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J. Biochem. 2010;147(6):793–798 doi:10.1093/jb/mvq044

JB THE JOURNAL OF BIOCHEMISTRY

JB Review

Regulatory mechanisms involved in the control of ubiquitin homeostasis

Received March 24, 2010; accepted April 14, 2010; published online April 23, 2010

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Ubiquitin (Ub) modification plays an essential role in the regulation of various cellular processes. Ub performs a remarkable array of cellular tasks through the production of a large number of ubiquitinated proteins; such tasks require many Ubs. Ubs are expressed abundantly from several Ub encoding genes, though not in excess. Rather, Ub expression is tightly regulated through various control mechanisms. Recent studies have shown that the cellular Ub level is regulated by balanced activities of deubiquitinating enzymes and their regulators. Here, we review the current understandings of the regulatory mechanisms that control Ub expression and its metabolism and maintain Ub homeostasis.

Keywords: deubiquitinating enzyme/endosome/homeostasis, stress response/ubiquitin.

Abbreviations: Dub, deubiquitinating enzyme; E1, ubiquitin-activating enzyme; E2, ubiquitin-conjugating enzyme; E3, ubiquitin ligase; *gad*, gracile axonal dystrophy; MVB, multivesicular body; Rfu1, Regulator for free ubiquitin chains 1; Ub, ubiquitin; Ub_3, free triubiquitin; Ub_4, free tetraubiquitin; *UBI1–3*, *UBI1*, *UBI2* and *UBI3*; UPS, ubiquitin-proteasome system.

Ubiquitin

Ubiquitination is a reversible post-translational modification of cellular proteins and is known to play central roles in the regulation of various cellular processes, such as protein degradation, protein trafficking, cell-cycle regulation, DNA repair, apoptosis and signal transduction (*1*, *2*). Ubiquitin (Ub) is a highly conserved 76 amino acid protein that covalently attaches to the lysine residues of target proteins via its carboxy-terminal glycine residue, forming an iso-peptide linkage, in an ATP-dependent fashion. The ubiquitination process is catalyzed by the sequential actions of three enzymes; a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2) and a ubiquitin ligase (E3). Since Ub itself contains seven lysines, it can attach repeatedly to other Ubs, allowing the formation of polyubiquitin chains. Therefore, Ub exists intracellularly either as a monomer, a substrate-conjugated polyubiquitin or monoubiquitin, or free (or unanchored) Ub chains, and there is a dynamic equilibrium among the three forms in the cell. The ubiquitination process can be reversed by deubiquitinating enzymes (Dubs), which are Ub-specific proteases. It is estimated that ~600 E3s and 100 Dubs exist in mammalian cells (*3*, *4*).

Among the various functions of Ub, the most characterized function is serving as a tag for selective proteolysis by the 26S proteasome. Multiple Ubs are covalently added to a substrate successively by E1, E2 and E3 enzymes, producing a substrate conjugated with polyubiquitin. The ubiquitinated substrates are recognized and degraded by the 26S proteasome after the polyubiquitin chain is processed off and recovered by Dubs.

In addition, ubiquitination is also critical in the vacuolar sorting process of both endocytic and biosynthetic membrane proteins (*2*, *5*). At the plasma membrane, Ub serves as a signal for endocytosis, and at the endosome, Ub serves as a signal to sort cargo proteins into the multivesicular body (MVB), which is a critical step to their transport to lysosomes. Ub is removed from the cargo by Dubs before its entry into the MVB.

Importance of Adequate Cellular Level of Ubiquitin

Ub is an abundant protein in eukaryotic cells constituting ~0.1–5% of total proteins, therefore it is assumed that it is redundantly expressed (*6*). However, due to its pervasive use and large number of substrates to be ubiquitinated in a cell, Ub does not seem to be produced in excess, rather the free pool of Ub is maintained at an adequate level depending on the cell conditions.

In yeast as well as in most higher eukaryotes, Ub is initially expressed in the form of different precursors: polyubiquitin, a linear fusion protein consisting of four or more Ub copies in a head-to-tail configuration, and fusion proteins between Ub and usually Ub_{L40} and Ub_{S27}, that are large and small essential ribosomal polypeptides, L40 and S27, respectively (*7*, *8*) (Fig. 1). These Ub precursors are cleaved by Dubs to release identical functional monomeric Ub units. In yeast, the single polyubiquitin gene, *UBI4*, is not required under vegetative conditions, suggesting that ribosome fusion Ub genes, *UBI1*, *UBI2 and UBI3* (*UBI1–3*) provides the bulk of Ub in the cell (*9*). However, cells lacking *UBI4* become sensitive to

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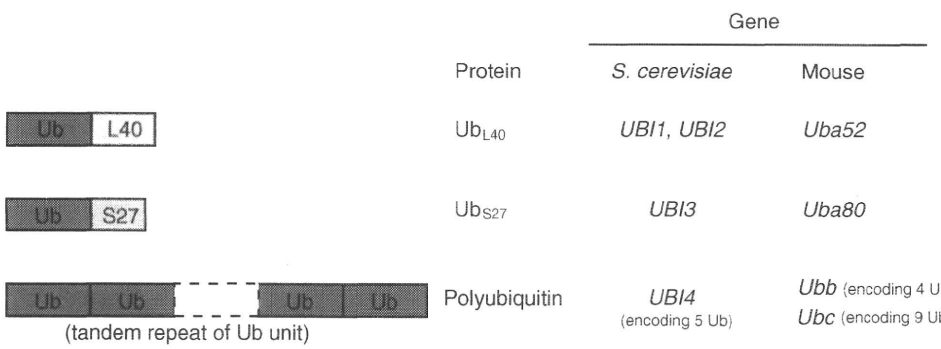

Fig. 1 Three Ub precursors and multiple Ub encoding genes in yeast *Saccharomyces cerevisiae* and mouse (see text for details).

various stresses including high temperature, starvation and amino acid analogs such as canavanine or L-threo-α-amino-β-chlorobutyric acid, and also defective in sporulation (*9*).

Mammals have four Ub genes, two of which encode polyubiquitin and the other two encode fusions with ribosomal proteins (*8*) (Fig. 1). The two polyubiquitin-encoding genes, *Ubb* and *Ubc*, express usually four and nine tandem repeats of Ub, respectively. Thus, the polyubiquitin genes seem redundant; however, the importance of the polyubiquitin-encoding genes was highlighted in the knockout mouse of either *Ubb* or *Ubc* (*6*, *10*, *11*). In the case of *Ubc*, disruption of *Ubc* in mice is embryonically lethal, possibly due to the lack of fetal liver proliferation at midgestation (*6*). Analysis of $Ubc^{-/-}$ mouse embryo fibroblasts showed 40% reduction in Ub level compared with the control. On the other hand, mice lacking *Ubb* are born normally at the expected Mendelian frequency (*10*). However, they are infertile due to the failure of progression of meiosis in germ cells (*10*). Consistently, significantly low Ub levels were found in the testis and germinal vesicle oocytes in 5-month-old mice whereas other organs were not significantly affected. Furthermore, the *Ubb* null mice develop adult-onset obesity due to the degeneration of hypothalamic neurons involved in the control of energy balance and feeding. Although the Ub level was not reduced in the whole brain, it was reduced in the hypothalamus by 30%. Therefore, a modest reduction in Ub level seems to cause infertility and neurodegeneration in mice.

Other than the mutations in Ub-encoding genes, mutations in several Dubs cause reduction of Ub and various defects. In yeast, deletion of Dub encoding genes, including *DOA4* and *UBP6*, can reduce the amount of monomeric Ub (*12–15*). These mutants are sensitive to canavanine, and the defects are compensated by expression of excess Ub. In mice, UCH-L1 is an abundant brain-specific Dub, constituting ~1–5% of the total proteins in the brain (*16*). The mutation in UCH-L1 is responsible for gracile axonal dystrophy (*gad*) in mice (*17*). The mice develop synaptic dysfunction and degeneration of neurons. In the brain of *gad* mice, monomeric Ub level is reduced by 20–30% compared with control mice, suggesting that a low level of Ub is a possible cause of the disease (*16*). Since UCH-L1 binds Ub, it is suggested that UCH-L1 stabilizes Ub or prevent Ub from degradation (*16*). Similarly, ataxia (ax^J) mutation, a spontaneous recessive mutation, is caused by reduced expression of Usp14, a homolog of yeast Ubp6 (*18*, *19*). The ax^J mice develop neurological dysfunctions including progressive motor system abnormalities, ataxia, loss of movement and premature death. In the ax^J mice, monomeric Ub level is reduced by 30–40%.

Curiously, not only a small amount of Ub but also Ub surplus is not beneficial to cells. In yeast, overexpression of Ub renders cells sensitive to certain kinds of stresses such as treatment with cadmium, arsenite and paromycin (*20*). In addition, overexpression of Ub worsens cell growth when it is introduced in mutants of ubiquitin-proteasome system (UPS)-related genes, such as *cdc48* temperature-sensitive mutant (*21*). Such mutants exhibit accumulation of ubiquitinated proteins, which could consequently lead to further accumulation of cytotoxic ubiquitinated proteins (*21*, *22*).

Regulatory Mechanism of Ubiquitin Homeostasis

Since keeping adequate amount of Ub is essential for a balanced cell function, cells have different regulatory systems to maintain Ub homeostasis by utilizing various machineries.

Transcriptional regulation of Ub-encoding genes

One of the regulatory mechanism for Ub level is operated at the level of transcription of Ub-encoding genes. In budding yeast, among the four Ub-encoding genes, *UBI1-4*, transcription of *UBI4*, a polyubiquitin gene, is heat inducible (*9*). Similarly, in higher organisms, transcription of polyubiquitin gene is stress inducible (*23*, *24*). In yeast, *UBI4* contains classical heat-shock elements (HSE) and stress-responsive elements in the promoter. Since multiple Ubs are produced efficiently by a single round of transcription–translation from a polyubiquitin gene, it is beneficial that a cell increases polyubiquitin gene expression under a state of emergency such as stress conditions. However, the mRNA levels of *UBI1-3* encoding ribosome-Ub fusions are likely to be repressed by stresses or conditions that induce *UBI4*, and there is evidence that the *UBI1-3* mRNA expression patterns are similar to those of ribosome subunits (*25*). Therefore, the exact net effect of these

opposing regulatory mechanisms on Ub production remains unclear. In yeast, upon heat shock, the level of monomeric Ub slightly increases for a short period and then decreases, probably due to the massive ubiquitination reactions at heat shock [(*9*) and Y. Kimura, unpublished results].

Regulation by the change of proteasome composition

As described, Ubp6 and its mammalian homolog Usp14 are Dubs associated with the proteasome (*26*). By binding reversibly to the proteasome via its Ubl domain, Ubp6 disassembles polyubiquitinated substrate proteins that are taken to the proteasome, and recovers Ub moiety from proteasomal degradation. Its Dub activity is enhanced by binding to the proteasome (*27*). The reduction of Ub observed in deletion or mutations of yeast Ubp6 and mouse Usp14 is explained by the lack of Ub recovery and that the unrecovered Ub is degraded along with the substrate by the proteasome (Degradation of Ub section) (*27*). Interestingly, Ubp6 inhibits the proteasome non-catalytically and decreases the overall flux of ubiquitinated proteins through the proteasome (*28*).

The regulation of Ubp6 expression is related to the level of Ub (*29*). In response to Ub deficiency, transcription of *UBP6* is increased, resulting in the production of more Ubp6. The increase in Ubp6 leads to an increase in Ubp6-associated proteasomes, which serves to retrieve Ub. Therefore, the versatility of proteasome content acts as a regulatory mechanism for Ub homeostasis. The mechanism of the transcriptional increase of Ubp6 upon Ub deficiency is unknown: it is not mediated by Rpn4, a transcription factor that regulates the expression of a set of proteasome subunits. Thus, the existence of an alternative transcriptional pathway is possible, which senses and responds to Ub deficiency.

Regulation by deubiquitinating enzyme and its regulators

Recent studies identified another mechanism involved in the regulation of monomeric Ub level: Ub level is regulated by the balanced action of Dub and its regulators (*21*, *30*) (Fig. 2). Rfu1 (regulator for free ubiquitin chains 1), a previously uncharacterized protein, was isolated initially as a multi-copy suppressor of *cdc48-3* temperature-sensitive mutant (*21*). Interestingly, cells that lack Rfu1 showed accumulation of monomeric Ub and reduced level of free (or unanchored) Ub chains, whereas overexpression of Rfu1 was associated with the opposite effects; reduction of monomeric Ub and accumulation of free Ub chains. These results suggest that Rfu1 inhibits the production of monomeric Ub and promotes the formation of free Ub chains. It turned out that the target of Rfu1 was Doa4, which is an endosome-localized Dub (*31*). Doa4 deubiquitinates cargo proteins at the endosome to retrieve Ub before cargo proteins are delivered to MVB (*31–33*). In addition, Doa4 is involved in Ub homeostasis, since lack of Doa4 was associated with accumulation of free Ub chains or small Ub species and reduction of monomeric Ub, which is the opposite effect of Rfu1 depletion (*12*). Subsequently, it was shown that Rfu1 interacts with Doa4 both *in vitro* and *in vivo*, and that recombinant Rfu1 inhibits the Dub activity of Doa4, indicating that Rfu1 is an inhibitor of Doa4 (*21*). Interestingly, Doa4 is recruited to the endosome and its activity is stimulated by another factor, Bro1, a class E Vps protein (*30*, *34*). In the absence of Bro1, Doa4 localization of the endosome is lost, and the Ub profile of the Δ*bro1* mutant is quite similar to that of Doa4-negative cells (*35*). Therefore, Doa4 is regulated by an activator (Bro1) and an inhibitor (Rfu1), indicating that there must be balanced regulation of Doa4 between Rfu1 and Bro1. It is speculated that Rfu1 may act on Doa4 to inhibit its activity on the endosome after recruitment of Doa4 to the endosome by Bro1.

Cellular stresses such as heat shock causes accumulation of misfolded proteins and these proteins should be ubiquitinated and degraded by the 26S proteasome. It was discovered that free Ub chains rapidly disappear at heat shock (Fig. 3) (*21*). At the same time, it was shown that Rfu1 decreases whereas Doa4 increases, producing more Doa4, which is free of Rfu1 (*21*). Since the lack of Doa4 as well as overexpression of

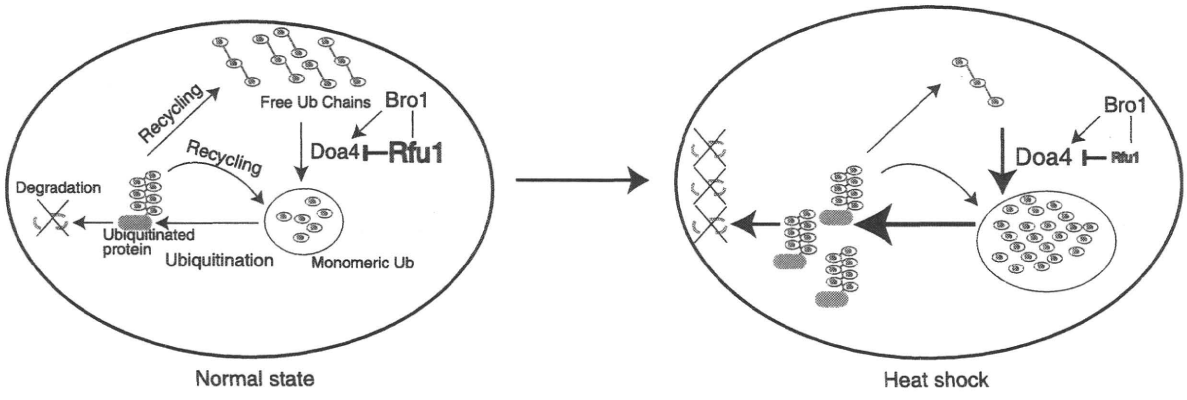

Fig. 2 Model of mechanisms involved in Ub homeostasis through Doa4, Bro1 and Rfu1, at normal state (left) and at heat shock (right). Under normal conditions, Rfu1 inhibits Doa4 activity, and excess Ub may be stored in the form of free Ub chains. Since Doa4 is activated by Bro1, Doa4 is controlled by a balance between activators and inhibitors. Heat shock results in a decrease in Rfu1 and increase in Doa4, favoring production of monomeric Ub from free Ub chains by Doa4. Physical interaction between Rfu1 and Bro1 is detected.

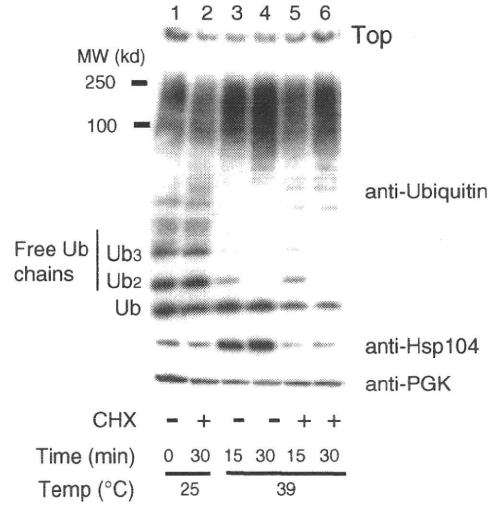

Fig. 3 Disappearance of free Ub chains upon heat shock in the absence and presence of CHX. Cells in early log phase at 25°C were treated or untreated with CHX and immediately placed at 25 or 39°C for the indicated time intervals. Top panel, anti-Ub; middle, anti-Hsp104; bottom, anti-phosphoglycerate kinase (PGK) as control for protein loading.

Rfu1 inhibits the heat shock-induced disappearance of free Ub chains, it was suggested that Doa4 and Rfu1 act on free Ub chains to produce monomeric Ub at heat shock. Thus, free Ub chain may function as a Ub reservoir that allows maintenance of monomer Ub at adequate levels under normal conditions and a rapid supply for ubiquitination of substrates under stress conditions (Fig. 2). The disappearance of free Ub chains occurs in the presence of cycloheximide (CHX), indicating that it does not require new protein synthesis (Fig. 3). Therefore, this regulatory system would be beneficial for a cell to produce monomeric Ub quickly to cope with emergency states such as heat shock.

Other factors involved in Ub homeostasis

In addition to Doa4 and Ubp6, several Dubs are likely to be involved in the regulation of Ub homeostasis. In yeast, Ubp14, a homolog of mammalian isopeptidase T, acts preferentially on free Ub chains, but cannot act on polyubiquitin conjugated with protein (*36*). Cells lacking Ubp14 show accumulation of free Ub chains, however, the mutants have a normal level of monomeric Ub (*37*). Cells lacking Ubp3, Ubp8 and Ubp10 accumulate free tetraubiquitin (Ub_4), free triubiquitin (Ub_3) and both Ub_3 and Ub_4, respectively (*15*).

Ufd3 (Doa1), a homolog of PLAP (phospholipase A2-activating protein) in mammals, was originally isolated as a factor required for the degradation of a ubiquitin-fusion degradation (UFD) substrate (Ub-Pro-β-galactosidase) in yeast (*38*, *39*). In addition to its role in protein degradation in the UPS, Ufd3 functions in the DNA damage response, and in the targeting of ubiquitinated membrane proteins to MVB (*40*, *41*). Ufd3 binds to Ub, Cdc48 and Hse1, a component of the proteins required for MVB sorting (*39–44*). Consistent with its function in MVB sorting, some Ufd3 localize to the endosomes (*40*). Interestingly, cells lacking Ufd3 show depletion of monomeric Ub and accumulation of Ub_3 (*38*, *41*). Since no enzymatic activities have been reported for Ufd3, the reason why deletion of *UFD3* results in the loss of Ub homeostasis is unknown at present. Recent studies showed that interaction of Ufd3 with Cdc48 is required for the maintenance of the Ub level (*44*). Since *UFD3* genetically interacts with various Dub encoding genes, including *UBP4*, *7*, *8*, *10*, *12* and *14*, Ufd3 may regulate these Dub activities through Cdc48 (*41*). Moreover, it is tantalizing that several factors known to be involved in Ub homeostasis, including Doa4, Rfu1, Bro1 and Ufd3 are localized in the endosomes. Hence, it is conceivable that the endosome may function not only as protein sorting factory but also as regulator of Ub homeostasis.

In a mutant of Rsp5, an E3 in yeast, reduced level of Ub is observed upon heat shock and it was shown that the reduced level of protein synthesis is sustained in *rsp1* mutant at heat shock (*45*).

Degradation of Ub

Ub is considered a stable protein based on its heat-stable physicochemical property and its globular structure, and indeed there have been reports that the half life of Ub is rather long [see review (*46*)]. For example, pulse-chase studies show that Ub turns over with a half-life of 28–31 h in cultures of human lung fibroblasts (*47*). One study using erythrocyte-mediated microinjection of ^{125}I-proteins showed that the Ub half life is 320 h in human fibroblasts, whereas that of BSA and lysozyme are only 20 and 22 h, respectively (*48*). However, there are several reports that Ub appears to be metabolized rather rapidly in the cell. A pulse-chase experiment showed that Ub half-life is 9 h in mouse leukemia cells (*49*). In yeast, CHX treatment experiments indicated a long Ub half-life, but only 2 h when estimated by the promoter-shut off experiment using galactose-inducible promoter (*13*, *50*). Such difference in Ub half-life time probably reflects differences between organisms or differences in susceptibility to change to experimental conditions. Finally, a recent study using in-cell nuclear magnetic resonance spectroscopy suggested destabilization of Ub in the cell (*51*).

With regard to degradation of Ub, Ub is degraded by the 26S proteasome and three different degradation processes have been described (*52*, *53*) [reviewed in (*46*)]. First, experiments using ^{125}I-Ub and proteasome inhibitor indicated Ub is degraded along with its conjugated substrate. When the ubiquitinated substrate is processed by the 26S proteasome, Ub are recovered by Dubs such as Ubp6 and Rpn11. However, it is possible that Ub at the most proximal part of the polyubiquitin chain is degraded along with the substrate. Second, monomeric Ub with an extension tail longer than 20 residues at the C-terminus are effectively degraded by the 26S proteasome without further modification (*52*, *53*). It is proposed that the tail allows the Ub-tail to reach the catalytic site of the proteasome. Such an unusual Ub derivative is indeed naturally produced; UBB^{+1}, which is produced by a misreading of *Ubb*

transcript. UBB^{+1} is implicated in the pathogenesis of an early onset form of Alzheimer's disease (*54*). In the third process, monomeric Ub is degraded after Ub itself is ubiquitinated (*53*).

The vacuole or lysosome, another major degradation machinery in the cell, may be involved also in the degradation of Ub. Ub localization in the vacuole/lysosome is reported in various species (*55*, *56*). Moreover, in yeast *doa4* cells in which Ub is not recovered and transported to the vacuole along with cargo proteins, Ub is rapidly degraded (*13*). However, the introduction of mutation in *PEP4*, which encodes a vacuolar protease, prevents the rapid degradation of Ub (*13*).

Conclusion

Various and complex regulatory mechanisms operate in the cell to maintain stable Ub levels. Thus, there are probably other yet unidentified mechanisms involved in the control of Ub homeostasis, *e.g.* one that involves Ufd3. Identification of various mechanisms involved in Ub homeostasis would enhance our understanding of the pathogenesis of various conformational diseases such as neurodegenerative diseases since the UPS plays an important role in the prevention of such diseases.

Funding

This work was supported by Takeda Science Foundation and by Grants-in-Aid for Specially Promoted Research from the Ministry of Education, Culture, Science and Technology of Japan.

Conflict of interest

None declared.

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A Phase I Study of Aromatic L-Amino Acid Decarboxylase Gene Therapy for Parkinson's Disease

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Gene transfer of dopamine-synthesizing enzymes into the striatal neurons has led to behavioral recovery in animal models of Parkinson's disease (PD). We evaluated the safety, tolerability, and potential efficacy of adeno-associated virus (AAV) vector–mediated gene delivery of aromatic L-amino acid decarboxylase (AADC) into the putamen of PD patients. Six PD patients were evaluated at baseline and at 6 months, using multiple measures, including the Unified Parkinson's Disease Rating Scale (UPDRS), motor state diaries, and positron emission tomography (PET) with 6-[^{18}F]fluoro-L-*m*-tyrosine (FMT), a tracer for AADC. The short-duration response to levodopa was measured in three patients. The procedure was well tolerated. Six months after surgery, motor functions in the OFF-medication state improved an average of 46% based on the UPDRS scores, without apparent changes in the short-duration response to levodopa. PET revealed a 56% increase in FMT activity, which persisted up to 96 weeks. Our findings provide class IV evidence regarding the safety and efficacy of AADC gene therapy and warrant further evaluation in a randomized, controlled, phase 2 setting.

Received 25 January 2010; accepted 5 June 2010; published online 6 July 2010. doi:10.1038/mt.2010.135

INTRODUCTION

Dopamine replacement has been the standard pharmacotherapy for motor impairment in Parkinson's disease (PD). Although virtually all patients benefit from levodopa at an early stage of the disease, severe loss of nigrostriatal nerve terminals in advanced PD leads to profoundly decreased activities of dopamine-synthesizing enzymes, including aromatic L-amino acid decarboxylase (AADC), an essential enzyme that converts levodopa to dopamine. Failure to respond to levodopa therapy may result from a reduction in AADC activity, decreased dopamine storage capacity in synaptic vesicles, postsynaptic changes in striatal output neurons, and abnormalities of nondopaminergic neurotransmitter systems.[1,2] Systemic administration of high-dose levodopa enhances oscillations in motor performance and complications, including hallucinations, due to dopaminergic stimulation of the mesolimbic system.

One potential treatment for advanced PD is gene therapy to restore striatum-selective dopamine production. In addition to AADC, tyrosine hydroxylase, which converts L-tyrosine to levodopa, and guanosine triphosphate cyclohydrolase I, which catalyzes biosynthesis of the essential tyrosine hydroxylase cofactor, tetrahydrobiopterine, are necessary for efficient synthesis of dopamine.[2] Viral vector–mediated gene transfer of these dopamine-synthesizing enzymes has been shown to achieve behavioral recovery in animal PD models, with efficient transduction of striatal neurons that escape degeneration.[3–6] When tyrosine hydroxylase and guanosine triphosphate cyclohydrolase I are expressed in the striatum, levodopa can be synthesized continuously. This strategy would be useful for reducing motor fluctuations associated with intermittent levodopa intake. Gene transfer of AADC alone in combination with oral levodopa administration would be a safer strategy for initial clinical trials. In the latter approach, the patients still need to take levodopa to control motor symptoms, but excess production of dopamine could be avoided by reducing the dose of levodopa. We assessed the safety, tolerability, and the potential efficacy of intraputaminal infusion of recombinant adeno-associated virus (AAV) serotype 2 vector encoding human AADC (AAV-hAADC-2) in patients with mid- to late-stage PD. We also examined whether the short-duration response to levodopa, the antiparkinsonian response that parallels the plasma levodopa levels, would change after gene therapy.[7]

RESULTS

Patient disposition and baseline characteristics

Six patients (4 men, 2 women), mean age 60 (range, 51–68) years, were enrolled (Table 1). The mean disease duration was 10 (range, 5–18) years, and time on levodopa was 9.3 (range, 5–15) years. The average baseline daily levodopa and levodopa equivalent doses were 642 and 808 mg, respectively.

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Table 1 Patients' baseline characteristics

Subject	Age (years)	Sex	Disease duration (years)	Time on levodopa (years)	Levodopa dose (mg)	Levodopa equivalents (mg)
A-1	51	M	11	9	600	900
A-2	63	M	9	9	450	650
A-3	66	F	7	7	500	700
A-4	58	M	11	11	700	700
A-5	68	F	18	15	1,000	1,100
A-6	56	M	5	5	600	800
Mean (SD)	60 (6.5)	67% M	10 (4.5)	9.3 (3.4)	642 (196)	808 (169)

Abbreviations: F, female; M, male.
Patients are listed in the order in which they received treatment. Levodopa equivalents were estimated as follows: 100 mg of levodopa with a dopa-decarboxylase inhibitor is equivalent to 0.8 mg talipexole, 1 mg pergolide, 1 mg pramipexole, and 1.5 mg cabergoline.

Primary end point

The procedure was well tolerated. All patients completed all protocol-defined visits. One patient (patient A-2) had a venous hemorrhage in the right frontal lobe just below a burr hole that was found on CT scan 3 days after infusion. The patient used his left arm less frequently than his right arm for 3 weeks; this was assumed to reflect mild frontal lobe dysfunction and resolved completely. Mild, transient headache around the burr holes was present for 2 days after surgery in all patients. There were no significant laboratory test abnormalities. All patients had mildly increased titers of anti-AAV2-neutralizing antibodies 6 months after treatment, which tended toward baseline concentrations thereafter (**Table 2**).

Table 2 Changes in neutralizing AAV2 antibody titers in sera following gene therapy

Subject	Pre	2 weeks	6 months	1 year
A-1	1:2	1:4	1:4	1:4
A-2	<1	1:32	1:4	1:2
A-3	1:32	1:64	1:64	1:32
A-4	1:32	1:32	1:256	1:64
A-5	1:4	1:32	1:32	1:32
A-6	<1	1:16	1:32	1:32

Abbreviations: AAV, adeno-associated virus.
Titers are determined by *in vitro* assay and represented as "1:" dilutions.

Clinical evaluations

The clinical results are summarized in **Table 3**. Intraputaminal AAV-hAADC-2 infusion significantly improved both total and motor scores of the unified Parkinson's disease rating scale (UPDRS) in the OFF state. Five of six patients showed substantial improvement in UPDRS motor ratings in the OFF state (**Figure 1**). Changes in the UPDRS ON state and the percent of ON state hours in a day were not significant. One patient with relatively mild motor symptoms at baseline did not improve on UPDRS (A-3 in **Figure 1**). However, this patient showed a remarkable increase in mobile time as measured by the diaries (28% at baseline to 58% at 6 months after gene transfer; **Figure 2**). The daily dose of levodopa was unchanged in two patients (A-2 and A-5) and reduced in three patients (A-1, A-3, and A-5) at 6 months. Patient A-6, who had daytime sleepiness, preferred to reduce pramipexole instead of levodopa after gene therapy.

The last three patients underwent the levodopa test after our institutional review board confirmed the safety of AADC gene transfer in the first three patients. The short-duration response to levodopa did not change significantly after gene therapy in these three patients, though UPDRS motor scores at 6 months showed slight improvement at 30 minutes in patient 5 and at 120 minutes in patient 4 after levodopa intake (**Figure 3**). Significantly higher peak plasma levodopa concentrations were observed in these two patients after gene therapy.

The mini-mental state examination (MMSE) and geriatric depression scale (GDS) scores did not change significantly.

Table 3 Clinical outcomes of six patients

	Baseline	6 months	*P* value
UPDRS Total OFF	53 (12.4)	38 (10.1)	0.049*
UPDRS Total ON	15 (7.2)	10.7 (2.9)	0.262
UPDRS Part III (Motor) OFF	25.3 (9.4)	13.7 (6.0)	0.024*
UPDRS Part III (Motor) ON	5.2 (4.6)	1.8 (1.5)	0.120
Percent day spent in mobile state	48.8 (12.9)	55.4 (14.8)	0.348
Daily levodopa equivalents dose, mg	808 (169)	707 (233)	0.097

Abbreviations: OFF, off-medication state; ON, on-medication state; UPDRS, Unified Parkinson's Disease Rating Scale.
Data are presented as means (SD). The UPDRS scores in each patient did not change during the 2 months of the screening period.
*$P < 0.05$.

PET analysis

PET imaging revealed increased 6-[^{18}F]fluoro-L-*m*-tyrosine (FMT), a tracer for AADC, activity 4 weeks postoperatively, which persisted at 6-month evaluation (**Figure 4**). The mean increase in FMT uptake from baseline in the combined (right and left) putamen at 24 weeks was 56%. Two patients (A-1 and A-2) who had PET scans 96 weeks after surgery showed persistently increased FMT uptake. In these two patients, motor performance in the OFF state also maintained its improvement at 96 weeks.

DISCUSSION

Extensive preclinical studies on both rodent and nonhuman primate models of PD have shown that AAV vectors can express exogenous genes for a long time in the brain target areas without

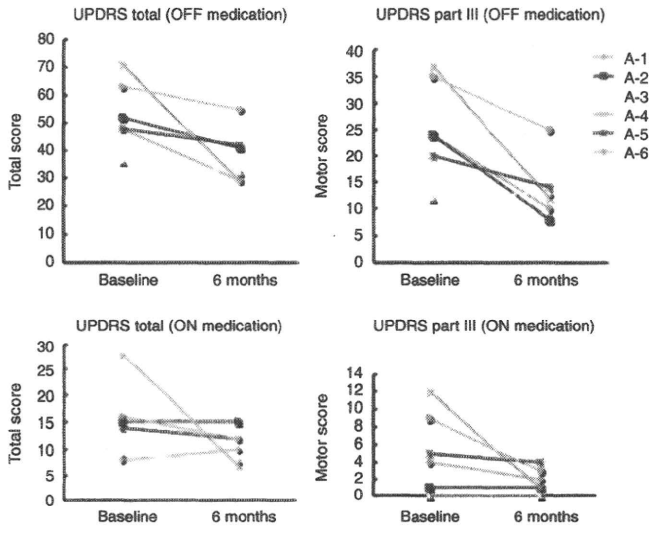

Figure 1 Changes in UPDRS scores. Absolute changes in scores from baseline to 6 months for individual patients. OFF, off-medication state; ON, on-medication state; UPDRS, Unified Parkinson's Disease Rating Scale.

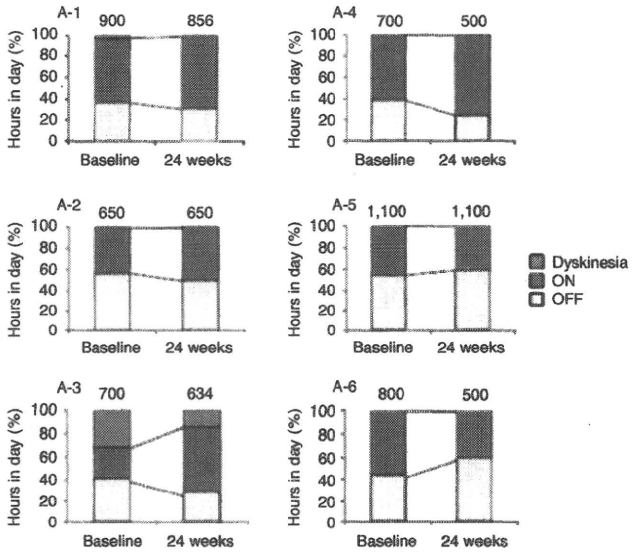

Figure 2 Evaluation of patients' diaries and daily doses of levodopa equivalents. For each 30-minute interval throughout the day, the patients recorded whether they were mobile (ON), immobile (OFF), or asleep. They also recorded the time with troublesome dyskinesias (Dyskinesia). The graph shows the percentage of hours in a day spent in each condition at baseline and at 6 months. The numbers on the bars indicate the mean daily doses of levodopa equivalents (mg). OFF, off-medication state; ON, on-medication state.

significant toxicity.[3,4,6,8,9] Recently, three phase I clinical trials of gene therapy for advanced PD demonstrated that AAV vector–mediated gene delivery into the subthalamic nucleus or putamen was safe and tolerable.[10–13] In this study, the safety of the AAV vectors for clinical use in the human brain was confirmed. Although one patient developed a venous hemorrhage in the subcortical white matter along the trajectory, it is well known that cerebral bleeding occasionally occurs in association with surgical procedures for deep brain stimulation in which electrodes are inserted into the basal ganglia through the frontal lobe white matter.[14,15] PET imaging in this patient showed that putaminal AADC expression was not affected by the subcortical venous hemorrhage and persisted up to 96 weeks. Thus, the venous hemorrhage was probably due to the surgical procedure and not gene transduction.

Although the present trial was a small, open-label study, and the nonblinded, uncontrolled analysis limits the interpretation, the initial efficacy outcomes are encouraging. Our patients showed improved motor performance in the OFF state. Levodopa has a relatively short plasma half-life (60–90 minutes), and antiparkinsonian effects observed after levodopa administration have generally been recognized as short- and long-duration responses. The short-duration response roughly parallels the plasma levodopa concentrations and is thought to be closely linked to dyskinesia, whereas the long-duration response builds up over weeks and improves trough (worst) motor performance in the OFF state.[7] Because the pattern of the short-duration response to levodopa did not change after gene therapy in our patients, the beneficial effect on the OFF state appears to be attributed to augmentation of the long-term response to levodopa.[16] In the preclinical studies with animal models of PD, AAV vectors mainly transduced medium spiny neurons that have dopamine receptors, and extracellular dopamine was increased in the striatum after administration of levodopa.[5,17] The mechanism underlying the long-duration response is not sufficiently understood, and future study is necessary to determine how nonphysiologic production of dopamine in the striatal neurons could enhance the response. It has been reported that the sustained long-duration response to levodopa is greater in patients treated with higher single doses of levodopa.[18] Thus, it is likely that increased dopamine in the putamen after gene transfer may enhance the stable long-duration response. Motor fluctuations in PD are associated with increased response to levodopa with a deeper trough in motor performance, rather than shortening of the response. Improving trough or OFF state motor function by augmenting the long-term response would likely reduce motor fluctuation.[16] Two of three patients in whom the short-duration response to levodopa was studied showed increased peak plasma levodopa concentrations after gene therapy. This finding may simply reflect variable absorbance of levodopa, and it remains to be elucidated whether changes in gastrointestinal absorption could be related to better motor performance in the OFF state.[19]

Activities and levels of AADC mRNA and protein are profoundly reduced in advanced PD,[2] but there are still several types of AADC-containing cells in the striatum, such as serotonin neurons, intrinsic dopamine neurons, AADC-containing "D" neurons, and glial cells.[20] These cells may act as a local source of dopamine. However, dopamine produced in nondopamine cells may not be taken up into dopamine cells and stored in synaptic vesicles, as dopamine transporter and vesicular monoamine transporter 2 are also reduced in advanced PD. The functional efficacy of dopamine produced from exogenous levodopa in these cells may be limited, at least in primates.[2,3] Striatal output neurons, main targets in AADC gene therapy, play a principal role in dopamine modulation of motor function in the basal ganglia. Dopamine synthesized in the striatal neurons themselves may more easily stimulate both synaptic and extrasynaptic receptors.

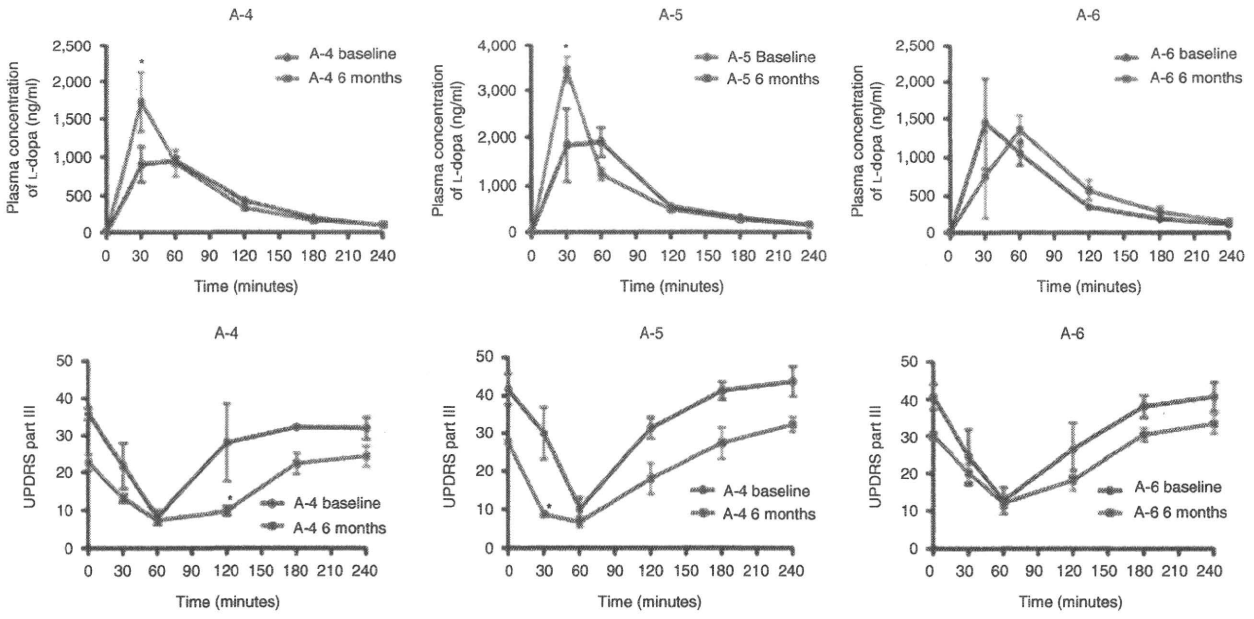

Figure 3 Short-duration response to levodopa. Comparison of short-duration response to levodopa before (blue) and after gene therapy (brown) in three patients (A-4, A-5, and A-6). Patients took 100 mg of levodopa with 25 mg benserazide orally after 20 hours without dopaminergic medication. Values represent means and SE of three trials. Upper panels: plasma levodopa levels; lower panels: Unified Parkinson's Disease Rating Scale motor scores. $^*P < 0.05$.

Results of a similar phase I protocol were reported recently for the 10 patients treated with AAV-hAADC-2 (ref. 10). That study used the same vector preparations as this study. The subjects were divided into two groups that received the same or one-third dose of the vector used in this study, respectively. Although the present patients had slightly milder initial symptoms, the patients treated with the same dose of vector in the two studies showed similar improvement in the OFF state and putaminal FMT uptake on PET. These findings provide independent confirmation of the safety, tolerability, and potential efficacy of AADC gene therapy. Future studies focusing on optimal vector dosing and defining the relationship between vector dose and clinical effects are necessary.[21]

In conclusion, these data indicate that AAV vector–mediated gene transfer of AADC is safe and may benefit advanced PD patients.

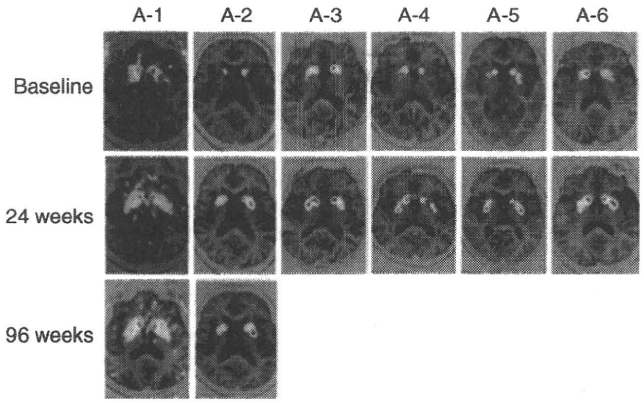

Figure 4 FMT-PET images. Axial images at the level of the putamen are shown before and 24 weeks after gene therapy for all six patients. Increased FMT uptake persisted until 96 weeks in two patients. The 4-week images are not shown because they are similar to the 24-week images. FMT, 6-[^{18}F]fluoro-L-*m*-tyrosine; PET, positron emission tomography.

MATERIALS AND METHODS

Study design. The protocol and consent forms were approved by the institutional review board. The protocol was also reviewed by the committee of the Ministry of Health, Labour and Welfare of Japan. A data safety monitoring board reviewed the ongoing study. All subjects reviewed the consent form and provided their written, informed consent.

This 24-week, phase I, open-label study was primarily designed to evaluate the safety and tolerability of intraputaminal AAV-hAADC-2 infusion in idiopathic PD. Patients were evaluated preoperatively and monthly postoperatively for 6 months, using multiple measures, including the UPDRS, motor state diaries, the MMSE, the short form of the GDS, and laboratory tests. The UPDRS was done in the practically defined OFF state 12 hours after withdrawal of all antiparkinsonian medications, and in the ON state 1 hour after administration of the usual morning dose of medication. Motor scores for the UPDRS can range from 0 to 56, with higher scores indicating poorer function. Using diaries that separated the day into half-hour segments, the patients recorded their mobility during the 4 days before admission and for another 4 days at 6 months after admission. They were trained to rate their condition as sleeping, immobile, mobile without troublesome dyskinesias, or mobile with troublesome dyskinesias. The total number of hours spent in each of these categories was calculated, and the differences between the baseline and the 6-month scores were compared between the groups.

The short-duration response to levodopa was evaluated in three patients (patients 4–6) at baseline and 6 months after gene transfer; they took 100 mg of levodopa orally with 25 mg benserazide after 20 hours without dopaminergic medication. Motor symptoms based on UPDRS motor (part III) and plasma levodopa concentrations were assessed at baseline and 30 minutes, 1, 2, 3, and 4 hours after levodopa intake.

Patients. The main entry criteria were: age 45–75 years; diagnosis of moderate to advanced PD, defined as Hoehn and Yahr Stage IV and UPDRS in the practically defined OFF condition of at least 20; at least

5 years of levodopa therapy; a minimum 8-point improvement in the UPDRS motor score after levodopa intake; and motor complications not satisfactorily controlled with medical therapy. The main exclusion criteria were atypical parkinsonism, dementia (MMSE score <20), and previous neurosurgical treatment for PD.

Vector and stereotaxic infusion. The vector used in this trial was a recombinant AAV2 with an expression cassette consisting of a human cytomegalovirus immediate-early promoter, followed by the human growth hormone first intron, complementary DNA of human AADC, and simian virus 40 polyadenylation signal sequence.[3–6] Clinical grade AAV-hAADC-2 was manufactured by Avigen (Alameda, CA) and provided by Genzyme (Boston, MA). The patients received AAV-hAADC-2 *via* bilateral intraputaminal infusions. Two target points were determined in the putamen that were sufficiently separated from each other in dorsolateral directions and identified on a magnetic resonance image. One burr hole was trepanned in each side of the cranial bone, through which the vector was injected into the two target points *via* the two-track insertion route. The vector-containing solution was prepared to a concentration of 1.5×10^{12} vector genome/ml, and 50 µl per point of the solution were injected at 1 µl/min; each patient received 3×10^{11} vector genome of AAV-hAADC-2.

Neutralizing antibody titers against AAV2 were determined by measuring β-galactosidase activities in HEK293 cells transduced with 5×10^3 vector genome/cell of AAV2 vectors expressing β-galactosidase in various dilutions of sera.[22]

PET. The AADC expression level in the putamen was assessed on PET imaging with FMT 6 days before surgery and 1 and 6 months after gene transfer. All patients stopped dopaminergic medications 18 hours before PET and took 2.5 mg/kg of carbidopa orally 1 hour before FMT injection. Subsequently, 0.12 mCi/kg of FMT in saline were infused into an antecubital vein, and a 90-minute dynamic acquisition sequence was obtained. The PET and magnetic resonance imaging data were co-registered with a fusion processing program (Syntegra; Philips, Amsterdam, The Netherlands) to produce the fusion images. Radioactivities within volumes of interest drawn in the putamen and occipital lobe were calculated between 80 and 90 minutes after tracer injection. A change in putaminal FMT uptake from baseline to 24 weeks was assessed using the putaminal–occipital ratio of radioactivities.

Statistical analysis. Values at baseline and 6 months after gene transfer were compared using Student's *t*-test (paired analyses). A two-sided *P* value <0.05 was taken to indicate significant differences. Two-way analysis of variance with Bonferroni correction of *P* values was used for the short-duration response to levodopa.

ACKNOWLEDGMENTS

This study was supported by grants from the Japanese Government: a grant-in-aid from the Research Committee of CNS Degenerative Diseases *via* the MHLW and grants from the Ministry of Education, Culture, Sports, Science and Technology. We thank Hiroshi Ichinose and Toshiharu Nagatsu for their helpful comments, and Naomi Takino, Hitomi Miyauchi, Keiko Ayabe, and Tetsuo Ito for their technical assistance. We also thank Avigen and Genzyme for providing clinical grade AAV vector.

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Journal of the Neurological Sciences 298 (2010) 78–84

Contents lists available at ScienceDirect

Journal of the Neurological Sciences

journal homepage: www.elsevier.com/locate/jns

Temporal trends and geographic clusters of mortality from amyotrophic lateral sclerosis in Japan, 1995–2004

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ARTICLE INFO

Article history:
Received 29 March 2010
Received in revised form 5 August 2010
Accepted 5 August 2010

Keywords:
Amyotrophic lateral sclerosis
ALS
Motor neuron disease
Mortality
Trends
Cluster analysis
Epidemiology
Japan

ABSTRACT

The present study examined temporal trends and geographic clustering of amyotrophic lateral sclerosis (ALS) mortality in Japan, during 1995–2004, using vital statistics based on death certificates. ALS was usually diagnosed by neurologists according to clinical guidelines that complied with the El Escorial Criteria. The underlying cause of death for ALS was coded as G12.2A. Regression analysis was used to examine temporal trends. Spatial scan statistic was used to detect any area of elevated risk as a cluster. A total of 12,173 (6864 male and 5309 female) ALS deaths were reported. Annual crude mortality rate per 100,000 population was 1.07 (1.26 for males and 0.89 for females) in 2004. Although the overall temporal trend was stable, the trend increased in the 70+ years age group (p for trend, <0.001 in males and <0.05 in females), while it declined in the under 70 years age group (p for trend, <0.01 for both sexes). Male preponderance and M/F ratio remained nearly constant over time. Three clusters were detected: two ($p<0.005$ in males and $p<0.05$ in females) in northeast and one ($p<0.05$ in males) in west-central Japan. Further research is needed to clarify contributing factors for the observed trends and clusters in ALS mortality.

1. Introduction

Amyotrophic lateral sclerosis (ALS) is still a fatal neurodegenerative disease characterized by the selective loss of upper and lower motor neurons. Five to 10% of cases of ALS are familial; the others are believed to be sporadic [1]. While advances have been made in identifying disease-causing genes for familial ALS, very little is known about susceptibility genes or other risk factors for sporadic ALS. Many putative environmental risk factors (i.e., heavy metals, solvents, electrical and electromagnetic fields, poliovirus, mechanical trauma, heavy physical activity, cigarette smoking, and diet) have been previously reported; however, age and a family history are the only established risk factors for ALS [1–4].

Variation in mortality over time and by geographic location, sex and ethnicity can often be a source of etiological clues [5]. The mortality rate from motor neuron disease (MND), of which ALS accounts for 85% or more [1,5], was reported to have steadily increased from the 1950s to the 1990s in western countries [3,5–10]. In some European countries and the United States, a greater increase in ALS mortality was observed in females than in males in the past 30 years, causing a decrease in the male to female (M/F) ratio [5,8–10].

Contrary to the trends noted in many other countries, Japan has shown an unusual pattern of mortality from MND for decades. The age-adjusted MND mortality rate rose from the mid 1950s, peaking in the early 1960s, and declined in the early 1970s [11,12]. Thereafter, the rate slowly increased to that in the early 1950s for a period of 20 years between 1970 and 1990 [11]. A recent study reported that it decreased from 1995 through 2001, and the M/F ratio slightly increased [13].

The Western Pacific form of ALS, referred to as ALS and parkinsonism-dementia complex (ALS/PDC), was identified in the 1950s in three distinct geographic isolates: Guam, western New Guinea and the Kii Peninsula of Japan [14,15]. Over the past four decades, the incidence of ALS/PDC has markedly declined in Guam [14]. On the other hand, a continuing high prevalence and incidence in Hohara in Mie prefecture [16] and Kozagawa in Wakayama prefecture [17] in the Kii peninsula are reported, although they temporarily declined in the 1980s.

In this study we examined the national ALS mortality data of Japan to reveal the recent temporal trends in ALS mortality and investigated whether or not any geographic clusters of ALS deaths exist in particular regions.

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doi:10.1016/j.jns.2010.08.004

2. Methods

The World Federation of Neurology Research Group on Neuromuscular Diseases published the El Escorial Criteria (EEC) in 1994 [18]. Based on these criteria, the research committee on ALS of Japan has updated the guidelines for diagnosis and treatments of ALS, and recommended it for clinicians and researchers [19]. ALS was usually diagnosed by neurologists using guidelines that complied with the El Escorial Criteria.

In 1995, the Statistics and Information Department of the Ministry of Health, Labour and Welfare, Japan, changed the coding for disease classification from that in the ninth version of International Classification of Diseases (ICD-9) to that in the tenth version (ICD-10). ICD-9 and ICD-10 designated MND with the respective four-digit codes of 3352 and G12.2. For the vital statistics of Japan after the year 1995, they added the capital letters A and B to G12.2 to differentiate ALS from other MND: G12.2A for ALS and G12.2B for primary lateral sclerosis, progressive bulbar palsy, spinal muscular atrophy, and other or unspecified MND. They followed the international rules for mortality statistics based on the underlying cause of death. The underlying cause of death was defined by the World Health Organization as the disease or injury that initiated the train of morbid events directly leading to death or the circumstances of the accident or violence that produced the fatal injury.

The national ALS mortality data from 1995 through 2004 were used for our present study. The data used were taken from the national mortality database of vital statistics based on death certificates, after obtaining permission for use from the Statistics and Information Department. The variables in the data file included the codes of the underlying cause of death for ALS along with age at death, sex, and place of residence where the deceased had lived. It did not contain any personal identifiable information (e.g., individuals' names or residential addresses).

The total number of deaths due to ALS from 1995 through 2004 was counted, and age-specific mortality rates were calculated according to 5-year age intervals. Annual crude mortality rates in 1995–2004 were calculated as the number of ALS deaths per million persons per year on the basis of the Japanese population for the respective year.

To examine temporal trends in deaths from ALS, annual age-adjusted mortality rates were calculated by the direct method using the total population of the 2005 census as a standard population. Linear regression analysis was used to examine temporal trends in mortality as well as M/F ratios in individual years as a continuous variable. Joinpoint regression analysis was used to provide annual percentage changes (APC) with 95% confidence intervals and p values for trends [20,21]. It was also performed in the groups of age at death, <70 and 70+ years. Statistical significance was determined as a p value for trend less than 0.05.

A cluster is defined as a geographically bounded group of occurrences of sufficient size and concentration to be unlikely to have occurred by chance without any assumptions about the shape or form of the cluster [22]. To detect clusters, the flexible spatial scan statistic [23–26] was used in our present study. It can detect clusters of any size and form located anywhere in the study region, whether or not they cross administrative borders. The most likely cluster can be detected as that with the maximum likelihood. P values were obtained using Monte Carlo hypothesis testing, comparing the test statistic from the observed data set with the test statistics from 999 random data sets generated under the null hypothesis of no clustering. Statistical significance was determined as a p value less than 0.05.

The flexible spatial scan statistic can be applied to geographically aggregated data. So, to eliminate the effects of age, we employed this statistic using the number of observed and expected deaths from ALS based on the vital statistics for each of the secondary medical care zones (SMCZ). At the time of this study, there were 359 SMCZs for medical care planning, each of which consisted of neighboring municipalities in all 47 prefectures of Japan, according to the Medical Service Law.

3. Results

A total of 12,173 (6864 male and 5309 female) ALS deaths were reported in Japan in the period between 1995 and 2004. Fig. 1 shows mortality rates due to ALS by 5-year age groups. The age-specific mortality rates rose steadily with age group up to the age of 75–79 years and then sharply declined for those aged 80 years and older.

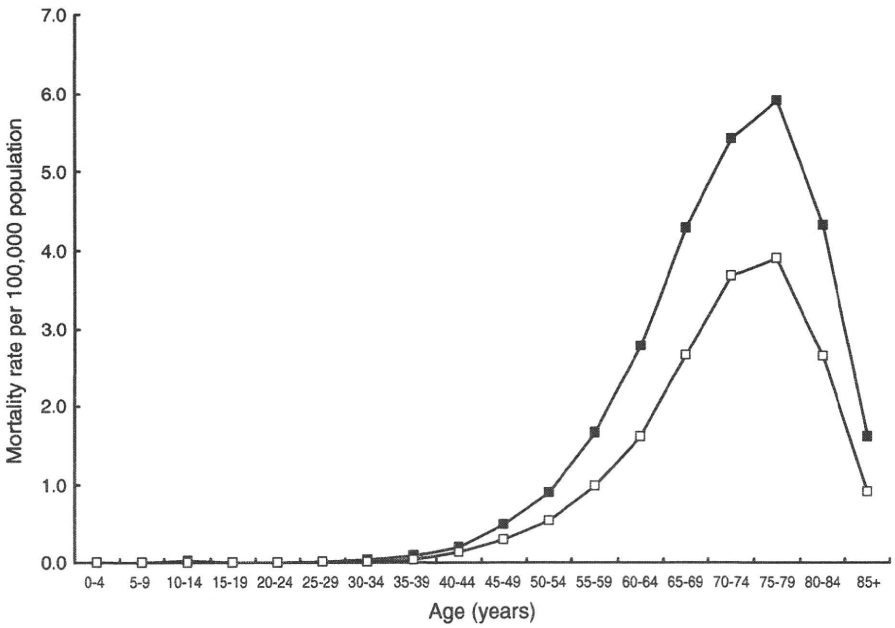

Fig. 1. Age-specific mortality rates from amyotrophic lateral sclerosis (ALS) by sex, Japan, 1995–2004 (■ = males and □ = females).

Table 1
Annual mortality rate per 100,000 population from amyotrophic lateral sclerosis (ALS), Japan, 1995–2004.

Year	Number of deaths			Crude mortality rate			Age-adjusted mortality rate[a]		
	Total	Male	Female	Total	Male	Female	Male	Female	M/F ratio
1995	1031	579	452	0.82	0.94	0.71	1.27	0.81	1.56
1996	1166	658	508	0.93	1.07	0.79	1.43	0.90	1.59
1997	1144	634	510	0.91	1.03	0.79	1.34	0.88	1.52
1998	1179	666	513	0.93	1.08	0.79	1.38	0.86	1.60
1999	1181	673	508	0.93	1.09	0.78	1.36	0.83	1.64
2000	1202	666	536	0.95	1.07	0.83	1.33	0.86	1.55
2001	1225	692	533	0.96	1.11	0.82	1.34	0.85	1.57
2002	1337	761	576	1.05	1.22	0.88	1.43	0.88	1.64
2003	1338	749	589	1.05	1.20	0.90	1.38	0.87	1.58
2004	1370	786	584	1.07	1.26	0.89	1.41	0.85	1.67

[a] Direct age-adjusted to the year 2005 census population. *P* values for trends are 0.200 for males, 0.899 for females and 0.122 for M/F ratios.

Annual crude mortality rates per 100,000 population increased from 0.94 to 1.26 for males and from 0.71 to 0.89 for females. After adjustment by age, ALS mortality rates have been stable during this period in both males and females. Mortality rates were higher for males than for females over the entire period. The M/F ratios remained almost constant (Table 1).

After stratification by age, age-adjusted mortality rates showed completely different temporal trends in the age groups of <70 years and 70+years (Figs. 2 and 3). Age-adjusted mortality rates have significantly increased in the older age group (p for trend, <0.001 in males and <0.05 in females), while they have significantly decreased in the younger age group (p for trend, <0.01 for both sexes).

The most likely, second, and third clusters are shown in Table 2, and each cluster is located on maps in Figs. 4 and 5. For males, two statistically significant clusters were identified, but both clusters were detected in different regions from the Kii Peninsula. In a part of the Kii Peninsula including Hohara and Kozagawa villages, which have been well known hyperendemic ALS foci for many years, a third cluster containing 236 observed deaths was detected, but it was not statistically significant. For females, one statistically significant cluster was identified. It was adjacent to the most likely cluster found in males, and some areas of these two clusters overlapped.

4. Discussion

Previous studies have reported a rise and fall in mortality from MND or ALS in Japan over the past five decades [11–13]. Our present study demonstrated that the recent temporal trend in age-adjusted mortality rate due to ALS was stable during the 10-year period between 1995 and 2004, since the introduction of ECC and ICD-10. When stratified by age into <70 and 70+years at death, we found opposite directions in age-adjusted mortality rates for the two groups: a clear upward trend in the older age group and a downward trend in the younger age group. This pattern is unique compared to those reported from previous studies [2,3,5,6,8–13]. In the United States, an increasing trend is seen for all ages except age 45 to 54 years [5]. In the United Kingdom and Norway, the trend is upward in the 60+ or 65+years age group but it is static in the under 60 years age group [8,10].

The increased ALS mortality in the older age group is common to all studies. It is noteworthy that in our present study there was a decreasing trend in the under 70 years age group. One of the hypotheses explaining this phenomenon is that age at onset may be chronologically delayed with time. According to a 10-year prospective population-based study conducted in Italy, mean onset age (SD) has become slightly higher: 64.2(11.2)years in 1995–1999 and 65.4 (11.1)years in 2000–2004 [27]. Although the data are cross-sectional in Japan, there is a substantial difference in age at onset between 61.8 (12.2)years in 1989–1999 [17] and 65.4(10.7)years in 2003–2006 [28]. As another hypothetical explanation, it may be that survival duration has been prolonged by the improvement of treatment such as pharmacotherapy (e.g., rilusole) combined with nutritional and respiratory support [1]. Remarkable progress in home-visit nursing care has been made in Japan, with the universal availability of

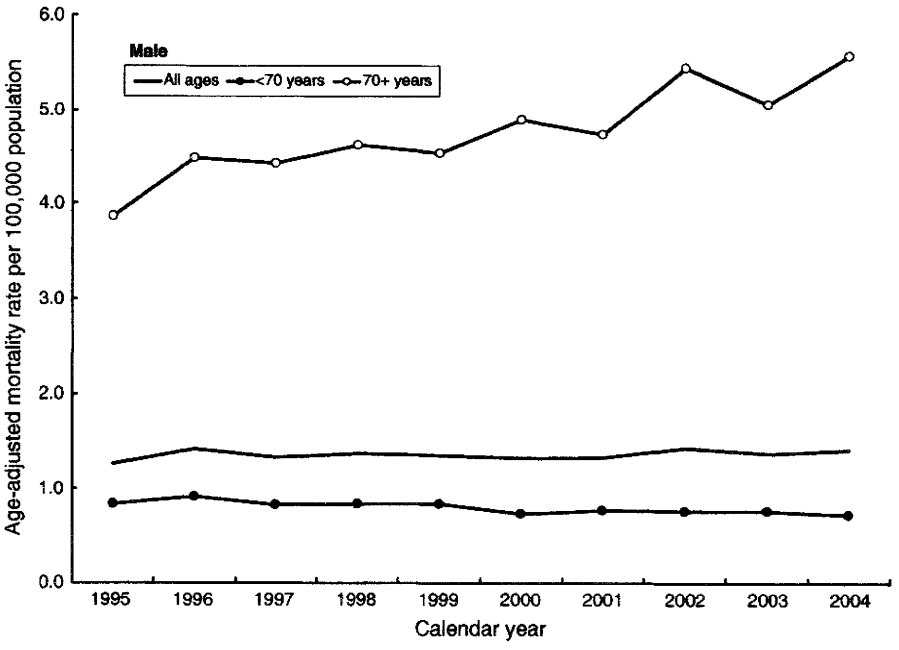

Fig. 2. Age-adjusted mortality rates from amyotrophic lateral sclerosis (ALS) in males, Japan, 1995–2004. The annual percentage changes (95% confidence intervals) were 0.5(−0.3, 1.4), −2.1(−3.2, −1.0) and +3.3(2.1, 4.5) for all age groups, age group of <70 years and age group of 70+years, respectively.

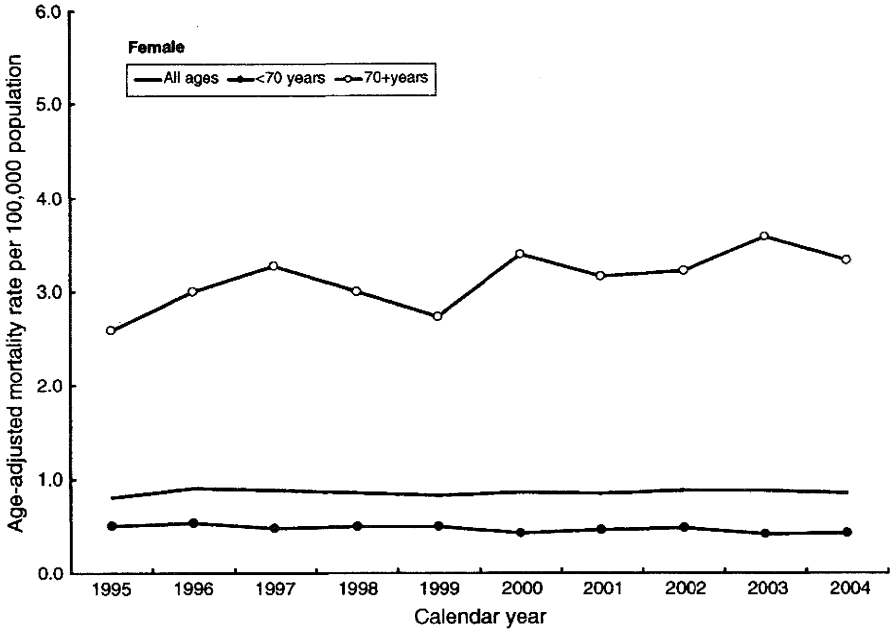

Fig. 3. Age-adjusted mortality rates from amyotrophic lateral sclerosis (ALS) in females, Japan, 1995–2004. The annual percentage changes (95% confidence intervals) were 0.0 (−0.7, 0.8), −2.3(−3.6, −0.9) and +2.3(0.3, 4.1) for all age groups, age group of <70 years and age group of 70+ years, respectively.

mechanical ventilatory support for ALS patients. A very high proportion of Japanese ALS patients (29.3%) received tracheotomy positive pressure ventilation (TPPV) compared to those in North America and Europe (2.1–5.4%) [28–33]. Based on the large-scale registered data on patients with ALS in Japan, about 40% of those under TPPV received care at home and almost 30% survived 9 years or more after the introduction of TPPV [28]. In our clinical experience, younger patients and their families are more likely to choose TPPV than older ones (unpublished). As a result, younger patients could survive longer. This may lead to prolongation of the age at death, particularly for younger patients, which may cause mortality to decrease in younger patients and consequently increase in older patients. To determine the duration of survival, both age at symptom-onset and age at death are required, but our present study lacked the former. Further research is needed to justify the aforementioned hypotheses.

Regarding the M/F ratio of ALS mortality over time, we found male preponderance that remained stable in our present study. This finding is inconsistent with the reported narrowing of the male to female gap in mortality [4,8–10] and incidence [2–4,34]. As some authors pointed out, the reduced M/F ratio may be partly caused by the changing lifestyle (e.g., smoking) of women, which has become more similar to that of men, in western countries [3,4,34]. According to OECD health data, for example, the prevalence of smoking has become similar for both sexes in western countries, but it is still markedly different between sexes in Japan (58.8% for males and 15.2% for females in 1995; 46.9% for males and 13.2% for females in 2004) [35]. In addition, we have to take into account gender difference in socioeconomic status that might affect patients' choice of treatment and long-term care. But we could not examine this factor because of the limited data in our current study. Further investigation is required to verify potential factors explaining the M/F ratio of ALS mortality over time.

The present study identified three statistically significant clusters of ALS mortality. Two (A1 in Fig. 4 and B1 in Fig. 5), which partially overlapped, were located in the northeastern part of the mainland of Japan. As shown in Fig. 4, one more cluster (A2) was geographically separated from the other (A1), at a short distance from the Kii Peninsula. In the Kii Peninsula, a cluster (A3) was observed but it did not reach statistical significance at the SMCZ level. Recent epidemiological surveys reported a continuing high incidence and prevalence in the subfoci of Hohara and Kozagawa villages of the Kii Peninsula [16,17,36]. Our cluster analysis may not be able to detect clustering in the two villages, which are much smaller units of area than SMCZ. These four clusters (A1–A3 and B1) are promising candidate areas for further analytical studies on ALS.

The limitations of our present study should be taken into account. One is case ascertainment for ALS based on the underlying cause of

Table 2
Geographic clusters of ALS mortality detected using flexible spatial statistics, Japan, 1995–2004.

Cluster	Approximate cluster location[a]	Number of SMCZs[b]	Expected cases	Observed cases	Relative risk	*p* value
Males (N=6864)						
Most likely cluster	A1: a part of Niigata–Gunma–Nagano–Fukushima	12	115.70	181	1.56	0
Second cluster	A2: a part of Hyogo–Kyoto–Osaka	7	182.16	254	1.39	0.03
Third cluster	A3: a part of Kii Peninsula (Wakayama–Mie–Nara–Osaka)	11	178.34	236	1.32	0.36
Females (N=5309)						
Most likely cluster	B1: a part of Gunma–Tochigi–Saitama	12	118.67	178	1.50	0.02
Second cluster	B2: a part of Hokkaido Island	11	172.54	231	1.34	0.26
Third cluster	B3: a part of Aichi–Gifu–Shizuoka–Shiga	12	276.71	384	1.26	0.3

[a] The clusters of A1–A3 and B1–B3 correspond to those on maps in Figs. 4 and 5, respectively.
[b] Abbreviated secondary medical care zones.

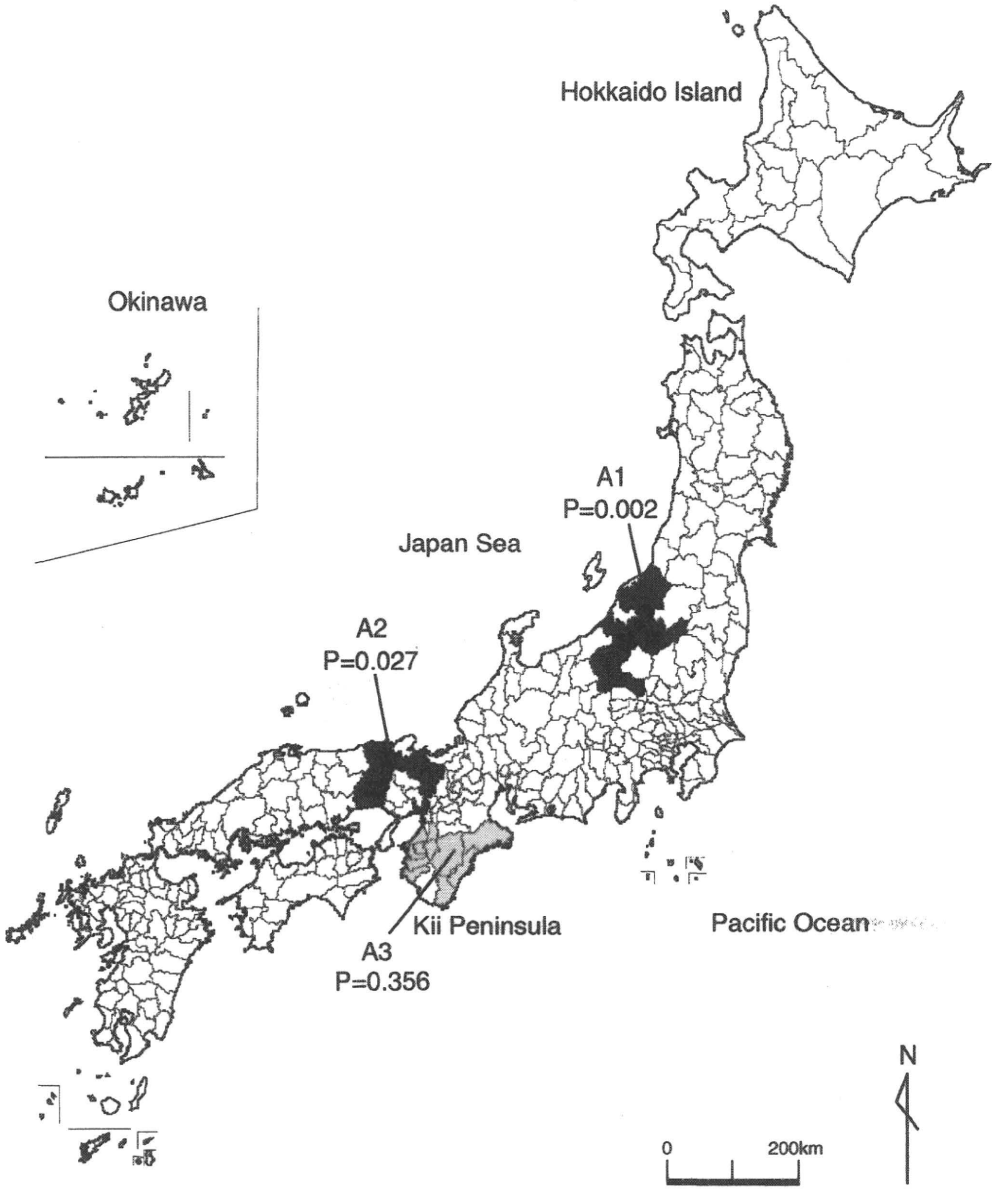

Fig. 4. Geographic clusters of age-adjusted deaths from amyotrophic lateral sclerosis (ALS) for males, Japan, 1995–2004. A1, A2 and A3 are the most likely, second and third geographic clusters, respectively (black (A1 and A2) and gray (A3) clusters with and without statistical significance, respectively).

death recorded on death certificates. This is not so serious, however, considering the following points: (1) ALS was usually diagnosed by neurologists, (2) the number of neurologists was large (e.g., 8555 as of March 31, 2009), and (3) neurologists have generally followed the clinical guidelines based on ECC since the recommendation of its use by the research committee on ALS of Japan [18,19]. The other is regional disparity affecting geographic clustering (e.g., number of neurologists and access to specialized medical care). Taking the following points together, it seems that the degree of regional disparity is small. The average number of neurology clinics/departments was 4.75 per 100,000 population [37]. The areas with higher or lower concentrations of neurology clinics/departments were not consistent with the locations in which the clusters of ALS mortality were detected in our present study. Basically, all ALS patients have been guaranteed free medical access by the provision of financial aid from universal medical insurance since 1961, countermeasures against intractable diseases including ALS since 1972, and nursing-care insurance since 2000.

In conclusion, we have provided new evidence of ALS mortality in Japan during the 10-year period between 1995 and 2004: 1) The overall temporal trend in age-adjusted ALS mortality is stable. 2) The trend is going up in the 70+ years age group while it is going down in the under 70 years age group. 3) Male preponderance and M/F ratio remain nearly constant. 4) Some geographic clusters are detected. Current thinking on complex diseases like ALS is that multiple genetic and environmental factors contribute to disease liability [38]. Further research is needed to clarify contributing factors for the observed trends and clusters in ALS mortality.

Acknowledgements

This study was supported by grants from Health and Labour Sciences Research, Research Committees on Epidemiology of and CNS Degenerative Diseases of Intractable Diseases from the Ministry of Health, Labour and Welfare, Japan.

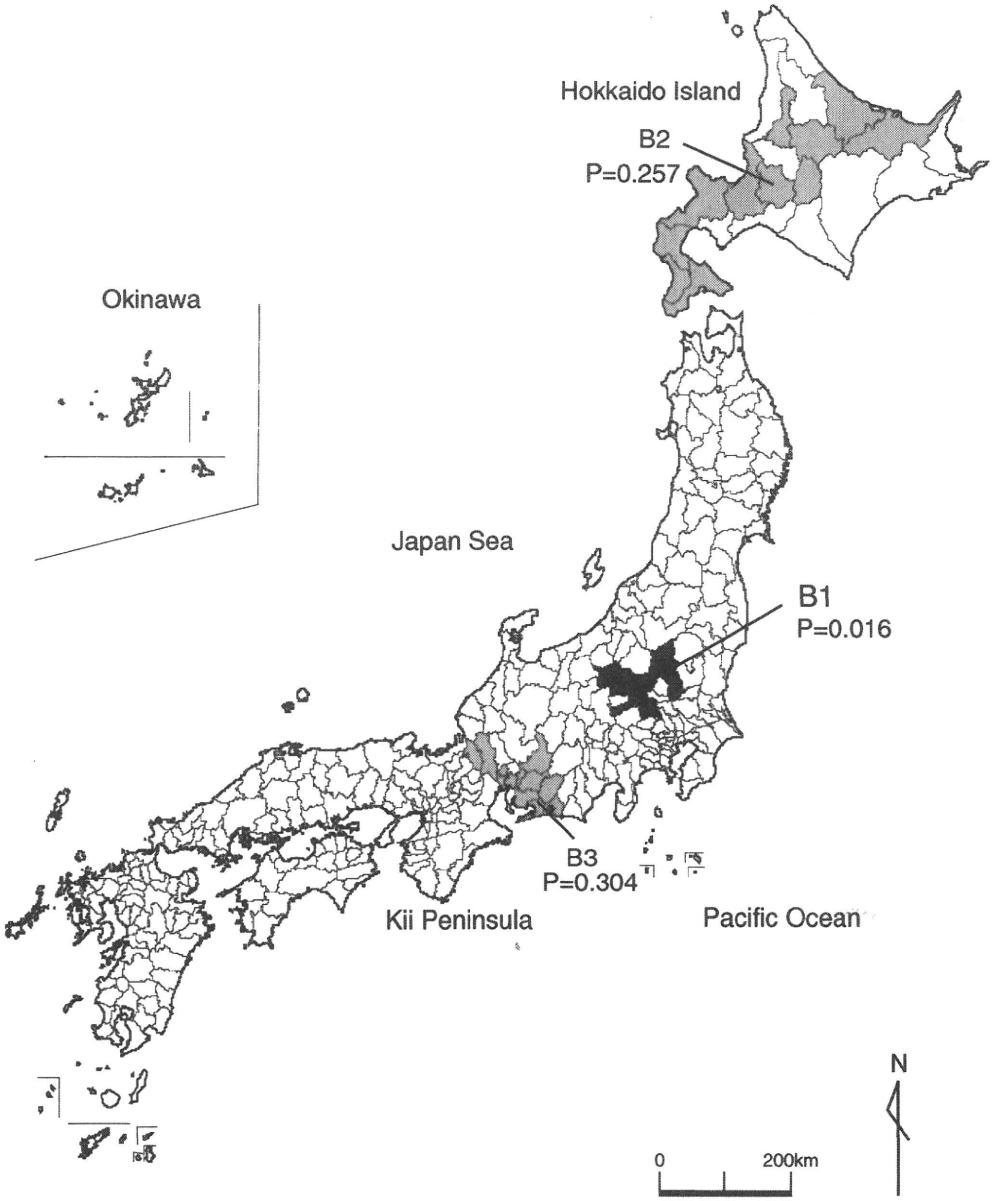

Fig. 5. Geographic clusters of age-adjusted deaths from amyotrophic lateral sclerosis (ALS) for females, Japan, 1995–2004. B1, B2 and B3 are the most likely, second and third geographic clusters, respectively (black (B1) and gray (B2 and B3) clusters with and without statistical significance, respectively).

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Behavioural Neurology 23 (2010) 153–158
DOI 10.3233/BEN-2010-0276
IOS Press

Is lesion of Exner's area linked to progressive agraphia in amyotrophic lateral sclerosis with dementia? An autopsy case report

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Abstract. Agraphia, as a neuropsychological symptom of ALS, especially ALS with dementia (ALS-D), has recently attracted more attention. However, the brain lesion responsible has not been identified. Here we present an autopsy case of ALS-D of a patient with obvious agraphia, without aphasia, that also presented cerebrospinal degeneration with TDP-43-pathology compatible with ALS-D. Of the pre-motor frontal lobe cortices, degeneration and immuno-histochemical pathology were most obvious in the caudal area of the left middle frontal gyrus, or Exner's area. Assuring this area plays a pivotal role in the kanji and kana formation used in writing the Japanese language, this case of ALS-D showed both agraphia and Exner's area stressed pathological lesions. It may thus be the first case to indicate an intimate relationship between the neuropsychological symptoms and an associated lesion for ALS-D.

Keywords: Amyotrophic lateral sclerosis with dementia (ALS-D), fronto-temporal lobar degeneration with TDP-43 proteinopathy (FTLD-TDP), progressive agraphia, Exner's area

1. Introduction

Amyotrophic lateral sclerosis with dementia (ALS-D) is a nosological condition presenting motor neuron disease (MND) and dementia. The clinical features of dementia in ALS-D are of the frontal lobe type, and ALS-D is located within a framework of fronto-temporal lobar degeneration (FTLD) [1]. Although analysis of language function in ALS-D is considered difficult to carry out, due to severe bulbar palsy, we previously reported that writing disorder may exist in the early stage [2]. Another report, in Japanese, also describes writing disorder in a patient with ALS-D [3]. Here we describe an autopsied case of ALS-D, presenting progressive agraphia without aphasia, and discuss its clinicopathological relationship.

2. Case history

A 73 year-old Japanese woman, with a history of breast cancer, was admitted to our hospital with speech disorder and personality change. Her family history provided no clues as to onset of these changes. Six months before admission, she became disoriented about dates and resistant to correction, and after three months, while playing cards with her family, she began missing her turn and became argumentative about continuing. At about the same time, her speech began to be slurred and became difficult, and her activities were slow down.

On admission, she showed no abnormality except for evidence of the surgery for breast cancer. She was cooperative during the neurological examination and

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ISSN 0953-4180/10/$27.50