer the conclusion, these data suggest an existence of another factor(s) to modulate age at onset of the disease. Contrary to cases with H46R mutation, the disease progression was rapid in family-3 (1.6 ± 0.5 years) while reduction of the SOD activity in the cultured lymphoblast of the patient was mild (74.8% of control). These results also suggest that another factor could affect the course of the disease. Identification of such factor(s) might have a potential to modify age at onset and course of the disease.

The I104F locates in the VI Greek key, and therefore, would be expected to make Cu/Zn SOD protein unstable (Deng et al. 1993). In fact, the Cu/Zn SOD activity of this patient was 43.1% of controls. In the same Greek key, three other mutations (L106V, I112T, and I113T) were reported in FALS patients (Deng et al. 1993; Esteban et al. 1994). However, the Cu/Zn SOD activity was not reported with those mutations. On the other hand, other mutations in exon 4 such as G93A and E100G that are located near the VI Greek key showed the Cu/Zn SOD activities of 35.8% and 35.9%, respectively (Deng et al. 1993). Rainero et al. (1994) showed variable ages of the onset in one family with a mutation in exon 2 (G41S), but the clinical phenotype was rather uniform. It would be interesting that a recent study with transgenic mice with G93A mutation showed some clinical variations among the mice (Gurney et al. 1994). Compared to FALS families with H46R and L84V mutations, the patients of FALS with V148I mutation showed a younger onset and a considerable clinical variety, especially in the first symptoms. Although the patients with mutations at dimer contact or the near showed relatively rapid progression (Deng et al. 1993; Rosen et al. 1993, 1994), such a considerable variety of the symptoms has not been reported with a mutation of the Cu/Zn SOD gene.

SOD removes superoxide radicals produced under normal and pathological conditions (Halliwell and Gutteridge 1985; Fridovich 1986). Therefore, free radical-related mechanism has been suggested in the pathogenesis of the disease (Mitchell et al. 1993). However, analyses of large number (222–240) of Caucasian FALS families suggest that only 14 to 20% of FALS carrys mutations in about 20 out of 153 amino acids of SOD protein (International Symposium on Superoxide Dismutase and Free Radicals in ALS and Neuro-Degeneration 1994). We have identified four different point mutations in the Cu/Zn SOD gene in 5 out of 7 FALS families (71%). In this study, some unique clinical features of FALS were observed in five Japanese FALS families with Cu/Zn SOD mutations. Lower

TABLE 1. *Summary of the Cu/Zn SOD mutations and clinical features*

	Family-1	Family-2	Family-3	Family-4	Family-5
Mutation	H46R (CAT→CGT)	H46R (CAT→CGT)	L84V (TTG→GTG)	I104F (ATC→TTC)	V148I (GTA→ATA)
Mutated exon	2	2	4	4	5
Mutation site	Cu binding	Cu binding	IV active site loop	VI Greek key	Dimer contact
Clinical characteristics	Mild course and uniform symptom	Mild course and uniform symptom	Sex difference of age at onset	Variable age at onset	Young onset and variable first symptom
Penetrance	Complete	Complete	Complete	Reduced ?	Complete
Age at onset[a]	49.6±10.9 (n=10)	48.0±9.5 (n=14)	53.8±15.3 (n=5)	33.0±20.7 (n=4)	28.0±3.8 (n=4)
Duration (years)[a]	15.8± 9.9 (n=5)	16.8±6.8 (n=9)	1.6± 0.5 (n=5)	21.3±11.8 (n=3)	1.8±0.5 (n=3)
First symptom[b]	Legs (all)	Legs (all)	Hands (all)	Legs (3)/hands (1)	Bulbar (2)/hands (2)/legs (1)
Upper motor sign					
Hyperreflexia[c]	40%	33%	+ (n=1)	75%	25%
Babinski sign	Negative	Negative	Negative	Negative	Negative
Lower motor sign	100%	100%	+ (n=1)	100%	100%
Bulbar palsy[c]	30%	33%	+ (n=1)	100%	50%
Others	—	—	—	Ophthalmoparesis and sensory disturbance	—
Creatine kinase	Normal	Normal	Normal	Moderately increased	Normal (3) or increased (1)
Muscle biopsy	Neurogenic change	n.o.	n.o.	n.o.	Neurogenic change
SOD activity[a]	75.9±11.0% (n=4)	71.4±14.6% (n=4)	74.8%[d] (n=1)	43.1% (n=1)	56.3 and 59.0% (n=2)

[a]Values of age at onset, duration, and a part of SOD activity are expressed as mean±S.D., and SOD activity is shown as % of age- and sex-matched control.

[b]Parentheses show number of patients in the family.

[c]Values are shown as % of the patients in each family, and can be varied based on incorporating not only evident sign but also suspicious or not.

[d]SOD activity was measured with cultured fibroblast because of death of the patient.

n.o., not obtained.

motor sign was evident in all cases of the present families, and Babinski sign was not observed in any cases, but hyperreflexia varied from 0 to 100% among patients with the different mutations. Mean age at onset, duration, and frequency of bulbar palsy also varied between mutations. Of interest, familial ALS with different mutations of the Cu/Zn SOD gene showed each clinical characteristics. However, there was not a good correlation between SOD activity and the severity of illness. Even though the age at onset was much younger in family-5 with greater reduction of SOD activity than family-3, duration of illness was the same between the families (Table 1). In addition, family-4 had the lowest SOD activity with the longest duration of illness. A recent report suggested that decrease of SOD activity was not related to develop symptom in homozygously mutated patients with FALS (Andersen et al. 1995). Thus, gain, but not loss, of the function of SOD may be associated with the development of the disease. Furthermore, considerable clinical variety of the symptoms in FALS families suggest that not only mutations of the Cu/Zn SOD gene but unidentified other factor(s) might also be involved in the occurrence and course of FALS. In fact, an abnormality of additional gene involving neurofilament has been reported (Figlewicz et al. 1994). Further analyses may be required to elucidate the effect of each mutation on the expression of the disease.

Analysis of SCA1-related CAG trinucleotide expansion

Spinocerebellar ataxia type 1 (SCA1) is an autosomal dominant neurodegenerative disorder characterized by cerebellar ataxia, ophthalmoparesis, and pyramidal signs (Schut 1950). Neuropathological findings in SCA1 have defined atrophy of the brainstem and cerebellum with loss of neurons in the inferior olives, pontine nuclei, and Purkinje cells, as well as degeneration of the spinocerebellar tracts and dentate nuclei (Zoghbi et al. 1988). The SCA1 gene was linked to the HLA locus on the short arm of chromosome 6 (Yakura et al. 1974), and more detailed genetic and physical mapping showed the localization at 6p22-p23 region (Zoghbi et al. 1991). Recent studies have identified an unstable trinucleotide CAG repeat expansion as the mutation causing SCA1 (Orr et al. 1993). Mutations with unstable triplet repeat expansions occur in some neurological disorders such as X-linked spinal and bulbar muscular atrophy (SBMA), fragile X syndrome, myotonic dystrophy (DM), Huntington's disease (HD), hereditary dentatorubropallidoluysian atrophy (DRPLA), and Machado-Joseph disease (MJD). The increase in the severity of the phenotype and the earlier age at onset in later generations is known as "anticipation". Increasing size of the triplet repeats in successive generations suggests a molecular basis for anticipation in the above disorders. The SCA1 gene is expressed in the brain, in the kidneys, and, to a considerable degree, in skeletal muscle, but the function of the SCA1 encoded protein, called ataxin-1, still remains unknown.

In the present study, families with autosomal dominant cerebellar ataxia of

Menzel type in the northeast of Japan (the area in Japan) showing the highest frequency of SCA1 were studied, and the correlation between clinical features and the number of CAG repeats examined was examined in SCA1 patients in Japanese kindreds. To investigate the mitotic and meiotic stability of the CAG repeat on the chromosomes bearing SCA1 genes, we analyzed the numbers of CAG trinucleotide repeats in different tissues, including the brain, muscle, blood lymphocytes and sperm, as well as analyzing mRNA for SCA1 to determine whether the expanded SCA1 allele is transcribed in organs of affected individuals.

Case report and DNA analysis

Thirty-eight affected members of 25 families with Menzel type of hereditary ataxia were clinically examined, and their DNAs were analyzed to determine if they had the expanded SCA1 allele or not. Other hereditary or non-hereditary types of spinocerebellar degeneration (SCD) were also examined in relation to the number of CAG repeats. Cerebellar signs such as ataxia, dysarthria and dysmetria; ocular findings such as ophthalmoparesis, slow saccades and nystagmus; atrophy of the face, tongue and skeletal muscles; pyramidal tract signs; involuntary movement; epilepsy; the autonomic nervous system; and dementia were clinically evaluated in each patient to compare clinical characteristics between patients with and without SCA1 mutations.

DNA was extracted from peripheral blood lymphocytes of the above family members with informed consent and from 50 healthy control individuals by a standard protocol. To assess CAG trinucleotide repeat expansion, PCR was performed.

For an estimation of accurate repeat numbers, a fluorescein isothiocyanate (FITC)-labeled primer of Rep-2 was used for PCR amplification, then an aliquot of the product was electrophoresed. The data were processed with fragment analysis software (Fragment Manager, Pharmacia) using a mixture of FITC-labeled fragments (50–500 base pairs in length) according to our previous method (Aoki et al. 1994a). Sperm was obtained from two affected men. Muscle specimens were obtained from two patients (a man and a woman) who showed marked muscle wasting. Blood, sperm and muscle specimens were simultaneously available in one of the above men. Brain tissues had been removed from one patient within 5 hr after death. Muscle and brain samples were immediately frozen in dry ice-isopentane or in liquid nitrogen, and kept at $-70^{\circ}C$ until analysis. From the above samples, DNA was extracted, and SCA1 alleles were examined with PCR.

Out of the 38 members of the 25 families with Menzel type of hereditary ataxia, 20 patients in 12 families had expanded SCA1 allele. Other autosomal dominant cerebellar ataxias such as Holmes type of hereditary ataxia (8 patients in 5 families) and DRPLA (4 patients in 3 families) revealed normal alleles for SCA1. Moreover, non-hereditary type of SCD such as olivopontocerebellar atro-

TABLE 2. *Clinical features and the numbers of CAG repeats in 20 SCAI patients*

Patient	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16*	17	18	19	20	
Repeat number	58	58	51	49	49	49	47	47	47	47	47	46	45	45	45	44	44	43	42	42	47.3± 4.4
Duration (years)	9	3	6	11	9	6	22	13	10	10	9	18	11	11	10	20	19	20	15	6	11.9± 5.4
Age at onset (years)	19	20	26	40	45	36	32	35	43	30	51	45	50	36	35	46	37	52	55	55	39.4±10.7
Sex	M	M	M	F	F	F	M	M	F	M	M	F	M	M	M	M	F	M	M	M	M14/F6
Ataxia, limb	+	±	+	+	+	+	+	+	+	±	+	+	+	+	+	+	+	+	+	+	90%
trunk	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	100%
Ophthalmoparesis	−	+	−	−	+	+	+	+	+	−	−	+	±	−	−	+	+	+	+	+	60%
Slow saccades	−	−	+	−	−	+	+	+	−	−	−	+	−	−	−	−	+	+	+	−	40%
Nystagmus	+	+	−	+	−	±	±	+	+	−	±	+	±	±	+	+	+	+	±	±	−	45%
Dysphagia	±	±	−	±	+	+	+	+	+	−	−	±	−	+	±	+	+	+	+	+	55%
Amyotrophy	+	−	−	−	+	+	+	+	−	−	−	−	−	+	−	+	−	+	+	−	45%
Hyperreflexia	+	−	+	+	+	+	+	+	+	+	−	−	−	+	+	+	+	+	+	−	75%
Babinski sign	−	−	−	+	+	±	+	−	+	+	−	+	+	±	−	+	+	±	±	±	45%
Disturbance of sphincter control	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	+	+	−	15%

"M" means male patients, "F" means females. "+" indicates the symptom is present, "−" that it is absent, and "±" that its presence is equivocal. Repeat numbers of expanded alleles from lymphocyte DNAs are shown except for case 16. The values of mean±S.D. of the repeat number, duration and onset age are shown on the right.

*Case 16 is an autopsied case. A blood sample was not available in this case; the repeat number was measured by the DNA extracted from the cerebellar cortex.

phy (24 patients) and late cortical cerebellar atrophy (7 patients) also showed a normal range of CAG repeats. The repeat size was 47.3 ± 4.4 (mean $\pm$ S.D., $n=20$) with a range of 42–58. Sizes of normal allele were 28.8 ± 0.4 (mean $\pm$ S.D., $n=20$), ranging from 26 to 32. The CAG repeat number was well fitted to the normal distribution when logarithmic transformation was performed. The age at onset was well correlated with the number of CAG repeats with a correlation coefficient (r) of 0.81 ($p<0.0001$, $n=20$) indicating that 63% ($r^2=0.63$) of the variation in the age at onset can be accounted for by the number of CAG repeats on the disease chromosome. The regression curve was also estimated on the transformed data. The patients with SCA1 allele were clinically characterized by relatively high frequency of hyperreflexia, ophthalmoparesis and dysphagia with a relatively low frequency of disturbance of sphincter control. Urinary incontinence was present in one patient, and pollakisuria was present in two patients. Dementia was not clearly observed, while some mental symptoms such as restlessness and emotional lability were seen in one patient (patient 16) in the advanced stage. Involuntary movement was rare, though neck dystonia and chorea in the forearm were noted in patient 12, and spasmodic torticollis and tremor in the right hand were observed in patient 14. Some patients had marked muscle wasting with a relatively increased number of CAG repeats and duration of the disease. Patients with juvenile onset (at ages under 18 years) were not observed in this study. Clinical summaries are shown in Table 2.

The number of CAG repeats of the muscle was 58 in patient 1, and 49 in patient 6, and the size of the expansion was the same as that of lymphocyte DNA. In contrast, the repeat numbers of the sperms were higher than that of lymphocyte DNA. The repeat lengths of the sperm were 62 (patient 1) and 54 (patient 3),

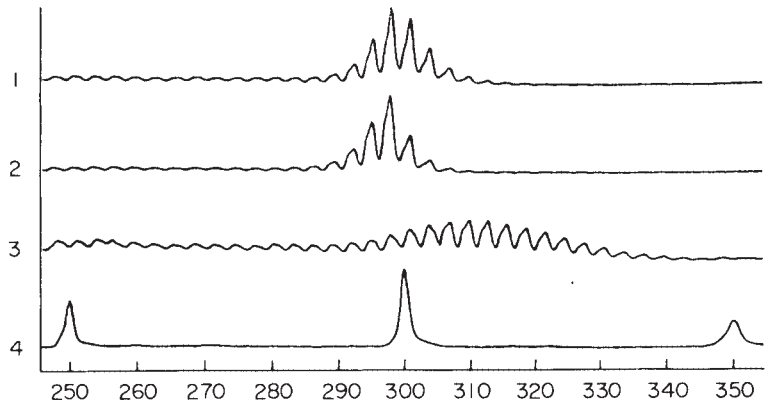

Fig. 3. Sizes of the CAG trinucleotide repeat in different tissues from a male SCA1 patient. The CAG repeat number of the lymphocytes (lane 1) was 58, the same as that of muscle (lane 2), whereas that of sperm (lane 3) was increased up to 62. Size standard is shown in lane 4, and scale indicates the sizes of PCR products. The repeat number was calculated from the size of the PCR products. Note the presence of multiple peaks in each tissue, and variability is most prominent in sperm (lane 3).

whereas those of lymphocytes were 58 (patient 1) and 51 (patient 3). DNAs from lymphocyte, muscle and sperm were simultaneously available in one patient (patient 1, Fig. 3). Blood DNA samples of the children of the sperm donors were not available for analysis of the number of CAG repeats, because they had no children. The CAG repeat size of cerebral cortex was 45, and that of cerebellar cortex was 44, in the autopsied patient (patient 16). A blood sample from this patient was not available.

Analysis of biopsied muscles (patient 1) showed that the size of each myofiber was slightly small, and shrunk fibers were observed in 0.1% of the population. However, grouped atrophy or small angulated fibers were not observed. Neither fiber-type grouping nor atrophy was shown by ATPase staining. Internal nuclei were observed in 1% of the fibers. Neither ragged-red fibers nor necrotic fibers were present. These findings were similar to those of disused atrophy. The findings of patient 6 were similar

In an analysis of the autopsied patient (patient 16), the cerebrum showed only mild atrophy, while the pons and cerebellum showed remarkable or moderate atrophy, respectively. Histological examination revealed marked loss of Purkinje cells with Bergman's gliosis, while the molecular layer and granular layer were relatively unaffected in the cerebellar cortex. There were marked neuronal loss and gliosis in the dentate nuclei and inferior olivary nuclei. Reduction of the neurons in pontine nuclei was present, but was not so severe. The nuclei of the abducens nerve were moderately degenerated. In the spinal cord, almost all of the large motor neurons of anterior horn and the principal neurons of Clark's dorsal nuclei had disappeared, and the spinocerebellar tract and dorsal column were also moderately affected. In contrast to the biopsied patients, muscle specimens showed typical neurogenic changes (data not shown).

Discussion

SCD is relatively prevalent in the Tohoku and Hokkaido districts of Japan. The mean prevalence of SCD (containing both hereditary and non-hereditary cases) in Japan is 8.4 per 100,000 population, while those in Miyagi Prefecture is 10.1, in Yamagata Prefecture, 12.4, and in the Hokkaido district, 13.1 per 100,000 population (Kameya et al. 1995). Ancestors of SCA1 pedigree first reported to be linked to the HLA locus in Hokkaido were thought to be immigrants mainly from Miyagi and Yamagata Prefectures about 100 years ago, in the Meiji era. Thus, most families, which have proved to be SCA1 by genetic linkage study in Japan, are suspected to have originated in this part of the Tohoku district. In HD and MJD, the linkage disequilibrium is observed with haplotype analysis of adjacent loci; this may suggest a founder effect (Takiyama et al. 1993; Barron et al. 1994). We could not examine the haplotypes of SCA1 patients both in the Tohoku and in the Hokkaido districts to see if they are related. The present study showed that about half of the patients with Menzel-type hereditary SCD carried the SCA1

mutation in this area. Clinical characteristics of SCA1 are a relatively high frequency of hyperreflexia, ophthalmoparesis, and dysphagia. Involuntary movements were rare. These findings are similar to those of MJD type II (Coutinho and Andrade 1978). It is difficult to distinguish MJD from SCA1 by clinical examination alone, although slow saccade is rare, and disturbance of sphincter control is relatively frequent in patients with MJD compared with those with SCA1.

A significant correlation between age at onset and the number of CAG repeats was present; the relationship seems to be similar with that in the Caucasian population (Orr et al. 1993) in contrast to DRPLA. The correlation between the age at onset and the repeat size and the parental origin effect occurs in Japanese DRPLA families, whereas in an African-American family (Haw River syndrome) there was little difference in the size of the expanded repeat among affected individuals (Burke et al. 1994). A previous report showed that patients with juvenile onset of SCA1 typically inherited the disease gene from an affected father (Chung et al. 1993; Orr et al. 1993). Although no juvenile patients were included in our study, the ages at onset of the patients with paternal transmission were relatively younger than those with maternal transmission. Loss of the CAT interruption between the CAG repeat tracts may be involved in the conversion of a stable allele to an unstable allele predisposing it to further expansion of the CAG repeat (Chung et al. 1993). The number of repeats was the same in lymphocytes and muscle DNA, whereas that in sperm was significantly greater, even though the number of patients examined was small. Increased levels of the variation of the CAG repeats as well as increased sizes of the CAG repeats also occurs in HD (Telenius et al. 1994). This may provide an explanation for the mechanism of paternal anticipation in SCA1.

The neuropathological findings in SCA1 can be summarized as follows: mild to moderate neuronal loss in the Purkinje cell layer, dentate nuclei and pontine nuclei; obvious reduction of neurons in the inferior olivary nuclei as well as marked degeneration of spinocerebellar tracts. Motor neurons in the cranial nerves and the anterior horn of the spinal cord are often involved. The pathological findings in one of the present patient (patient 16) were consistent with those reported previously. The muscle specimen from the autopsied patient revealed apparent neurogenic changes as shown by marked loss of motor neurons in the anterior horn of the spinal cord. The present study demonstrated relatively high prevalence of SCA1 based on an increase of CAG repeats in the Tohoku district in Japan, and the clinical characteristics were related to the CAG expansions. The germline repeats showed greater heterogeneity with larger size suggesting mitotic stability and meiotic instability of the CAG repeat in SCA1. This may provide us with a possible explanation for the molecular basis of paternal anticipation of the disease. Furthermore, the SCA1 gene was expressed from both normal and expanded alleles in muscles of affected individuals indicating a

gain-of-function effect of the mutation.

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