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ORIGINAL ARTICLE

α-Synuclein Pathology in the Amyotrophic Lateral Sclerosis/ Parkinsonism Dementia Complex in the Kii Peninsula, Japan

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Abstract

α-Synuclein pathology was examined in the brains and spinal cords of 10 patients with amyotrophic lateral sclerosis (ALS)/parkinsonism-dementia complex (PDC) in the Kii Peninsula, Japan. Various types of phosphorylated α-synuclein–positive structures including neuronal cytoplasmic inclusions, dystrophic neurites, and glial cytoplasmic inclusions were found in all ALS/PDC cases. There were phosphorylated α-synuclein–positive neurons in 8 cases (80%), and the amygdala was most severely affected. Phosphorylated α-synuclein was distributed mainly in the limbic system and brainstem; tau pathology was more prevalent than α-synuclein pathology in most affected areas. In the substantia nigra, periaqueductal gray, locus coeruleus, raphe nuclei, dorsal nucleus of the vagus nerve, hypoglossal nucleus or ventral horn, and intermediolateral nucleus of the spinal cord, α-synuclein pathology was more predominant than tau pathology in only 1 or 2 patients. Phosphorylated α-synuclein– positive structures were not found in the molecular layer of the cerebellum. Phosphorylated α-synuclein frequently colocalized with tau in neuron cell bodies, neurites, and glia. Immunoblots of sarkosyl-insoluble fractions extracted from the brain of 1 patient showed a triplet of α-synuclein–immunoreactive bands that were ubiquitinated. These results suggest that interaction between tau and α-synuclein be involved in the pathogenesis of Kii ALS/PDC.

Key Words: α-Synuclein, Amyotrophic lateral sclerosis, Guam, Kii Peninsula, Parkinsonism-dementia complex, Tau.

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INTRODUCTION

Amyotrophic lateral sclerosis/parkinsonism-dementia complex (ALS/PDC) is a neurodegenerative disease endemic to Guam and the Kii Peninsula of Japan (1–3). The clinical picture of ALS/PDC is a unique combination of parkinsonism, dementia, and symptoms of upper and lower motor neuronal dysfunction. Neuropathologic findings of ALS/PDC include numerous neurofibrillary tangles (NFTs) associated with nerve cell loss in the cerebral cortex and brainstem in addition to ALS pathology. Our investigation of the topographical distribution of NFTs suggested that ALS and PDC in the Kii Peninsula comprise part of a spectrum of tauopathies (4).

α-Synuclein is a presynaptic protein. Phosphorylated α-synuclein is the main component of Lewy bodies (LBs) that are characteristic of Parkinson disease and dementia with LBs (DLB), and of the glial cytoplasmic inclusions found in multiple system atrophy (5, 6). In Guamanian patients with PDC, α-synuclein–positive structures have been detected in the amygdala in approximately 40% of cases (7, 8) and in the cerebellum in more than 60% of cases (9). In this report, we examined phosphorylated α-synuclein immunoreactivity in the brains and spinal cords from 10 patients with ALS/PDC from the Kii Peninsula (Kii ALS/PDC) and analyzed biochemical aspects of α-synuclein from 1 patient.

MATERIALS AND METHODS

Cases

We examined 10 patients with neuropathologically verified Kii ALS/PDC (mean age, 69.1 years; range, 60–77 years). Demographic features and clinical manifestations are listed in Table 1. This study was approved by the ethics committee of Mie University Graduate School of Medicine. Informed consent was obtained from the patients or their families.

Neuropathology and Immunohistochemistry

The brains and spinal cords were fixed in formalin solution for 2 to 3 weeks. The brains were sliced into coronal sections and the spinal cords were sliced axially. Paraffin-embedded samples were cut into 9-μm-thick sections for hematoxylin and eosin, Klüver-Barrera, and Gallyas-Braak staining. All histologic samples showed numerous NFTs

TABLE 1. Clinical Data

Case No.	Age, y	Sex	Duration of Illness, y	Phenotype		
				A	P	D
1	70	F	13	+	−	−
2	63	F	5	+	−	−
3	66	F	3	+	−	−
4	65	M	3	+	−	+
5	77	M	7	+	−	+
6	70	F	8	+	+	+
7	60	F	7	+	+	+
8	76	F	6	+	+	+
9	70	F	14	−	+	+
10	74	M	6	−	+	+

A, Amyotrophic lateral sclerosis; P, parkinsonism, D, dementia; F, female; M, male; −, absent; +, present.

without senile plaques. Nerve cell loss was chiefly in the temporal cortex, frontal cortex, and the nuclei of the brainstem. Loss of anterior horn cells and degeneration of pyramidal tracts were common features.

Six-micrometer-thick sections were prepared for immunohistochemical studies. Immunostaining was performed using the avidin-biotin-peroxidase complex (ABC) method with a Vectastain ABC kit (Vector Laboratories, Burlingame, CA). The antibodies used and their dilutions were as follows: anti-phosphorylated α-synuclein antibody specifically recognizes phosphorylation at Ser-129 (PSer129, 1:5000; monoclonal; Wako, Osaka, Japan) and anti–phosphorylated tau antibody (AT8, 1:100; monoclonal, Innogenetics, Ghent, Belgium). Regions selected for evaluation are shown in Table 2. To evaluate the phosphorylated α-synuclein–positive structures and phosphorylated tau-positive structures, scores ranging from (−) to (+++) were assigned according to the number of structures in the area of maximum density. Phosphorylated α-synuclein–positive and phosphorylated tau-positive neurons were counted in 100× microscopic fields. Densities of phosphorylated α-synuclein–positive were scored as follows: −, 0; ±, 1; +, 2 to 5; ++, 6 to 10; +++, more than 10/field. Densities of phosphorylated tau-positive neurons were scored as follows: −, 0; +, 1 to 10; ++, 11 to 20; +++, more than 20/field. Colocalization of phosphorylated α-synuclein and phosphorylated tau was determined in sections double-labeled with PSer129 antibody and AT8 antibody using immunofluorescent substrate (Alexa Fluor 488 and 546; Life Technologies, Carlsbad, CA).

Western Blot

Sarkosyl-insoluble α-synuclein was extracted from the hippocampus of case 10. Sarkosyl-insoluble α-synuclein was prepared as previously described (10, 11) with slight modifications. Briefly, frozen brain tissue samples were homogenized in a 20-fold volume of buffer A (10 mmol/L Tris, pH 7.5, 1 mmol/L EGTA, 1 mmol/L dithiothreitol, 10% sucrose) containing 1% Triton X-100, incubated for 30 minutes at 37°C and spun at 100,000 × *g* for 30 minutes at 25°C. The resultant pellets were subsequently homogenized in buffer A containing 1% sarkosyl, incubated at 37°C for 30 minutes, and centrifuged 100,000 × *g* for 30 minutes. The sarkosyl-insoluble pellet was homogenized in 4 volumes of buffer A containing 1% CHAPS and spun at 100,000 × *g* for 20 minutes. The pellet was sonicated in 1 volume of 8 mol/L urea and spun at 100,000 × *g* for 20 minutes. The supernatant was mixed with an equal volume of 2× SDS sample buffer and treated at 100°C for 3 minutes. Aliquots of the samples were separated on a 10% or 15% sodium dodecyl sulfate (SDS)–polyacrylamide gel and transferred to a polyvinylidene difluoride membrane. The membrane was then probed with PSer129 (1:2000) and phosphorylation-independent anti–α-synuclein antibody (Syn 102: mouse monoclonal antibody, epitope location on α-synuclein residues 131–140) (12).

In vitro ubiquitination of α-synuclein was performed as previously described (12) with minor modifications. Briefly, 20 μg of human recombinant α-synuclein and 2 μg of ubiquitin (derived from bovine blood cells) or methylated ubiquitin were incubated with an ubiquitin ligase fraction (Fraction II) from rabbit reticulocytes at 37°C for 2 hours in a buffer containing 50 mmol/L Tris-HCl (pH 9.0), 2 mmol/L ATP, 5 mmol/L $MgCl_2$, and 1 mmol/L dithiothreitol. The conjugation reaction was stopped by boiling the samples in an equal volume of SDS sample buffer followed by separation of the components by SDS–polyacrylamide gel electrophoresis. To examine the ubiquitinated state of α-synuclein, we compared the mobilities of α-synuclein derived from Kii ALS/PDC, ubiquitinated, and unubiquitinated recombinant α-synuclein by SDS–polyacrylamide gel electrophoresis using anti-ubiquitin monoclonal antibody 1510 (anti-Ub 1510) (12). Ubiquitinated and unubiquitinated recombinant α-synuclein were identified by labeling with PSer129 and Syn102.

RESULTS

A representative image of a neuron with LBs and adjacent neurons with NFTs is shown in Figure 1A. Various types of phosphorylated α-synuclein–positive structures, including neuronal cytoplasmic inclusions and LBs (Fig. 1B), Lewy neurites (Fig. 1C), and glial cytoplasmic inclusions (Fig. 1D), were found in all ALS/PDC cases. There were no neuronal intranuclear inclusions. Phosphorylated α-synuclein–positive neurons were found in 8 (80%) of the 10 cases. Phosphorylated α-synuclein–positive neurons were

TABLE 2. Topographical Distribution of α-Synuclein–Positive Neurons and Tau-Positive Neurons

			Case 1	Case 2	Case 3	Case 4	Case 5	Case 6	Case 7	Case 8	Case 9	Case 10
Diagnosis:			**ALS**	**ALS**	**ALS**	**ALS With D**	**ALS With D**	**PDC**	**PDC**	**PDC**	**PDC**	**PDC**
Sex			**F**	**F**	**F**	**M**	**M**	**F**	**F**	**F**	**F**	**M**
Age, y			**70**	**63**	**66**	**65**	**77**	**70**	**60**	**76**	**70**	**75**
Frontal cortex BA8/9		Tau	−	+	+	++	NA	++	+	++	+	−
		αS	−	−	NA	−	−	−	−	−	−	−
Cingulate gyrus BA24		Tau	−	+	NA	+	NA	NA	−	+	++	−
		αS	−	−	NA	−	−	−	−	−	−	−
Insula		Tau	+	++	+	++	+	++	++	+	++	+
		αS	−	−	NA	−	−	−	−	−	−	+
Parietal cortex BA40		Tau	NA	NA	NA	+	NA	NA	NA	+	−	+
		αS	−	−	NA	−	NA	NA	NA	NA	−	±
Temporal cortex BA21		Tau	+	+++	+	+	+++	+++	++	++	++	+
		αS	−	−	−	−	−	−	−	±	−	+
Hippocampus (Ammon horn)		Tau	+++	+++	+	+++	+++	+++	+++	+++	+++	+++
		αS	−	−	−	−	−	−	++	+	−	++
Meynert nucleus		Tau	+	+	NA	++	++	++	+++	NA	+	+
		αS	−	−	NA	−	−	−	+++	NA	−	−
Caudate nucleus		Tau	−	+	NA	+	+	NA	±	+	+	−
		αS	−	−	NA	−	−	−	+	−	+	−
Putamen		Tau	+	++	NA	+	+	NA	±	+	+	+
		αS	−	−	NA	−	−	−	+	−	−	−
Pallidum		Tau	−	+	NA	+	+	NA	±	NA	+	+
		αS	−	−	NA	−	−	−	+	NA	−	−
Transentorhinal cortex BA28		Tau	++	+++	+	++	++	+++	+++	+++	++	+++
		αS	−	−	−	−	−	−	+	++	−	±
Motor cortex		Tau	NA	+	NA	+	+	++	+	+	++	NA
		αS	NA	NA	NA	−	−	−	−	−	−	−
Thalamus		Tau	−	+	+	+	−	NA	++	+	++	+
		αS	−	NA	NA	−	−	NA	−	NA	−	−
Subthalamic nucleus		Tau	−	+	NA	+	NA	NA	NA	+	+	+
		αS	−	NA	NA	−	NA	NA	NA	NA	±	±
Amygdala		Tau	+++	+++	+++	+++	+++	++	+++	++	+++	+++
		αS	−	−	−	+	+	+	++	+	±	+++
Parahippocampus		Tau	+++	+++	+	++	+++	+++	+++	+++	+++	+++
		αS	−	−	−	−	−	−	++	+	−	+++
Cerebellum	Molecular layer	Tau	−	−	−	−	−	−	+	−	−	−
		αS	−	−	−	−	−	−	−	−	−	−
	Dentate nucleus	Tau	−	+	−	+	+	+	++	+	+++	+
		αS	−	−	−	+	−	−	−	+	−	−
	White matter	Tau	−	−	−	−	−	+	−	−	+	−
		αS	−	−	−	−	−	−	−	±	−	−
Midbrain	Substantia nigra	Tau	−	+	+	+	+	++	+	+++	+++	++
		αS	++	−	−	+	−	−	++	+	−	++
	Periaqueductal gray	Tau	+	+++	+++	+++	+	+	++	+++	+++	+++
		αS	+	−	−	+	−	−	+++	++	−	++
Pons	Locus coeruleus	Tau	+	++	+	+	+	+	+	+++	+++	+++
		αS	+++	−	−	+++	−	−	−	+++	−	+++
	Raphe nuclei	Tau	+	+	+	+	+	+	+	+	+++	+++
		αS	−	−	−	+	−	−	−	+++	−	++
	Pontine nucleus	Tau	−	−	−	−	−	−	−	+	+++	−
		αS	−	−	−	−	−	−	−	−	−	−

(Continued on next page)

TABLE 2. *(Continued)*

			Case 1	Case 2	Case 3	Case 4	Case 5	Case 6	Case 7	Case 8	Case 9	Case 10
Diagnosis:			ALS	ALS	ALS	ALS With D	ALS With D	PDC	PDC	PDC	PDC	PDC
Sex			F	F	F	M	M	F	F	F	F	M
Age, y	70		63	66	65	77	70	60	76	70	75	
Medulla	Dorsal nucleus of vagus nerve	Tau	+	+	−	−	−	+	−	−	−	+
		αS	+	−	−	+++	−	−	−	−	−	+++
	Hypoglossal nucleus	Tau	+	±	−	−	−	+	+	−	+	−
		αS	−	−	−	−	−	−	−	−	−	+
	Inferior olivary nucleus	Tau	−	−	−	−	−	+	+	−	+	++
		αS	−	−	−	−	−	−	−	−	−	+
spinal cord	Ventral horn	Tau	−	−	−	NA	−	−	+	+	++	+
		αS	−	−	−	NA	−	−	−	++	−	+
	Intermediolateral nucleus	Tau	−	−	+	NA	−	−	+	+	+	−
		αS	±	−	−	NA	−	−	±	−	−	+++

α-Synuclein (αS)–positive neurons were counted in microscope fields at a magnification of 100× and the density of α-synuclein positive neurons was scored as follows: −, 0; ±, 1; +, 2 to 5; ++, 6 to 10; +++, more than 10. The density of tau-positive neurons was scored at a magnification of 100× as follows: to , 0; +, 1 to 10; ++, 11 to 20; +++, more than 20.

mainly detected in the amygdala (70%); substantia nigra, periaqueductal gray (50%); locus coeruleus (40%); and hippocampus, transentorhinal cortex, parahippocampus, raphe nucleus, dorsal vagal nucleus, and intermediolateral nucleus of the spinal cord (30%) (Table 2). There were no phosphorylated α-synuclein–positive structures in the molecular layer of the cerebellum in any of the 10 cases. Tau-positive neurons were abundant in most areas examined. Phosphorylated α-synuclein–positive neurons outnumbered tau-positive neurons in the substantia nigra, locus coeruleus, and dorsal nucleus of the vagus nerve in a few patients, and periaqueductal gray, raphe nucleus, spinal ventral horn, and spinal intermediolateral nucleus in only 1 or 2 patients (Table 2). Semiquantitative evaluation suggested that the densities of

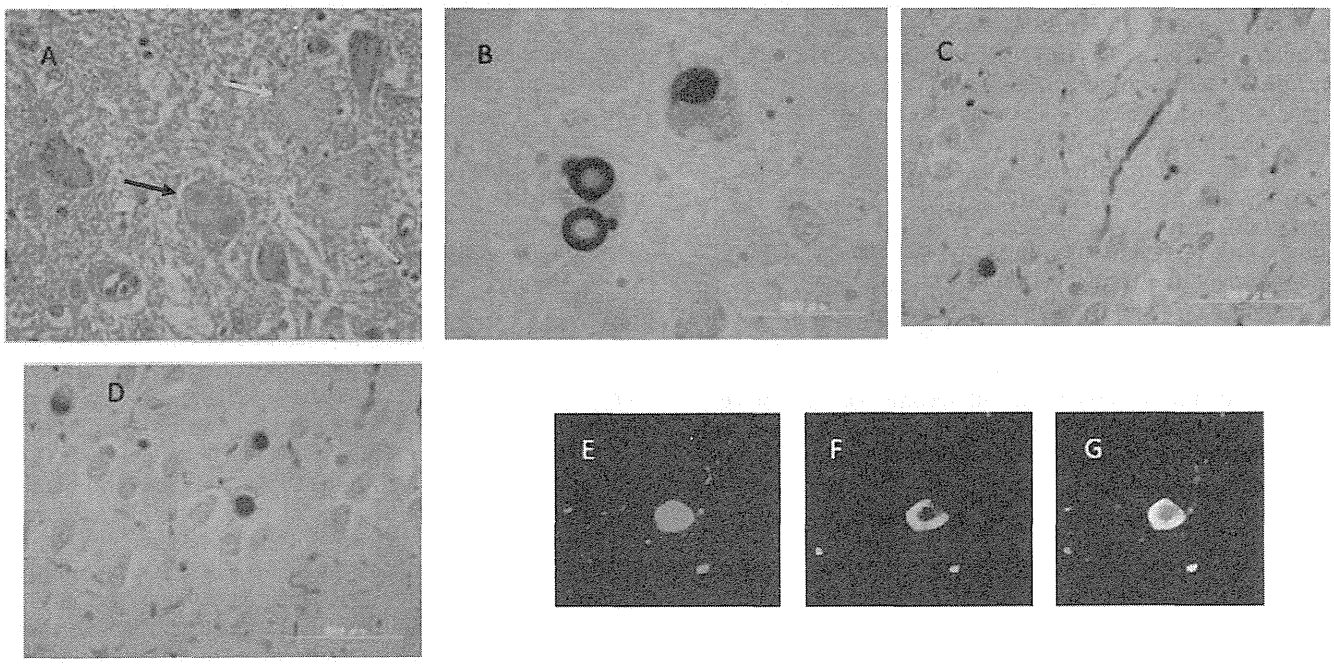

FIGURE 1. Hematoxylin and eosin staining, immunostaining using an antibody against phosphorylated α-synuclein (PSer129), and double immunofluorescence with PSer129 and anti-phosphorylated tau (AT8) antibodies. **(A)** Lewy bodies (LBs) (black arrow) and neurofibrillary tangles (NFTs) (yellow arrows) in the locus coeruleus (hematoxylin and eosin stain). **(B)** LBs and cytoplasmic round inclusion in the locus coeruleus (PSer129). **(C)** Lewy neurites in the amygdala (PSer129). **(D)** Glial cytoplasmic inclusion in the amygdala (PSer129). **(E–G)** Double immunofluorescence labeling showing the coexistence of α-synuclein (PSer129, Alexa 488: green) **(E)**, tau (AT8, Alexa 546: red) **(F)**, and their merged image **(G)**.

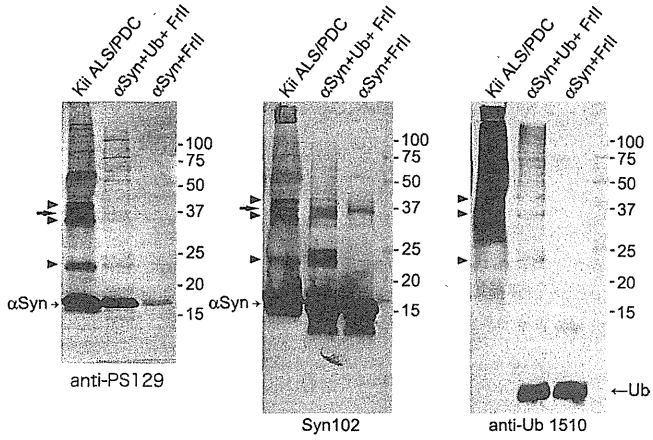

FIGURE 2. Sarkosyl-insoluble fractions from case 10 with Kii amyotrophic lateral sclerosis/parkinsonism dementia complex (ALS/PDC), ubiquitinated recombinant α-synuclein (αSyn + Ub + Frll), and nonubiquitinated recombinant α-synuclein (αSyn + Frll). Samples were immunoblotted with a phosphorylation-dependent anti–α-synuclein antibody (anti-PSer129) (PS129), a phosphorylation-independent anti–α-synuclein antibody (Syn 102), and an anti-ubiquitin (Ub) antibody (anti-Ub 1510). Note that 24-, 32-, and 40-kDa bands are detected in the Kii ALS/PDC brain and αSyn + Ub + Frll with anti-PSer129, Syn 102, and anti-Ub 1510 (arrowheads). Arrow indicates an α-synuclein dimer, which was also detected in αSyn + Frll.

phosphorylated α-synuclein–positive neurons were not related to age or duration of illness but to the densities of tau-positive neurons. Colocalization of phosphorylated α- synuclein and phophorylated tau was observed in many neuronal cytoplasmic inclusions and neuropil threads in the amygdala, substantia nigra, periaqueductal gray, locus coeruleus (Figs. 1E–G), hippocampus, transentorhinal cortex, and parahippocampus.

Immunoblots of sarkosyl-insoluble fractions extracted from the brain of a Kii ALS/PDC patient showed a few α-synuclein–immunoreactive bands. The major immunoreactive band with an apparent molecular mass of 17 kDa and minor 24-, 32-, and 40-kDa bands migrating at a higher molecular mass range on the Tris–glycine gel system were immunoreactive with PSer129 and Syn102. The higher molecular mass of phosphorylated α-synuclein-related polypeptides suggested ubiquitination compared with recombinant α-synuclein, which was incubated with the ubiquitin ligase fraction with or without ubiquitin (Fig. 2), as previously reported in other synucleinopathies (12).

DISCUSSION

α-Synuclein–positive pathology has been identified in a variety of disorders with extensive tau pathology including sporadic Alzheimer disease (13, 14), familial Alzheimer disease (15), DLB (16), familial DLB (17), familial Parkinson disease associated with the α-synuclein A53T mutation (18), Down syndrome (19), neurodegeneration with brain iron accumulation (20–22), and Guam PDC (7–9). Colocalization of tau and α-synuclein was variable in these diseases. For example, extensive colocalization of tau and α-synuclein was reported in DLB (16) and familial DLB (17); the co-occurrence of tau and α-synuclein was variable in Guam PDC (7, 8).

In the present study, the frequency, distribution, and morphology of α-synuclein deposits are described in the brains and spinal cords of patients with Kii ALS/PDC for the first time. α-Synuclein deposits were observed mainly in the limbic system and brainstem; α-synuclein was phosphorylated and ubiquitinated. Tau-positive neurons were more abundant than α-synuclein–positive neurons in most areas examined, and there was extensive colocalization of tau and α-synuclein.

Although Kii ALS/PDC and Guam ALS/PDC share a number of clinical and neuropathologic features, it remains unclear whether they are identical. Yamazaki et al (7) examined α-synuclein–positive intraneuronal inclusions in the motor cortex, medial temporal lobe, and brainstem of 13 patients with Guam PDC using antibodies against non-phosphorylated α-synuclein; they found that 7 (54%) of 13 PDC patients showed α-synuclein–positive inclusions in at least 1 region of the brain. The authors concluded that the amygdala was most affected by α-synuclein pathology, in which α-synuclein was frequently colocalized with tau. Forman et al (8) reported that α-synuclein pathology of the amygdala in Guam ALS/PDC was present in 37% of 19 patients with PDC, but absent in patients with ALS, preclinical PDC, early PDC/ALS, clinical (pathology pending) PDC, PDC/ALS, and control Chamorro patients. The α-synuclein aggregates rarely colocalized within neurons harboring NFTs. On the basis of these findings, the authors suggested a possible interaction between tau and α-synuclein and tau deposits preceding α-synuclein deposits. Sebeo et al (9) reported that numerous α-synuclein–immunoreactive spherical structures in the molecular layer of the cerebellum were observed in 63.6% of Guam PDC patients. These structures were seen exclusively in patients showing α-synuclein pathology in the amygdala, and were much more pronounced in the hemisphere than in the vermis and were associated with Purkinje cells and Bergmann glia cells.

We found that in Kii ALS/PDC, α-synuclein pathology in the amygdala was absent in patients with ALS but was present in ALS patients with dementia and PDC. These results suggest that the α-synuclein pathology in the amygdala may have been induced by tau deposition and may be related to dementia in ALS/PDC. Because α-synuclein inclusions are not found in every brain with other tauopathies, tau in ALS/PDC cases might accelerate α-synuclein aggregation. The combination of misfolded α-synuclein and tau that occurs in ALS/PDC might promote cytotoxic protofibrils and accelerate protein deposits (23). We carefully searched the cerebellum of Kii ALS/PDC patients for similar α-synuclein–positive structures in the molecular layer but failed to find α-synuclein–positive pathology. In general, neuronal cell loss and tau deposits in the molecular layer are exceptional in Kii ALS/PDC. The cause of this discrepancy in α-synuclein pathology in the cerebellum between Guam ALS/PDC and Kii ALS/PDC might be clarified by using the same antibody and identical staining protocols in further studies.

In summary, α-synuclein–positive structures were common in both ALS and PDC and were mainly distributed

in the brainstem and limbic system. The amygdala was the most affected structure in Kii ALS/PDC. The interaction between tau and α-synuclein might modify the pathogenesis of Kii ALS/PDC.

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ORIGINAL ARTICLE

An increase in ALS incidence on the Kii Peninsula, 1960–2009: A possible link to change in drinking water source

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Abstract

We investigated changes in the incidence of amyotrophic lateral sclerosis (ALS) in the Koza/Kozagawa/Kushimoto area (K. area) in the Kii Peninsula, Japan in 1960–2009. Probable and definite ALS cases diagnosed using El Escorial criteria were collected during a five-decade period: period I–V, 1960–2009. Forty-three ALS patients matched the selection criteria in the overall K. area, including three patients on Oshima, a small island opposite the mainland K. area. The age- and gender-adjusted incidence of ALS in the overall K. area (standardized for the 2005 Japanese population) decreased from 5.47/100,000 (95% CI 1.86–9.08) in period I to 0.61/100,000 (95% CI −0.28–1.50) in period III, and then increased to 4.39/100,000 (95% CI 1.70–7.07) in period V. On Oshima, the age- and gender-adjusted incidence of ALS was 9.45/100,000 (95% CI −7.39–26.29) in period V. The present research indicates an increase of ALS incidence in the K. area, especially on Oshima. A limitation of this study was the small population.

Key words: *Focus area, Kii-ALS, incidence, new cluster*

Introduction

Amyotrophic lateral sclerosis (ALS) is a devastating adult-onset degenerating disease of unknown etiology of the motor neuron systems. The Koza, Kozagawa and Kushimoto (K.) area in the Kii Peninsula of Japan was reported to have a higher incidence of ALS in the 1950s than other areas of the world (1–5). Epidemiologic research showed that drinking water sourced from Kozagawa River in the K. area contained severely low levels of Ca and Mg, and Ca/Mg deficiency was speculated to have a role in the development of ALS in these areas (5,6). On Oshima, a small island municipally included in the K. area, the source of drinking water was changed from regional water to the Kozagawa River in 1975. To clarify whether ALS epidemiology on Oshima changed after altering the water source, we investigated changes in ALS incidence on Oshima and in the K. area in 1960–2009.

Methods

Area of investigation

The K. area (population: 23,357 in 2005 census, 430.3 km^2) is located in the southern part of Wakayama Prefecture of the Kii peninsula, Japan (Figure 1). Oshima (population: 1279 in 2005 census, 9.93 km^2) is an island opposite the K. area. The K. area has a local public healthcare and welfare center, two local public hospitals, two private hospitals and 19 medical clinics.

Incidence of ALS in 1960–2009

ALS patients on Oshima and overall in the K. area were enrolled from multiple sources, including hospital medical records and reports from local medical staff over a five-decade period: period I, 1960–1969;

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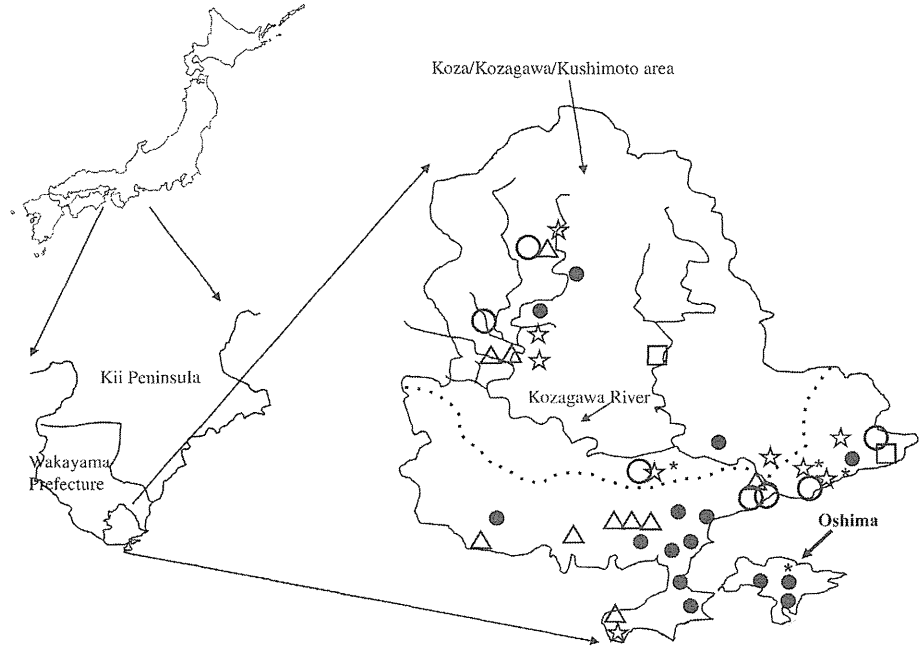

Figure 1. Geography of Koza/Kozagawa/Kushimoto area and Oshima, and distribution of patients with ALS. Patients with ALS between 1960 and 1969 (period I: ○), between 1970 and 1979 (period II: △): between 1980 and 1989 (period III: □), between 1990 and 1999 (period IV: ☆), and between 2000 and 2009 (period V: •) were plotted. *: ALS/PDC

period II, 1970–1979; period III, 1980–1989; period IV, 1990–1999; period V, 2000–2009. Regional physicians including neurologists in hospitals and clinics and the staff of the public healthcare and welfare center in the K. area were requested by our team to report all patients with possible motor neuron disease every year (7). To ensure complete case identification, Wakayama Prefecture's List of Patients with Intractable Disease certified by the Ministry of Health and Welfare of Japan was used. The selection criteria were as follows: 1) patients who our neurologists examined and diagnosed using the El Escorial criteria (8); 2) patients with probable or definite ALS who had been living in the K. area, including Oshima, for at least one year before diagnosis. Patients who showed Parkinsonism or dementia during the disease course (ALS/Parkinsonism dementia complex: ALS/PDC) were also included.

The present research was approved by the ethics committee of Wakayama Medical University and Kansai University of Health Sciences.

Statistics

A direct method was used to standardize the annual incidence rates by age and gender, using populations in the 2005 census in Japan: 127,537,189. Unpaired *t*-test was performed and two-sided $p<0.05$ was considered significant.

Results

Patient population and clinical characteristics

We enrolled 50 patients with definite or probable ALS in the K. area, including Oshima, in 1960–2009, and 43 patients matched the selection criteria (Table I)

Table I. Patients with ALS in the K. area, including Oshima in each period.

	Population[&]			ALS patients matched for the selection criteria				
Period	Overall K area	Oshima	Total ALS patients enrolled (*n*)	Overall K area	Oshima	Age at onset Mean (S.D.)	M/F ratio	FH *n* (%)
I	18,251[#]	2,823	7	7	0	56.1 (10.8)	2.5	0 (0)
II	32,128	2,095	12	10	0	55.3 (10.6)	1.5	1 (10)
III	29,732	1,756	4	2	0	59.0 (7.1)	2.0	0 (0)
IV	26,405	1,508	11	9	0	57.7 (11.5)	2.0	3 (33)
V	23,357	1,279	16	15	3	67.6 (12.3)*	0.88	2 (13)
Total			50	43	3		1.53	6 (14.0)

K.: Koza/Kozagawa/Kushimoto area; M/F: male/female ratio; FH: familial history.
[&]: populations in 1965, 1975, 1985, 1995 and 2005 census.
[#]: Koza/Kozagawa area.
*: $p<0.01$ when compared with the mean age at onset in period I.

(seven were excluded because they were living outside the K. area when diagnosed). Patient distribution was restricted to the mainland side of the K. area during periods I–IV. Three ALS patients were found on Oshima (two males and one female) in period V (Figure 1).

The mean age at onset in period V was the highest among the periods (Table I). The male: female ratio was low in period V compared to periods I–IV. The frequency of cases with a positive familial history in a detailed interview was 14.0%. Cu/Zn superoxide dismutase (SOD1) genes were analyzed in three of six familial cases; none had a SOD1 gene mutation. Three ALS/PDC patients were found in the K. area in period IV and one patient on Oshima in period V.

Incidence of ALS in 1960–2009

The mean annual crude ALS incidence in the K. area in period V was 6.42/100,000 and that on Oshima was 23.46/100,000. The age- and gender-adjusted ALS incidence in the K. area decreased from 5.47/100,000 (95% CI 1.86−9.08) in period I to 0.61/100,000 (95% CI−0.28−1.50) in period III and then increased to 4.39/100,000 (95% CI 1.70–7.07) in period V (Figure 2). On Oshima, the age- and gender-adjusted incidence of ALS was 9.45/100,000 (95% CI−7.39−26.29) in period V.

Discussion

The age- and gender-adjusted ALS incidence in the K. area decreased from 5.47 in 1960–1969 to 0.61 in 1980–1989, and then increased to 4.39 in 2000–2009. The declining trend of the male: female ratio in the past 10 years was comparable with other reports (9,10), and the recent increase of ALS incidence in females could be related to some type of environmental or cultural factor pertaining in females in this area and the increased confirmation of older female patients (11). The reason for the decline in 1980–1989 is not clear, but might be partially due to missing cases from the data set, emigration, and environmental, lifestyle and cultural changes. Recent reports of annual age-adjusted ALS incidences ranged from 0.42/100,000 (12) to 2.96/100,000 (13) in other areas in the world (14–17). Taken together, the age- and gender-adjusted incidence in the K. area and Oshima in 2000–2009 was higher than in other areas. On Oshima, no patient with ALS was found in previous research in 1946–1965 (18). It is noteworthy that a high ALS incidence was first found on Oshima in 2000–2009 after the drinking water source was changed to the Kozagawa River in 1975. The present research indicated an increase of ALS incidence in the K. area, especially on Oshima. A limitation of this study was the small population. Continuous study over a longer period is needed in this area.

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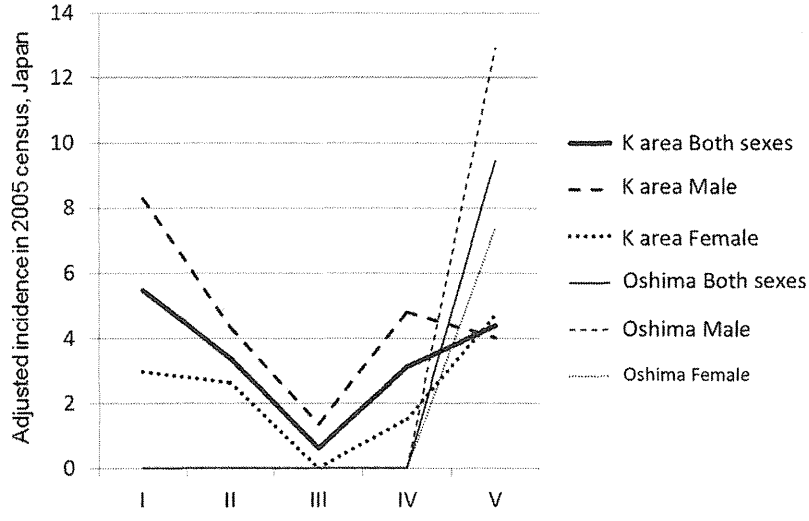

Figure 2. Changes in incidence of ALS in the overall Koza/Kozagawa/Kushimoto (K.) area and Oshima between 1960 and 2009. Comparing the incidence by the periods, the age- and gender-adjusted incidence of ALS in the overall K. area for the 2005 Japanese population was 5.47/100,000 (95% CI 1.86−9.08), male: 8.29/100,000 (95% CI 1.84−14.73), female: 2.98/100,000 (95% CI−0.69−6.66) in period I, markedly decreased to 0.61/100,000 (95% CI−0.28−1.50), male: 1.36/100,000 (95% CI−0.58−3.30), female: 0 in period III, but recently increased again to 4.39/100,000 (95% CI 1.70−7.07), male: 4.01/100,000 (95% CI 0.22−7.81), female: 4.71/100,000 (95% CI 0.93−8.49) in period V.

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Q05 パーキンソン病の発症に遺伝子の関与はあるのか？

結論から先に

◉遺伝子の関与は大きい！

◉パーキンソン病は，約5～10%に家族性パーキンソン病を認め，パーキンソン病の発症に遺伝子が少なからず関与していると考えられる。

◉これまでの解析で，単一遺伝子異常がパーキンソン病の5～30%以上で同定されてきている。ただし人種により頻度に差がある。

◉また，孤発性パーキンソン病では10～20%以上に発症の危険因子や保護的因子として働く遺伝的因子が明らかにされている。

◉パーキンソン病は，“単一遺伝子異常により，または多くの遺伝的因子を背景とし加齢因子，環境因子との相互作用により発症する多因子遺伝性疾患”と考えられてきている。

◉遺伝子，遺伝的因子の関与は少なく見積もってもパーキンソン病の20～30%で示唆され，今後ここ数年のうちにも次世代シークエンサーを用いた網羅的な遺伝子解析により，さらに多くの知見が蓄積されていくものと思われる。

1 パーキンソン病はどれくらいありふれた疾患か？ その病態・原因は？

□― パーキンソン病はアルツハイマー病についで2番目に多い神経変性疾患で，有病率は10万人に125人（1,000人に1人，0.1%）と考えられ，加齢を危険因子として65歳以上では約2%（50人に1人）がパーキンソン病に罹患するとの報告もあり，頻度の高い疾患といえます。今後高齢化社会が進むにつれ，ますます患者が増えていくものと考えられます。

□― パーキンソン病の大部分は孤発型ですが，約5～10%に家族性パーキンソン病を認め，パーキンソン病の発症に遺伝的因子が少なからず関与していると考えられます。

□― これまでのところパーキンソン病の根本の原因については不明ですが，黒質神経細胞が脱落し，1つの遺伝子異常により，または複数の遺伝的因子と加齢因子と環境因子との相互作用，組み合わせにより閾値を超えると発症する多因子疾患である，という考え方が主流となってきています。

❷ 環境因子についてはどのように考えられてきている？

□ 1980年代にヘロインの不純物MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) によるパーキンソニズム，黒質神経細胞脱落の報告があり，環境因子，ミトコンドリア機能異常がパーキンソン病の病態に関与することが明らかにされました[1]。

□ 一般の環境因子では，危険因子として頭部外傷歴，金属曝露，農薬などが，そして保護的因子としてカフェイン，喫煙が報告されています。

□ しかしながら，環境因子については曝露歴，曝露基準の評価が難しく，現時点でどれもパーキンソン病にどこまで関与しているのか，確定的な知見として明らかにされてはいません。

❸ 遺伝的因子についてはどのように考えられてきている？

□ 一方，アイスランドにおける全国民を対象とした遺伝子解析疫学調査により，50歳以上のパーキンソン病患者の配偶者の発症率に比べ同胞，子どもなど血縁者の発症危険率は有意に高く，パーキンソン病の発症には遺伝的因子が関わっていることが示唆されました。

□ 環境因子と遺伝的因子の関与を考える上で大切な知見として，一卵性双生児の研究があります。PET (positron emission tomography) での異常所見とも併せると，一卵性双生児間では疾患一致率は100%ではないものの，二卵性双生児の疾患一致率に比べ約3倍以上高いと報告されました。遺伝的因子を背景に，環境因子との相互作用のもとパーキンソン病が発症することが示唆されるとともに，遺伝子の関与がかなり大きいものと考えられました。

❹ 家族性パーキンソン病の原因遺伝子はどこまで明らかになっているの?

● α-synuclein (*PARK1*)

□ 遺伝的因子に関し，1997年 α-*synuclein* (*PARK1*) が常染色体優性遺伝性パーキンソン病 (ADPD) の家系からその原因遺伝子として同定され[2]，α-シヌクレイン (synuclein) はパーキンソン病の病理学的診断マーカーとしてのレビー (Lewy) 小体の主要構成成分であることが報告されました。

□ ついでα-*synuclein*の遺伝子重複による発現過剰は，レビー小体形成および臨床的重症度と相関することも報告され，パーキンソン病ではα-*synuclein*がレビー小体の形成および臨床像に大きく関わっていることが示唆されました。

parkin (*PARK2*)

□ α-*synuclein*の同定の翌1998年，2番目の原因遺伝子として常染色体劣性遺伝性パーキンソン病（ARPD）の家系から*parkin*（*PARK2*）がわが国で単離され[3]，ユビキチン・プロテアソーム系におけるユビキチンリガーゼとしての機能が報告されました。

□ Parkin蛋白の機能喪失，ユビキチン・プロテアソーム系の機能低下から発症に至る機序も考えられ，それらの発見はとても大きなインパクトとなりました。

□ そしてこれら家族性パーキンソン病の原因遺伝子のレビー小体の形成，パーキンソニズムの発症機序への関与について，分子遺伝学的研究が盛んに行われてきています。家族性パーキンソン病の遺伝子産物の分子機構の解明が，パーキンソン病の原因，発症機序の解明につながるものと期待されているわけです。

その他の原因遺伝子

□ その後ここ10年ばかりの間でも，家族性パーキンソン病研究の進歩は目覚ましいものがあり，新しい遺伝子座や遺伝子の報告が続いています。2011年には新規原因遺伝子として*VPS35*と*EIF4G1*が報告されました。

□ これまでに*PARK1*～*18*までが遺伝子座として報告され，ADPDとしてα-*synuclein*，*UCH-L1*，*LRRK2*，*VPS35*，*EIF4G1*，ARPDとして*parkin*，*PINK1*，*DJ-1*と計8個の原因遺伝子が報告されています。

□ そのほか*PARK*シリーズ，パーキンソン病関連遺伝子として，*ATP13A2*，*GIGYF2*，*HTRA2/OMI*，*PLA2G6*，*FBXO7*なども報告され（**表1**），これら遺伝子の発見により，遺伝子解析，蛋白の機能解析がさらに広がり，新しい知見がどんどん増えてきています。

5 孤発性パーキンソン病の原因遺伝子異常はどこまで明らかになっているの？

□ 家族性パーキンソン病のうち，メンデル遺伝形式に従う単一遺伝子異常によるパーキンソン病とともに，原因遺伝子変異の浸透率の低さのため，見かけ上孤発性に発症するパーキンソン病の一群があることがわかってきています。

表1 ▸ パーキンソン病関連遺伝子の一覧

	染色体上の位置	遺伝子	遺伝形式
PARK1	4q21-q23	*SNCA*（α-*synuclein*）	常優/感受性遺伝子
PARK2	6q25.2-q27	*PRKN*（*parkin*）	常劣
PARK3	p13	unknown	常優
PARK4	（4p15）	*SNCA*	常優
PARK5	4p14	*UCH-L1*	常優
PARK6	1p36-p35	*PINK1*	常劣
PARK7	1p36	*DJ-1*	常劣
PARK8	12p11.2-q13.1	*LRRK2*	常優/感受性遺伝子
PARK9	1p36	*ATP13A2*	常劣
PARK10	1p	unknown	感受性遺伝子
PARK11	2q37.1	*GIGYF2*	常優
PARK12	Xq21-q25	unknown	感受性遺伝子
PARK13	2p12	*HTRA2*（*OMI*）	常優
PARK14	22q13.1	*PLA2G6*	常劣
PARK15	22q12.3	*FBXO7*	常劣
PARK16	1q32	unknown	感受性遺伝子
PARK17	16q12	*VPS35*	常優
PARK18	3q27	*EIF4G1*	常優
	1q21	*GBA*	感受性遺伝子
	12q24.1	*ATXN2*（*SCA2*）	常優
	17q21.1	*MAPT*	常優/感受性遺伝子
	4p16	*GAK*	感受性遺伝子
	6p21.3	*HLA-DRB5*	感受性遺伝子
	4p15	*BST1*	感受性遺伝子

常優＝常染色体優性遺伝（AD）
常劣＝常染色体劣性遺伝（AR）

ADPDの場合

たとえば，ADPDの原因遺伝子のうちα-*synuclein*の2重複変異は孤発性パーキンソン病の約1～2%未満に認め，*LRRK2* G2019S変異も白人の1.6%を占めるとする報告があります。これらは浸透率が低い（遺伝子変異を持っていても発症しない）ことで説明されます。北アフリカ人では実にパーキンソン病の30～40%に*LRRK2* G2019S変異を認めたとの報告もあります。

ARPDの場合

ARPDの原因遺伝子のうち，*parkin*変異は45歳以下発症の孤発性パーキンソン病

の約20％を占め，*PINK1*では孤発性パーキンソン病の約1％というデータがあります。

- *parkin*，*PINK1*，*ATP13A2*，*PLA2G6*などのヘテロ変異が発症の原因になる，または危険因子になるかどうかは，いろいろな報告があり，まだはっきりとした結論が得られていませんが，ヘテロ変異例におけるPETでの異常所見も示されてきており，発症に関与している可能性も示唆されてきています。
- 孤発性パーキンソン病の解析症例の蓄積はまだ十分とはいえず，未知の遺伝子変異が数多く存在するものと思われます。

6 パーキンソン病の感受性遺伝子，危険因子となる遺伝的因子は？

- 病的遺伝子変異のみならず，α-*synuclein*や*LRRK2*のいくつかの一塩基多型（SNP）が孤発性パーキンソン病の危険因子となり，感受性遺伝子としても働くことが報告されてきており，家族性パーキンソン病と孤発性パーキンソン病の発症には共通の機構があると考えられます。
- ゲノムワイドの関連解析では，α-*synuclein*などいくつかの遺伝子が感受性遺伝子となることが示されてきています。さらには最近，常染色体劣性遺伝性疾患のGaucher病の原因遺伝子である*GBA*ヘテロ変異が，パーキンソン病の危険因子となることが報告されています。
- 日本人の孤発性パーキンソン病では，現在わかっている*LRRK2* G2385Rおよびいくつかの*GBA*ヘテロ変異だけでも，ともにパーキンソン病患者の約10％に認める頻度の高い危険因子であることがわかっています。
- また，*LRRK2*のほかの変異群が危険因子や保護的因子にもなっていることが，最近報告されました。
- 発症の原因とはならないものの危険因子や保護的因子となる，多くの遺伝的因子の組み合わせによる孤発性パーキンソン病の発症も考えられることから，パーキンソン病は“多因子遺伝性疾患”として考えられるようになってきています。

7 遺伝子からみたパーキンソン病の原因・病態研究の進歩

- 人種差はありますが，これまで蓄積された知見からは，全パーキンソン病のうち家族性パーキンソン病と考えられる症例は5～10％，その他孤発性パーキンソン病の中で単一原因遺伝子異常によるものは約5～20％，危険因子となる遺伝子異常

が明らかにされているものは約10～20%と見積もられます。

- すなわち，全パーキンソン病の少なくとも約20%，5人に1人は何らかの形で発症に関与する遺伝的因子が明らかにされており，もはや特発性ではないことが明らかにされているとも考えられます。
- これら遺伝子解析結果に基づき，関連遺伝子の正常機能および異常機能の解析，動物モデルの研究が進んでいます。必ずしも遺伝子異常がなくとも，神経変性のカスケードにおいてこれらの遺伝子産物が働いていることを示す知見も積み重ねられてきています。
- 神経変性過程におけるARPDの遺伝子産物そのもの(Parkin，PINK1，DJ-1など)のミトコンドリアへの関与も明らかにされつつあります。パーキンソン病の危険因子である加齢に伴い，ミトコンドリアDNAの欠失変異が多くなり黒質神経細胞障害をきたすことも想定されてきており，加齢因子と遺伝的因子が密接に関わっていることも示されてきています。
- このようにパーキンソン病の分子遺伝学的研究は日進月歩であり，その知見が孤発性パーキンソン病の発症メカニズムの解明に大きく貢献することが現実味を帯びてきており，今後もパーキンソン病の遺伝子からの研究が果たす役割は大きいといえます。

8 パーキンソン病におけるさらなる遺伝的因子の解明は可能？

- 十分可能であり，実際現在も短期間のうちに新たな知見がどんどん積み重ねられています。ただ，世界的にも網羅的大規模解析のデータにはまだまだ乏しい状況です。
- 人種差もありますが，これまでの多施設間の遺伝子解析結果からは，ADPDの大多数，ARPDの約40%が未知の遺伝子異常によって引き起こされていると考えられており，新規原因遺伝子の同定も含めた新たな研究の発展が望まれるところです。
- また，危険因子となる遺伝子変異や多型も複数わかってきており，ゲノムワイドの関連解析による感受性遺伝子の同定も世界中で行われてきました。さらに，次世代シークエンサーにより網羅的に高速に遺伝子解析がなされるようになってきており，新規原因遺伝子も同定されてきています。今後孤発性パーキンソン病における遺伝的因子の知見もますます重要になってくるものと思われ，積極的に解析されてきています。

9 パーキンソン病に対する遺伝子からのアプローチは日常診療から始まっている

□— パーキンソン病はcommon diseaseであり，臨床医が日常の外来で孤発性パーキンソン病はもちろん，家族性パーキンソン病（ADPD，ARPD）の患者さんに遭遇することも決して稀ではありません。忙しい診療の中でも，血族婚の有無など詳細な家族歴を聴取することは重要で，パーキンソン病の家系内で非発症者と思われる人に軽微なパーキンソニズムが潜在的に存在していることもあり，家系内メンバーの直接の診察所見に基づいた情報収集が有用になってくる場合もあります。

□— しかもADPDやARPDの中には浸透率が低い場合も多く，見かけ上孤発性と思われる症例でも遺伝子変異が認められる場合が少なくありません。したがって，家族歴がなくても遺伝性パーキンソン病といえることが少なからずあります。少なくともパーキンソン病の５人に１人に具体的な遺伝的因子が示されてきていますが，まだまだ未知の遺伝的因子は多いと考えられます。

10 １人の患者さんからパーキンソン病の原因，病態，治療についての研究へ，そしてまた臨床へ

□— したがって，１例１例の臨床情報に基づいた解析結果からclinical baseで遺伝的因子の知見を蓄積することは，今後もますます重要になってくるものと思われます。そのことにより，正確な早期診断，早期治療，予防的治療や，遺伝情報に基づき薬剤応答性，副作用の出やすさなどを考え，個人個人に最も合った形で治療法を決定するオーダーメイド医療の一般化が可能となってきます。

□— 今後，iPS細胞の研究の発展・応用も大変期待されます。

□— このようにpatient orientedということを原点とし，家族性および孤発性パーキンソン病のさらなる症例解析データの蓄積，解析研究の成果が多くの患者さんに還元できるよう，臨床に立ち返る形で診断治療に応用されてきています。

11 おわりに

□— 以上のように，パーキンソン病において，遺伝子，遺伝的因子は大きな発症の原因または背景となり，神経変性の共通経路で何らかの働きをしていることも明らかにされてきています。その知見から分子遺伝学的研究がさらに大きく発展し，パーキンソン病の根本の原因，発症機序の解明，治療の開発につながるのも，それほど遠

い将来のことではないと期待されます。

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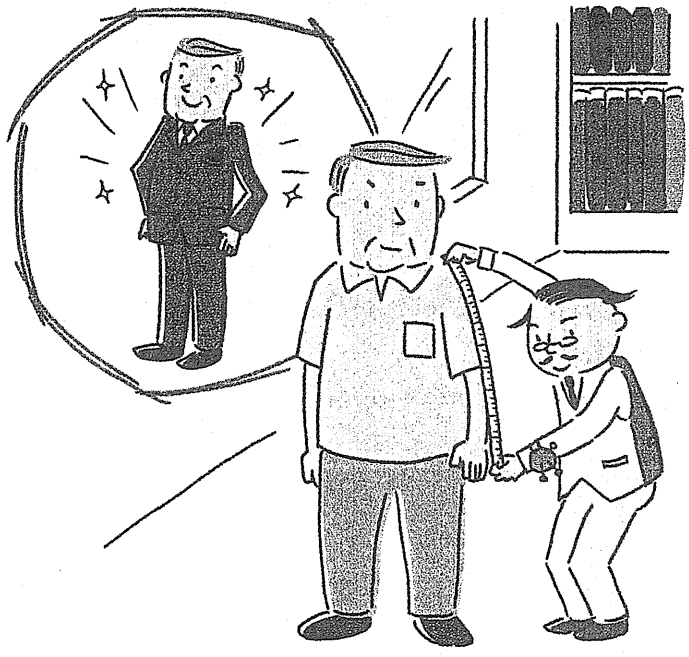

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Molecular analysis and biochemical classification of TDP-43 proteinopathy

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Amyotrophic lateral sclerosis and frontotemporal lobar degeneration with TAR DNA-binding protein of 43 kDa pathology are progressive neurodegenerative diseases that are characterized by intracytoplasmic aggregates of hyperphosphorylated TAR DNA-binding protein of 43 kDa. These TAR DNA-binding protein 43 proteinopathies can be classified into subtypes, which are closely correlated with clinicopathological phenotypes, although the differences in the molecular species of TAR DNA-binding protein 43 in these diseases and the biological significance thereof, remain to be clarified. Here, we have shown that although the banding patterns of abnormally phosphorylated C-terminal fragments of TAR DNA-binding protein 43 differ between the neuropathological subtypes, these are indistinguishable between multiple brain regions and spinal cord in individual patients. Immunoblot analysis of protease-resistant TAR DNA-binding protein 43 demonstrated that the fragment patterns represent different conformations of TAR DNA-binding protein 43 molecular species in the diseases. These results suggest a new clinicopathological classification of TAR DNA-binding protein 43 proteinopathies based on their molecular properties.

Keywords: amyotrophic lateral sclerosis; frontotemporal lobar degeneration; TDP-43; classification

Abbreviations: ALS = amyotrophic lateral sclerosis; FTLD = frontotemporal lobar degeneration; FTLD-TDP = frontotemporal lobar degeneration with TAR DNA-binding protein of 43 kDa pathology; TDP-43 = TAR DNA-binding protein of 43 kDa

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Introduction

Amyotrophic lateral sclerosis (ALS) and frontotemporal lobar degeneration with TDP-43 pathology (FTLD-TDP) are sporadic and familial neurodegenerative diseases characterized neuropathologically by intracytoplasmic aggregates of TAR DNA-binding protein of 43 kDa (TDP-43) (Arai *et al.*, 2006; Neumann *et al.*, 2006). In ALS, upper and lower motor neurons progressively degenerate. Neuropathologically, the TDP-43-positive structures appear as rounded or skein-like inclusions in the lower motor neurons. Similar TDP-43-positive inclusions are also observed in the prefrontal gyrus that contains the upper motor neurons. Moreover, TDP-43-positive glial cytoplasmic inclusions are found close to the upper and lower motor neurons in ALS (Tan *et al.*, 2007). In FTLD-TDP, TDP-43 pathology is distinguished into four histological subtypes (types A–D) based on the predominant type of TDP-43-positive structures present (Mackenzie *et al.*, 2011). Type A is characterized by numerous short dystrophic neurites and crescentic or oval neuronal cytoplasmic inclusions; type B has moderate numbers of neuronal cytoplasmic inclusions, throughout all cortical layers, but few dystrophic neurites; type C has a predominance of elongated dystrophic neurites in upper cortical layers, with few neuronal cytoplasmic inclusions; and type D refers to the pathology associated with inclusion body myopathy with early onset Paget disease and frontotemporal dementia caused by *VCP* mutations, characterized by numerous short dystrophic neurites and frequent lentiform neuronal intranuclear inclusions. There is a relationship between subtypes of TDP-43 pathology and clinical phenotype, and many cases of ALS and frontotemporal lobar degeneration (FTLD) are readily distinguished by each clinical symptom. However, some cases have symptoms of both ALS and FTLD. ALS with dementia refers to cases initially presenting with motor neuron disease becoming demented, whereas FTLD-motor neuron disease refers to cases presenting with cognitive impairment and subsequently developing motor neuron disease.

TDP-43 pathology is also present in a subset of familial ALS and FTLD due to mutations in *TARDBP* (Kabashi *et al.*, 2008; Sreedharan *et al.*, 2008), progranulin (*GRN*; Baker *et al.*, 2006) and *C9ORF72* (DeJesus-Hernandez *et al.*, 2011; Renton *et al.*, 2011) genes. Although most patients with mutations in *TARDBP* present with ALS, some present with FTLD (Gitcho *et al.*, 2009; Kovacs *et al.*, 2009). Cases with FTLD-TDP with *GRN* mutation often show type A pathology (Mackenzie *et al.*, 2006b; Cairns *et al.*, 2007b; Josephs *et al.*, 2007). The pathology of ALS and FTLD due to mutations in *C9ORF72* is heterogeneous: TDP-43 pathology overlaps between ALS and FTLD-TDP types A and B (Murray *et al.*, 2011). One large multicentre study of sporadic and familial FTLD-TDP showed broad overlap between the TDP-43 subtyping, especially between types A and B (Armstrong *et al.*, 2010). These overlaps might occur because current pathological classification may be inadequate, as it is based solely on the morphological assessment of certain subjective cortical regions. A more objective and unbiased classification is needed.

In this study, we have investigated a wide range of patients with various TDP-43 proteinopathies to investigate whether patterns of protease-resistant TDP-43 might indicate different TDP-43 strain types, and characterize the TDP-43 C-terminal banding patterns in multiple regions of the CNS, basing our approach on the method used for demonstration of prion strain variation and the aetiology of new variant Creutzfeldt–Jakob disease (Collinge *et al.*, 1996). We show at least three C-terminal banding patterns that distinguish diseases with TDP-43 proteinopathy and report that the banding pattern in individual patients is indistinguishable in different brain regions and spinal cord. Corresponding patterns of protease-resistant phosphorylated TDP-43 are also seen between the pathological phenotypes. As with the prion diseases, the present results suggest that the different conformation of abnormal TDP-43 deposits in the CNS in patients corresponding with various subtypes of TDP-43 proteinopathy, and that the conformation state of the abnormal TDP-43 protein may determine the pathological phenotype.

Materials and methods

Patients

Human brain tissues were obtained from the Brain Donation Programme at the University of Tsukuba (Japan), Tokyo Metropolitan Institute of Gerontology (Japan), National Shimofusa Mental Hospital (Japan) and the University of Manchester (UK). This study was approved by the local Research Ethics Committee. The subjects in this study included eight patients with ALS, five patients with FTLD-TDP type A, eight patients with FTLD-TDP type B, six patients with FTLD-TDP type C and two patients with Alzheimer's disease without TDP-43 pathology. All cases with ALS met the revised El Escorial criteria for ALS (Brooks, 1994) without dementia. All cases with FTLD-TDP fulfilled clinical diagnostic criteria of FTLD (Neary *et al.*, 1998), and classifications of TDP-43 subtype were made in accordance with published guidelines (Cairns *et al.*, 2007*a*; Mackenzie *et al.*, 2011). Four patients with FTLD-TDP type A were cases of familial FTLD-U with *GRN* mutations. One familial ALS case, one with type A, and two with type B had the GGGCC repeat expansion in *C9ORF72*. The age, gender, brain regions examined and clinical diagnosis are given in Table 1.

A fresh frozen tissue sample was taken and cut into two pieces. One piece was fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 2 days and was used for immunohistochemical analysis. The other piece was homogenized and used for immunoblot analysis. In principle, we took the precentral gyrus and lumbar part of the spinal cord in the ALS cases, and the frontal lobe in the FTLD-TDP cases, because TDP-43 pathology is always known to be prevalent in these regions (Tan *et al.*, 2007; Geser *et al.*, 2008, 2009). However, the spinal cord was not available in four cases with ALS, and both motor regions in two cases were not available. In these cases, the frontal lobe was examined instead. For ALS Cases 1, 3, 5 and ALS and FTLD-TDP type C Case 22, the whole of the cerebral hemisphere and brainstem were available as fresh frozen tissues. In these four cases, we took the multiple regions, as described in Table 1. Every tissue sample was examined immunohistochemically for TDP-43-positive lesions. All samples, except some from the cerebellar cortex, showed an accumulation of abnormal TDP-43-positive structures.

Immunoblotting

Sarkosyl-insoluble, urea-soluble fractions were extracted from each region as previously described (Arai *et al.*, 2006; Hasegawa *et al.*, 2008).