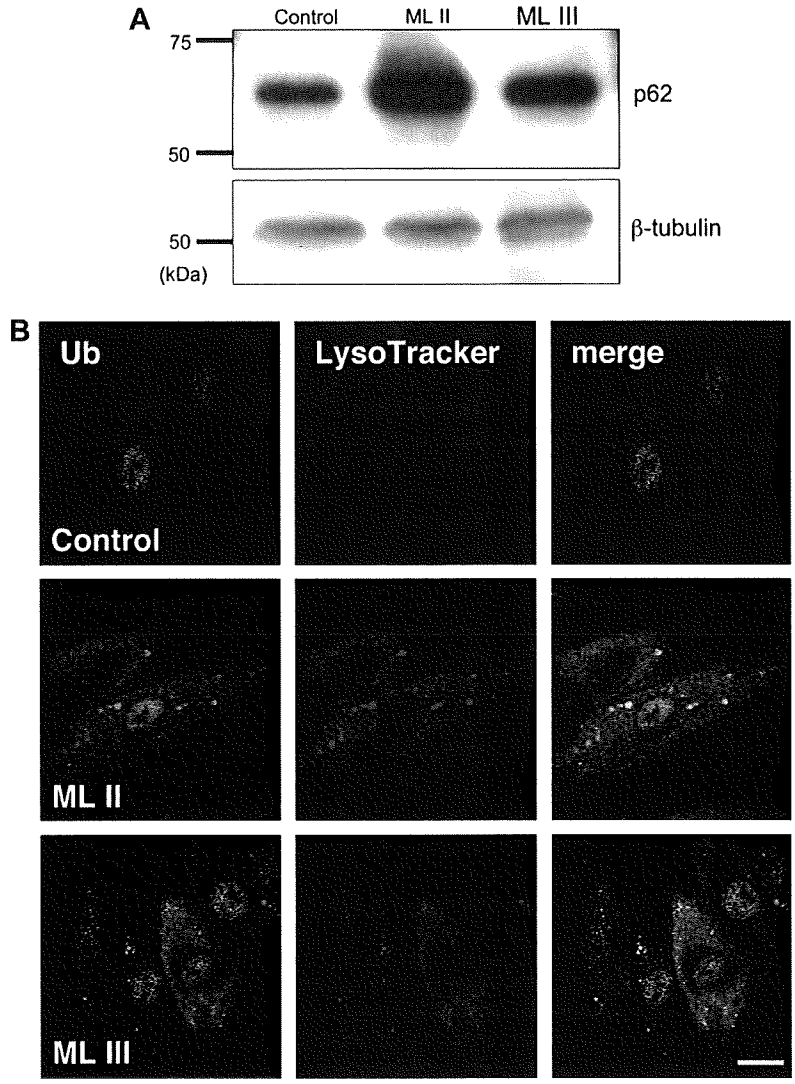

Fig. 2. Accumulation of p62 and ubiquitin-positive inclusions in ML fibroblasts. (A) Immunoblotting analysis with anti-p62. (B) Immunofluorescence showed significant accumulation of ubiquitin-positive inclusions colocalized with LysoTracker in ML cells. Bar = 10 μm.

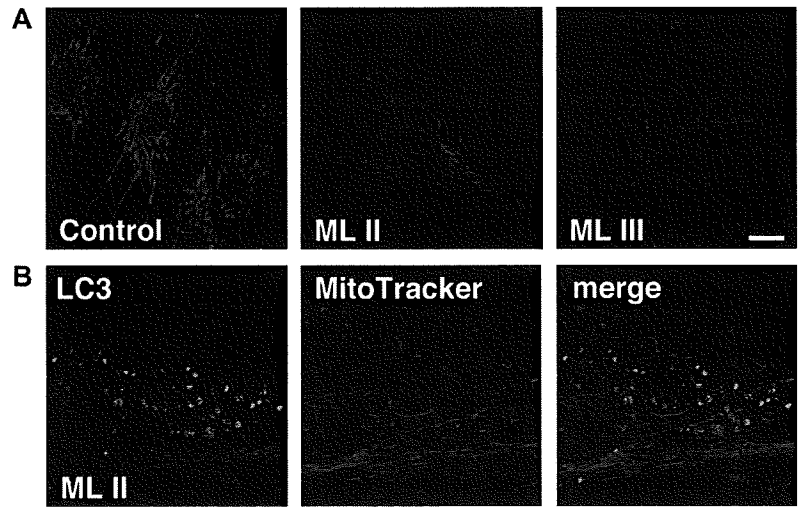

Fig. 3. Mitochondrial impairments in ML fibroblasts. (A) Morphological analysis with MitoTracker-labeled mitochondria. (B) Fluorescence staining with anti-LC3 and MitoTracker revealed that the proportion of tubular-shaped mitochondria was decreased in LC3-positive vesicle rich regions in ML cells. Bar = 10 μm.

ti-well plate reader (excitation 550 nm/emission 640 nm; PerSeptive Biosystems, Framingham, MA, USA).

3. Results

3.1. Increase in autophagosomes and autolysosomes in ML fibroblasts

First, the level of autophagy was assessed in ML fibroblasts. Using Western blotting analysis, the level of LC3-II protein, a membrane bound specific autophagosome marker [8], was found to be increased markedly in the lysate from ML II fibroblasts compared with that in control fibroblasts (Fig. 1A). A modest increase in LC3-II expression was also observed in the lysate from ML III cells. However, the level of beclin-1, a regulator of autophagy, was not elevated in ML II or III cells. The elevation of autophagosome formation was consistent with the observation in ML fibroblasts labeled with MDC, a selective marker for autolysosomes (Fig. 1B). The subcellular localization of autophagosomes was examined using confocal microscopy, and it was found that the number of LC3-positive structures was increased in the cytosol of ML II and III cells, and these LC3-positive structures, especially large vesicles, partly colocalized with LysoTracker and Lamp-2 positive vesicles (Fig. 1C and D). These large circular LC3-positive structures were often seen in ML II cells compared with control and ML III cells.

3.2. Accumulation of ubiquitinated proteins and p62 proteins in ML skin fibroblasts

Recently, p62 protein has been suggested to interact with ubiquitinated proteins and LC3, which may regulate the selective autophagic clearance of protein aggregates [13]. Next, we examined the levels of p62 and ubiquitinated proteins. Immunoblotting analysis showed p62 protein accumulated in the lysates from ML fibroblasts (Fig. 2A). Immunostaining confirmed that ubiquitin (Ub)-positive aggregates were co-labeled with LysoTracker-positive structures in ML cells (Fig. 2B).

3.3. Mitochondrial dysfunction and its restoration by inhibition of autophagy in ML II and III fibroblasts

Autophagic delivery to lysosomes has been shown to be the major pathway in mitochondrial turnover [8]. It was hypothesized that constitutive activation of autophagic formation could affect mitochondrial turnover and impair its function. To address this hypothesis, morphological analysis was performed using MitoTracker Red CMXRos, a membrane potential-dependent fluorescent dye. As shown in Fig. 3A, thick tubular structures of mitochondria were stained by MitoTracker in control cells, whereas thinner tubules and fragmented structures were observed ML II and III fibroblasts (Fig. 3A). These results were the same as in the previous report [14]. When cells were stained using MitoTracker and anti-LC3, MitoTracker-positive tubular structures were decreased near LC3-positive granules in the cytosol of ML fibroblasts, although these did not colocalize (Fig. 3B).

To determine whether the suppression of autophagy recover mitochondrial impairments in ML cells, the effect of 3-MA, an inhibitor of autophagosome formation [15], on ML skin fibroblasts was investigated. When cells were treated with 5 mM 3-MA for 16 h, MitoTracker-labeled mitochondria morphology showed no significant difference from the control cells, whereas thin or fragmented mitochondria seemed to be restored in ML cells (Fig. 4A). Impaired mitochondrial membrane potentials in the affected cells were repeatedly confirmed by staining with JC-1, another mitochondrial membrane sensor, and they recovered significantly after incubation with 3-MA (Fig. 4B).

3.4. Cathepsin B and D in ML II and III fibroblasts

In normal human fibroblasts, cathepsin B and D proteins were transported from the Golgi complex to the lysosome via M6P-dependent pathway [7]. Immunofluorescence analysis showed that cathepsin B and D proteins were colocalized with Lamp-2-positive structures in control fibroblast, whereas these proteins were observed diffuse and partly in the perinuclear, Golgi patterns

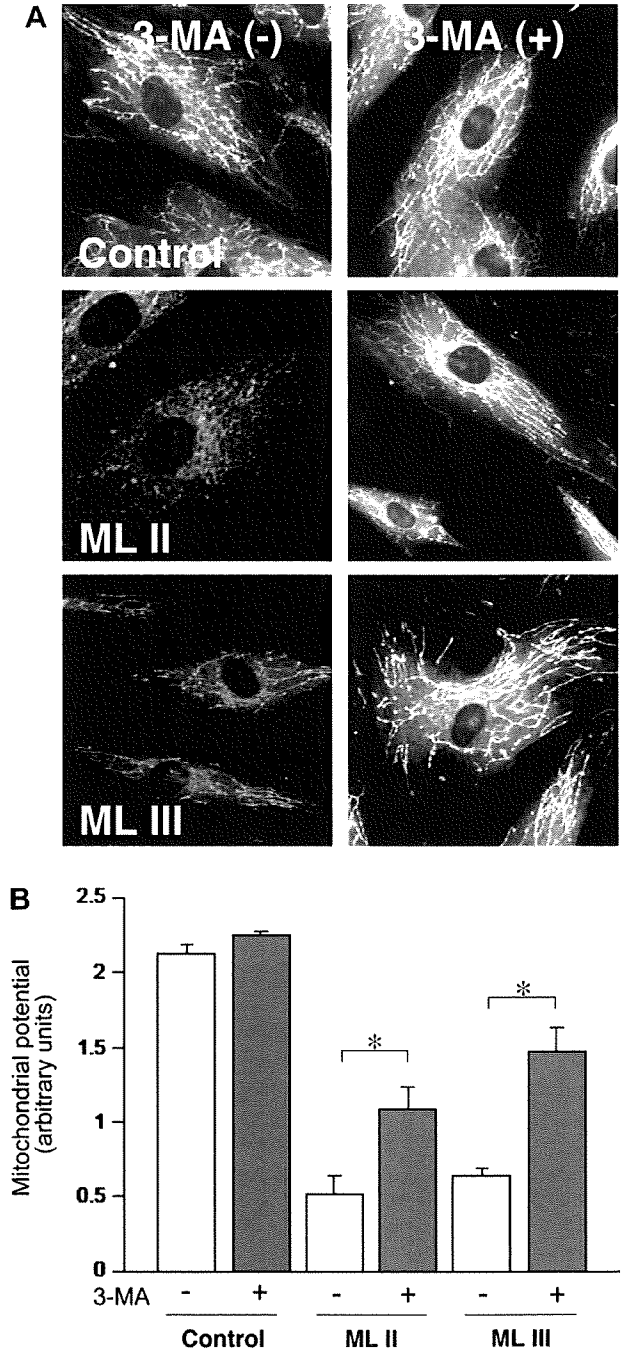

Fig. 4. Restoration of mitochondrial activity by autophagosome inhibitor. Cells were cultured with or without 5 mM 3-MA for 16 h and stained using MitoTracker. DMSO was used as a vehicle (A) MitoTracker staining. (B) The ratio of green and red fluorescence was determined from 10 independent images each of JC-1 labeled-cells. Values are means ± SEM by paired *t*-test. $^{*}p < 0.05$.

(Fig. 5A). The activity of cathepsin B clearly decreased in ML II and III cells, compared to the control (Fig. 5B). Inhibition of autophagy by 3-MA had no effects on subcellular localization of cathepsin B and D proteins (data not shown) and the activity of cathepsin B.

4. Discussion

In this study, accumulation of autolysosomes was observed in ML II and III skin fibroblasts using immunoblotting analysis and immunostaining with anti-LC3. Colocalization studies with LC3, LysoTracker, and Lamp-2 showed that fusion of autophagosomes with late endosomes/lysosomes was not blocked in ML cells. In addition, the accumulation of p62 and ubiquitinated proteins in ML cells suggested a decreased ability to degrade endogenous substrates for autophagy. These findings indicated the impairment of the clearance of autolysosomes in ML II and III skin fibroblasts. Autophagic impairments have been reported in other lysosomal storage disorders [16]. However, the induction of autophagy (marked by beclin-1 activation) differs between these diseases. Elevation of beclin-1 expression has been observed in several cholesterol and sphingolipid storage diseases [17] but not in ML II and III skin fibroblasts. Beclin-1 is thought to be a positive regulator of the autophagic pathway and it has been shown recently to have multiple functions by forming three different complexes with Vps34 [18]. ML II and III skin fibroblasts have many inclusion bodies filled with undegraded substrates, and these contents have been partially characterized [19]. It is possible that these storage materials complicatedly involves in the downstream pathways of autophagy.

Autophagy is a degradative pathway with major roles in the quality control of bulk cytosolic organelles at steady state [8]. In the present study, the numbers of enlarged vesicles, regarded as autolysosomes, increased remarkably in affected cells. Mitochondrial fragmentation and loss of membrane potential was observed in ML cells, and mitochondrial structure seemed to be excluded especially in autolysosome rich regions from the morphological results. There was a possibility that mitochondria are directly impaired by increased autophagosome formation, because inhibiting the formation of autophagosomes by treating with 3-MA for 16 h lead to the recovery of mitochondrial structure and membrane potential. Mitochondrial impairment in lysosomal storage diseases is considered as a secondary accumulation of

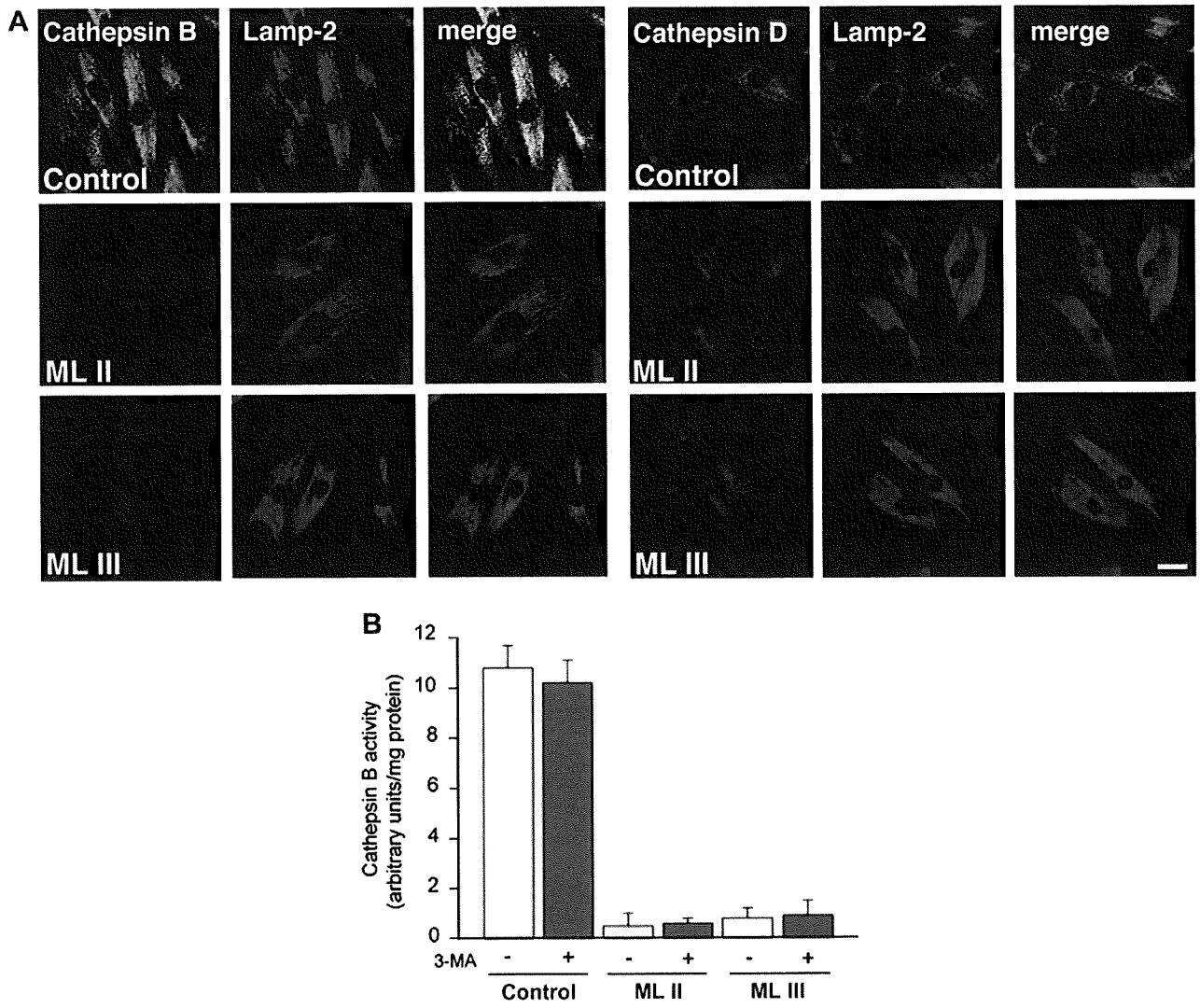

Fig. 5. Subcellar localization and activities of cathepsin B and D. (A) Immunofluorescence of cellular distribution of cathepsin B and D with Lamp-2. Bar = 10 μm. (B) Enzyme activity for cathepsin B. Values are means ± SEM by paired *t*-test.

abnormal mitochondria caused by the defective autophagic degradation pathways [16]. It is suggested that temporary mitochondrial recovery by blocking autophagy finally results in mitochondrial dysfunction over long periods through the secondary accumulation of abnormal mitochondria followed by cell death.

Cathepsin B and D are the main lysosomal aspartic proteases, which are translocated from the Golgi complex to the late endosomes and lysosomes via M6P-dependent manner [20]. In this study we showed defective cathepsin B and D in ML fibroblasts. Previous studies have reported that autophagy was involved in the pathogeneis of the mouse model of neuronal ceroid lipofuscinoses, which is caused by the mutation in cathepsin D or B/L gene [21]. We propose that the mechanism leading to autophagosome accumulation in ML fibroblasts may at least in part share the common pathway in neuronal ceroid lipofuscinoses. It is also indicated that the 3-MA effect to mitochondria is not derived from the restored cathepsins' activities nor normalization of targeting of cathepsins to lysosomes.

Mitochondrial dysfunction is associated with neurodegenerative and neuromuscular diseases [22,23]. According to these pathological conditions, mitochondrial dysfunction ultimately leads to apoptosis. However, cytochrome-c oxidase deficiency, aberrant mTOR signaling, or active cell death were not detected in steady state cultures of ML cells (data not shown), probably because cultured fibroblasts can dilute accumulating cytosolic contents by cell division. On the other hand, ML skin fibroblasts show very low viability against freezing stock. There is a possibility that mitochondrial function is partially compensated for by regular cell proliferation. Further studies are essential to examine the physiological relevance of these results in ML.

Furthermore, it was also found that the elevated levels of LC3, p62, and ubiquitinated proteins correlated with the clinical findings, though lysosomal enzyme activities or phosphotransferase activities did not correlate with the clinical phenotypes [24]. Recently, enzyme replacement therapy and bone marrow transplantation have been developed as possible therapies for lysosomal storage disorders [25,26]. The present findings may provide significant new insights regarding cellular phenotype and clinical phenotype correlations.

In conclusion, the present provides the first characterization of autophagic impairments accompanied by mitochondrial alterations in cultured ML II and III skin fibroblasts and these impairments were temporarily rescued by blocking autophagy. These findings raise the possibility of exploring new therapeutic options by modulating of inclusion body formation and autophagic impairments in ML II and ML III patients.

Acknowledgments

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A Validation Exercise on the New Consensus Criteria for Multiple System Atrophy

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Abstract: The revised (new) consensus clinical diagnostic criteria for multiple system atrophy (MSA) were published in 2008. To validate these criteria, we utilized the same cohort that we reported previously, which included 59 patients with a clinical diagnosis of MSA that was confirmed neuropathologically in 51 of them at the Queen Square Brain Bank for Neurological Disorders. At the *first* clinic visit, sensitivity with new consensus *possible* category was higher, and PPV marginally higher, than for clinical diagnosis and old consensus possible category. New consensus *probable* category showed marginally higher sensitivity than, and the same PPV as, old consensus probable category. At the *last* clinic visit, new consensus possible category had exactly the same sensitivity and only marginally higher PPV compared with old consensus possible category. New consensus probable category showed the same sensitivity and PPV as old consensus probable category. Our data indicate that in this case material the new consensus criteria for possible MSA could improve diagnostic accuracy at first neurological evaluation compared with the old consensus criteria. Prospective clinicopathological validation studies of the new consensus criteria, particularly incorporating in vivo structural and functional imaging results, are required to extend the current findings. © 2009 Movement Disorder Society

Key words: multiple system atrophy; diagnostic criteria; parkinsonism; cerebellar ataxia; autonomic failure

Multiple system atrophy (MSA) is a progressive sporadic neurodegenerative disease of undetermined etiology that causes parkinsonism and cerebellar, autonomic, and pyramidal dysfunction in varying combinations.[1] Neuropathologically, MSA is characterized by neuronal cell loss, astrogliosis, and oligodendroglial cytoplasmic inclusions in the striatonigral and olivopontocerebellar systems as well as the spinal cord (intermediolateral cell column and Onuf's nucleus).[2]

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The accuracy of clinical diagnosis of MSA has been suboptimal. In particular, MSA is commonly mistaken for PD owing to a number of overlapping features including asymmetry, and resting tremor and a positive levodopa (L-dopa) response in some patients. We previously reported a clinicopathological study on 59 cases with a clinical diagnosis of MSA when last assessed prior to death.[3] In 51 of these cases, the diagnosis of MSA was confirmed pathologically, and among the remaining eight, the most frequent false positive misdiagnosis was PD.[3] Application of either Quinn and coworkers[4] or old consensus criteria[5] was superior to actual clinical diagnosis made early in the disease; however, there was little difference at *last* clinic visit.[3]

In 2007, a second consensus meeting proposed new revised criteria, which were published in 2008.[6] The new criteria for *possible* MSA require either ataxia

or parkinsonism plus at least one feature suggesting autonomic failure *plus* one of either a number of clinical "red flags"[7] (including new ones—Babinski sign with hyperreflexia, stridor, rapidly progressive parkinsonism, postural instability within 3 years, or dysphagia within 5 years of motor onset) or for the first time also paraclinical investigation results—atrophy on MRI of putamen, middle cerebellar peduncle (MCP), pons or cerebellum (for MSA-P), or of putamen, MCP or pons (for MSA-C); putaminal (for MSA-C) and putaminal, midbrain, or cerebellar (for MSA-P) hypometabolism on FDG-PET scan; or presynaptic nigrostriatal dopaminergic denervation (for MSA-C) on SPECT or PET. In contrast, the criteria for *probable* MSA were kept essentially unchanged.

We have therefore assessed, in the same clinicopathological case series as previously studied,[3] whether the new consensus criteria improve clinical diagnostic accuracy.

PATIENTS AND METHODS

Among 806 patients with parkinsonism whose brains were donated to the Queen Square Brain Bank for Neurological Disorders between 1984 and 2000, we identified 59 consecutive cases with a clinical diagnosis of MSA when last assessed prior to death.[3] Clinical data abstracted from the patients' medical records included autonomic, cerebellar, pyramidal, and parkinsonian symptoms and signs. These enabled individual patients to be classified by old[5] and new[6] consensus criteria. Response to L-dopa was graded on a four-point scale, and initial as well as last recorded response was noted. The occurrence of symptoms and signs was determined at both the first and last visit to a neurologist. One of the authors (Y.O.) coded each feature mentioned in the case notes by the neurologist who was unaware of the neuropathological findings. If there was no mention of a feature, the feature was coded as absent.

Clinical and pathologic data from patient subgroups were compared using Student's *t*-test. The proportion of cases with each clinical feature was compared between true MSA and false positive cases using the χ^2 test for proportions for a two-by-two contingency table. To assess validity of each clinical feature as well as the diagnostic criteria, we chose sensitivity and positive predictive value (PPV) as our outcome measures. We report the results for both categories of diagnosis (probable or possible) for the two sets of consensus criteria. We do not report specificity as it is statistically imprecise owing to the small number of false positive cases. To compare the relative performance of different criteria, we calculated *P*-values using an exact McNemar's test and difference in proportions with 95% confidence intervals using the method described by Fleiss et al.[8]

RESULTS

The 59 MSA cases comprised 37 men and 22 women. Other details have been described elsewhere.[3] In summary, 51 cases were confirmed pathologically, resulting in a PPV for the last clinical diagnosis of 86%. The remaining eight cases (14%) had a false positive clinical diagnosis of MSA. Six of them had Lewy body PD with variable degrees of cortical Lewy body involvement, one case had PSP, and one cerebrovascular disease.

Table 1 compares the sensitivity and PPV of the old and new consensus criteria at both *first* and *last* clinic visits. The sensitivity and PPV of the clinical diagnosis made in the patients are also presented for comparison. Details of the L-dopa response at first visit were lacking in 16 cases. To avoid a loss in statistical power, the patients' first ever record of L-dopa response (on average 15 months, range 3–48, after the first visit) after the first visit was used. At the first clinic visit, sensitivity with new consensus possible category (41%) was higher than for clinical diagnosis (22%) and old consensus possible category (28%)—(difference in proportions 14%, 95% CI −2 to 29%, $P = 0.09$), but PPV was only marginally higher (95% vs. 92% and 93%). New consensus probable category showed marginally higher sensitivity (18%) than, and the same PPV (100%) as, old consensus probable category.

The upper half of Table 2 shows the sensitivity and PPV of the individual core signs and symptoms included in the new consensus criteria at first clinic

TABLE 1. *Sensitivity and PPV for the new and the old consensus criteria (n = 59:51/8 cases)*

	Sensitivity	95% CI	PPV	95% CI
First clinic visit				
Initial clinical diagnosis	22	13–35	92	65–99
Old consensus possible	28	17–41	93	70–99
New consensus possible	41	29–55	95	78–99
Old consensus probable	16	8–28	100	68–100
New consensus probable	18	10–30	100	70–100
Last clinic visit				
Last clinical diagnosis	100	93–100	86	75–94
Old consensus possible	92	82–97	86	74–92
New consensus possible	92	82–97	89	77–95
Old consensus probable	63	49–75	91	78–97
New consensus probable	63	49–75	91	78–97

Values are given as percentages.

TABLE 2. *Sensitivity and PPV for signs included in the old and the new consensus criteria (n = 59:51/8 cases)*

	Sensitivity	95% CI	PPV	95% CI
First clinic visit				
Initial clinical diagnosis	22	13–35	92	65–99
Old consensus autonomic and urinary—criterion	24	14–37	86	60–96
Old consensus autonomic and urinary—feature	35	24–49	90	70–97
Old consensus parkinsonism—criterion	78	65–88	85	72–93
Old consensus parkinsonism—feature	78	65–88	85	72–93
Old consensus poorly levodopa responsive parkinsonism	55	41–68	82	67–92
Old consensus cerebellar—criterion	14	7–26	100	65–100
Old consensus cerebellar—feature	28	17–41	100	79–100
Old consensus pyramidal dysfunction/signs	16	8–28	89	57–98
New consensus autonomic failure—probable	25	16–39	87	62–96
New consensus autonomic failure—possible	59	45–71	94	80–98
New consensus parkinsonism	78	65–88	85	72–93
New consensus poorly levodopa responsive parkinsonism	55	41–68	82	67–92
New consensus cerebellar syndrome	14	7–26	100	65–100
Last clinic visit				
Last clinical diagnosis	100	93–100	86	75–94
Old consensus autonomic and urinary—criterion	73	59–83	90	78–96
Old consensus autonomic and urinary—feature	86	74–93	88	76–94
Old consensus parkinsonism—criterion	92	82–97	85	74–92
Old consensus parkinsonism—feature	92	82–97	85	74–92
Old consensus poorly levodopa responsive parkinsonism	90	79–96	87	75–94
Old consensus cerebellar—criterion	39	27–53	100	84–100
Old consensus cerebellar—feature	71	57–81	90	77–96
Old consensus pyramidal dysfunction/signs	33	22–47	81	62–92
New consensus autonomic failure—probable	67	53–78	89	76–96
New consensus autonomic failure—possible	94	84–98	89	78–95
New consensus parkinsonism	92	82–97	85	74–92
New consensus poorly levodopa responsive parkinsonism	90	79–96	85	73–92
New consensus cerebellar syndrome	53	40–66	90	75–97

Values are given as percentages.

visit. The new consensus possible criteria for autonomic failure had higher sensitivity (59%) and PPV (94%) than the old consensus autonomic and urinary feature (35% and 90%)—(difference in proportions for sensitivity 24%, 95% CI 8–39%, $P = 0.004$), whereas the new consensus probable, compared with old consensus probable, criteria for autonomic failure (25 and 87%) did not. In terms of parkinsonism and cerebellar syndrome, there was no difference at all. The lower half of Table 2 shows the sensitivity and PPV of these signs and symptoms at last clinic visit. Although the new consensus probable criteria for autonomic failure did not improve sensitivity or PPV over the old consensus criteria, the new consensus possible criteria for autonomic failure slightly improved sensitivity and PPV (94 and 89%) over the old consensus autonomic and urinary feature (86 and 88%). In terms of parkinsonism, there was no change. The new consensus criteria for a cerebellar syndrome showed higher sensitivity (53%) but lower PPV (90%) than the old consensus cerebellar criteria (39 and 100%)—(difference in proportions for sensitivity 14%, 95% CI 2–25%, $P = 0.02$), though it was less sensitive than the old consensus cerebellar feature (71%, differences in proportions −18%, 95% CI −3 to −33%, $P = 0.01$)

We have set out the sensitivity and PPV for additional features of possible MSA at first clinic visit in Table 3.

DISCUSSION

We attempted to compare the validity of the old and the new revised consensus criteria for MSA in a large neuropathologically examined series of cases.

The new consensus criteria[6] incorporate several refinements for the diagnosis of possible MSA. They include autonomic disturbance as a mandatory feature for both probable or possible categories. In terms of definition of probable MSA, only one slight change has been made, in the cerebellar domain. On the other

TABLE 3. *Sensitivity and PPV for additional features of possible MSA at first clinic visit (n = 59:51/8 cases)*

First clinic visit	Sensitivity	95%CI	PPV	95%CI
Initial clinical diagnosis	22	13–35	92	65–99
Babinski sign with hyperreflexia	16	8–28	89	57–98
Stridor	4	1–13	100	34–100
Poor response to levodopa	51	38–64	81	65–91
Postural instability within 3 years after motor onset	27	17–41	100	79–100
Gait ataxia, cerebellar dysarthria, limb ataxia, or cerebellar oculomotor dysfunction	27	17–41	100	79–100
Dysphagia within 5 years of motor onset	6	2–16	100	44–100
Atrophy on MRI of putamen, middle cerebellar peduncle, pons, or cerebellum	0	nc	nc	nc
Hypometabolism on FDG-PET scan	No case			
Presynaptic nigrostriatal dopaminergic denervation on SPECT or PET	No case			
At least one of the additional features for possible MSA-P	67	53–78	85	71–93
At least one of the additional features for possible MSA-C	76	63–86	85	72–92

Values are given as percentages; nc, not calculated.

hand, for possible MSA, the new criteria employ two domains (ataxia or parkinsonism) associated with one or more features suggestive of autonomic dysfunction, plus one of many additional features.

At first visit, new consensus possible category had higher sensitivity (41%) than old consensus possible (28%), old or new consensus probable categories (16% and 18%), and initial clinical diagnosis (22%). New consensus probable category showed similar sensitivity over old consensus probable category (18% vs. 16%). At last visit, new consensus possible category showed the same sensitivity (92%), and very similar PPV (89%) compared with old consensus possible category (86%) or last clinical diagnosis (also 86%). New consensus probable category presented no change at all.

These data suggest that the new consensus criteria for possible MSA improve the diagnostic accuracy at first presentation to the neurologist, though due to the relatively small sample size we cannot exclude the possibility that this difference may have occurred by chance. Since the new criteria for possible MSA have been simplified, hopefully, they will be used more in routine clinical practice, thus enabling earlier recognition of patients. In contrast, the criteria for probable MSA have essentially remained the same, so their diagnostic accuracy has not changed.

As an individual feature, possible "autonomic failure" improved both sensitivity (59%) and PPV (94%) at first clinic visit. By last visit, its sensitivity (94%) was still almost as high, and its PPV (89%) higher than, last clinical diagnosis (100% and 86%). On the other hand, "cerebellar syndrome" in the new consensus criteria showed low sensitivity (14%) but high PPV (100%) at first visit, identical to the old consensus criterion. However, by last clinic visit, it was superior to the old cerebellar criterion but not as good as cerebellar feature in terms of sensitivity. Therefore, the value of the modified item "cerebellar syndrome" is dependent on whether patients are assessed early or later in the natural history of the disease.

With regard to additional features of possible MSA, postural instability within 3 years of motor onset had low sensitivity (27%), but optimal PPV (100%) at first clinic visit (Table 3). This feature and others such as stridor, dysphagia within 5 years of motor onset, and cerebellar signs (gait ataxia, cerebellar dysarthria, limb ataxia, or cerebellar oculomotor dysfunction) showed higher PPV than clinical diagnosis at first visit. Importantly, these clinical features were very similar to the ones identified by the EMSA study group as "red flag" categories.[7]

Although several developments and refinements have been introduced in the new consensus criteria, testing them against the same MSA patient group as previously reported showed limited improvement in diagnosing true MSA cases. Several factors may account for this, including limitations on the material and design of our study. Thus, our study design was retrospective as well as noncontemporaneous and some of the novel clinical features added to the criteria may have therefore been missed during the documentation in life by the patients' physicians of neurological history and examination. Furthermore, and importantly, the spectrum of neuroimaging studies undergone was variable: thus not all patients had received high-magnetic field MRI, and none had undergone

functional imaging studies during life. Although our methodology potentially introduces some interpretation bias, the data used in our previous study[3] of the old consensus criteria are identical to those used in this comparison. A prospective clinicopathological study that includes patients with mild or atypical presentations and that utilizes the additional clinical features and modern neuroimaging in the new criteria should hopefully yield substantially higher sensitivities at first neurological visit.

In conclusion, our study demonstrates that utilizing available clinical data the new consensus criteria for possible MSA improve clinical diagnostic accuracy compared with the old consensus criteria. These findings require confirmation in a prospective clinicopathological study.

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Cross-Sectional and Longitudinal Studies of Three-Dimensional Stereotactic Surface Projection SPECT Analysis in Parkinson's Disease

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Abstract: Although dementia is increasingly recognized as a common feature in Parkinson's disease (PD), its pathological substrate remains unknown. We conducted cross-sectional and longitudinal brain perfusion SPECT analyses to explore changes during the course of developing dementia in PD. Fifty-five patients originally diagnosed with PD were imaged in the cross-sectional study. Twenty-one of these, nine without dementia and 12 with dementia (PDD), were included in the longitudinal study to observe perfusion changes during the course of their disease. Data were analyzed using three-dimensional stereotactic surface projection SPECT analysis. The UK Parkinson's Disease Society Brain Bank criteria were used to diagnose PD and the revised criteria for the clinical diagnosis of dementia with Lewy bodies for PDD. The cross-sectional study showed that patients with PDD had significantly reduced perfusion in the right posterior cingulate, the right precuneus and the left posterior cingulate area. In the longitudinal study, significantly reduced perfusion was observed in the left anterior frontal gyrus in PD without dementia, and in the right inferior parietal lobule in those that developed PDD. We suggest that a relationship exists between developing dementia in PDD and reduced perfusion in the posterior parietal area. © 2009 Movement Disorder Society

Key words: Parkinson's disease; Parkinson's disease with dementia; SPECT

INTRODUCTION

Parkinson's disease (PD) is a chronic progressive disorder for which there is no cure or proven strategy for slowing the progression of the disease. Dementia, depression, and disability affect quality of life in these patients.[1] In one recent study, more than three quarters of a PD cohort developed dementia during an 8-year follow up period.[2] Another study showed an almost six-fold increased risk for dementia in PD patients, compared with subjects without PD.[3] In many cases, the clinical picture of dementia resembles dementia with Lewy bodies (DLB), with cognitive decline being associated with visual hallucinations (VH) and fluctuations in cognitive performances.[4] The term PD dementia (PDD) should be used to describe dementia that occurs only in the context of well-established PD.[5] In research settings in which a distinction is made between DLB and PDD, the 1-year rule should be used.[5] Recently, not only clinical diagnostic criteria for dementia associated with PD,[6] but also the diagnostic procedure for PDD[7] were published.

Using three-dimensional stereotactic surface projection (3D-SSP) single proton emission computed tomography (SPECT) analysis, we previously observed widespread hypoperfusion areas including the bilateral temporal bases, frontal bases, medial parietal lobes, parietal association areas, and visual cortices in PD patients without dementia.[8] Furthermore, hypoperfusion in the bilateral posterior cingulate areas exhibited a significant correlation with the presence of dementia.[8] Since then, the number of patients included in this

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study increased. In the present study, we first aimed to further investigate cross-sectional differences in brain perfusion in patients who had not developed dementia (that is PD without dementia) and in those who later developed dementia (that is PDD). Second, we aimed to apply the same 3D-SSP analysis to longitudinally investigate brain perfusion changes in the same individual during the disease course of PD.

SUBJECTS AND METHODS

Subjects and Clinical Assessments

This study is a part of an ongoing SPECT study with currently 55 patients, originally diagnosed as PD in the Department of Geriatrics, Cardiology and Neurology at Kochi Medical School. Subjects underwent detailed clinical history taking, physical, neurological, and neuropsychiatric examinations, and a standard blood screen for thyroid function, thiamine, B12, folate, and syphilis was conducted for demented patients. Further clinical investigations, including routine EEG, MRI, and ^{123}I-metaiodobenzylguanidine myocardial scintigraphy were performed for all patients, and if necessary, the head-up tilt test or cystometry were also conducted. Patients with either clinical or radiological evidence of stroke were excluded from the study.

Patients were followed up either monthly or bimonthly by specialized neurologists (YO and YM). Prospective standardized assessments for parkinsonism, presence of dementia, fluctuating cognition (FC), and repeated VH were conducted. Standardized schedules administered included the Mini-Mental State Examination, the revised-Hasegawa Dementia Scale, and the Unified Parkinson's Disease Rating Scale part III (UPDRS).[9] The UPDRS assessments were conducted during both the drug "ON" and "OFF" medication periods. Two of the PDD patients were unable to complete the neuropsychiatric examinations due to severe Parkinsonism and dementia. Diagnoses were made by consensus using the UK Parkinson's Disease Society Brain Bank criteria for PD[10] and the revised criteria for the clinical diagnosis of DLB for PDD.[5] The overall dementia diagnosis was made when a patient's cognitive decline was of a sufficient magnitude to interfere with normal social or occupational function,[5] for at least several months. Fluctuating cognition was considered positive if either the doctor or caregiver confirmed that the patient had demonstrated clear fluctuations in condition, for example, from the patient being mute or confused and unable to stand without assistance, to then being able to hold a normal conversation.[11]

TABLE 1. *Characteristics of patients in the cross-sectional study*

Characteristic	PD without D (n = 32)	PDD (n = 23)	*P* value
Age at onset (yr)	60.8 (11.4)	63.1 (7.9)	ns
Sex (male)	15	14	ns
Disease duration (yr)	9.2 (5.4)	9.6 (5.8)	ns
LED (mg)	361 (174)	355 (196)	ns
UPDRS-OFF	37 (13)	36 (15)[a]	ns
UPDRS-ON	23 (12)	27 (12)[a]	ns
HDSR	26 (4)	18 (7)	<0.001
MMSE	25 (5)	18 (7)	<0.001

Values in parenthesis are SD.

LED, levodopa equivalent dose; UPDRS, Unified Parkinson's Disease Rating Scale, MMSE, Mini-Mental State Examination; HDSR, Revised-Hasegawa Dementia Scale.

[a]Two of the 23 patients did not complete the UPDRS assessment.

The first, cross-sectional part of the study consisted of 55 patients whose original diagnosis was PD. Among them, 32 patients did not have dementia and 23 did, at the time of the SPECT scans. Table 1 shows the characteristics of the two groups. Patients in the PDD group had a diagnosis of PD for 9.6 ± 5.8 years prior to the diagnosis of PDD, and underwent the first SPECT scans an average of 0.4 ± 1.0 years later.

The second, longitudinal part of the study consisted of those 21 of the 55 patients who had undergone two SPECT scans at intervals during the disease course of PD. Table 2 shows the characteristics of this group. All of these patients had a diagnosis of PD (without dementia) when they underwent the first SPECT scans at baseline. Nine of them underwent a second SPECT scan (44 ± 29 months following the first) under the same diagnosis, with a disease duration of 12.2 ± 7.1 years. The other 12 patients developed dementia (i.e., PDD) 9.9 ± 7.3 years after the diagnosis of PD. They underwent a second SPECT scan 0.1 ± 0.4 years after the diagnosis of PDD, 43 ± 28 months following the first scan. All patients with PDD experienced repeated VH when the dopaminergic agents were titrated, and seven of the 12 PDD patients showed marked FC. In terms of medication, one was under low dose quetiapine and three were under donepezil. The other 34 patients were not entered into the longitudinal study; these included 11 patients who were already diagnosed with PDD at the first SPECT scans, and 23 PD without dementia patients who had not undergone a second SPECT scan within the given time period (age at onset of 63.5 ± 10.0 years old; disease duration of 8.0 ± 4.2 years).

TABLE 2. *Characteristics of patients in the longitudinal study*

Characteristics	No dementia (n = 9)	Developed dementia (n = 12)	*P* value
Age at onset (yr)	53.9 (12.5)	61.6 (7.8)	ns
Sex (male)	5	7	ns
Disease duration (yr)	12.2 (7.1)	9.9 (7.3)	ns
LED (mg)	452 (191)	319 (152)	ns
UPDRS-OFF	40 (13)	35 (16)[a]	ns
UPDRS-ON	20 (12)	29 (13)[a]	ns
HDSR	27 (2)	17 (8)	<0.01
MMSE	28 (2)	19 (8)	<0.01
Scan interval	44 (29)	43 (28)	ns

Values in parenthesis are SD.

LED, levodopa equivalent dose; UPDRS, Unified Parkinson's Disease Rating Scale, MMSE, Mini-Mental State Examination; HDSR, Revised-Hasegawa Dementia Scale.

[a]Two of the 12 patients did not complete the UPDRS assessment.

SPECT Data Acquisition and Image Analysis by 3D-SSP

SPECT data acquisition, 3D-SSP image analyses, the control database were as previously described.[8,12–14] Briefly, the subjects were imaged with a rotating triple-head gamma camera (TOSHIBA GCA 9300A/HG) fitted with a high-resolution fan-beam collimator, which permits a spatial resolution of 8.0-mm full width at half maximum. The imaging was started 10 minutes after intravenous injection of 111MBq (3mCi) of *N*-isopropyl-*p*-[123I]iodoamphetamine. No medication was stopped for scanning. In this article, pixel values of each individual image set were normalized to the global brain pixel value before the analysis as follows: normalized pixel value = (individual pixel value)/(individual global brain pixel mean value). The normalized activity of each patient was compared with the reference control database by means of a *Z* score. A *Z* score was calculated for each surface pixel: *Z* score = ([normal mean] − [individual mean]/[normal SD]). To assess the difference in regional patterns of rCBF between subgroups, two-sample *t*-test were performed on a pixel-by-pixel basis and then transformed to *Z* values by a probability integral transformation. Finally, subtraction scans were processed.

Statistical Analyses

SPECT data from 32 PD without dementia patients and 23 PDD patients were processed with NEUROSTAT. Unpaired *t*-tests were conducted using the 2tZ iSSP software, providing 3D-SSP image analyses from the eight projections.[12–14] These 3D-SSP images were thresholded at $P < 0.001$ ($Z > 3.1$). In the longitudinal study, two consecutive SPECT data from each of the 21 patients were processed with NEUROSTAT and paired *t*-tests were conducted using the 1tZ iSSP software. This software provides not only 3D-SSP image analyses from the eight projections, but also 3D-SSP maps with axial, coronal and sagittal views. These 3D-SSP maps were thresholded at $P < 0.001$ uncorrected in Figure 2. Regions were reported as significant if they had $P < 0.05$ corrected for multiple comparisons at cluster level. The Talairach Daemon was used to assign anatomical labels to voxel coordinates.[15] Descriptive statistics are presented as means plus-minus SD. We used JMP 6.0.3 for all statistical calculations on nonimage data.

RESULTS

Cross-Sectional Study

Figure 1 shows the comparison of brain perfusion data from PD without dementia patients and those from PDD patients. The PDD group had significantly reduced perfusion in the right posterior cingulate, the right precuneus and the left posterior cingulate area.

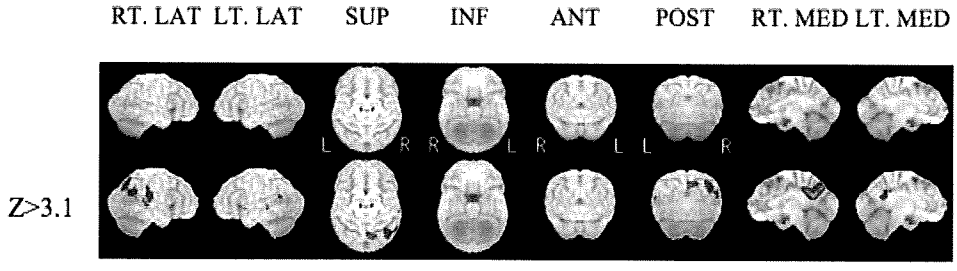

FIG. 1. Areas of reduced perfusion in Parkinson's disease with dementia patients compared to those without dementia in the cross-sectional study. Results of three-dimensional stereotactic surface projection SPECT analysis, where areas of significantly reduced perfusion are shown in pseudocolors. Reduced perfusion areas were found in the right posterior cingulate, the right precuneus, and the left posterior cingulate area. *Z* values and the eight projections are provided.

TABLE 3. *Brain regions showing significantly reduced cerebral blood flow changes in the longitudinal study*

Brain regions	BA	Talairach coordinates x	y	z	Peak Z-score
Patients who did not develop dementia (n = 9)					
L anterior frontal gyrus	25	6	21	2	4.85[a]
R posterior cingulate gyrus	31	−12	−33	29	4.28
L precuneus	7	12	−46	−47	4.23
L middle frontal gyrus	10	30	59	11	4.09
Patients who later developed dementia (n = 12)					
R inferior parietal lobule	40	−33	−46	40	4.45[a]
L posterior cingulate gyrus	31	19	−31	29	4.05

BA, Brodmann area.
[a]$P < 0.05$ corrected for multiple comparisons at cluster level.

Longitudinal Study

Table 3 shows the areas of significantly reduced perfusion during the course of PD, and Figure 2 is a 3D-SSP map corresponding to the data in the table. The nine PD patients who did not have dementia at the second SPECT scans (i.e., PD without dementia) had significantly reduced perfusion in the left anterior frontal gyrus (BA25, Fig. 2A). The 12 PD patients who had developed dementia by the time of the second SPECT scans (i.e., PDD) exhibited significantly reduced perfusion in the right inferior parietal lobule (BA 40, Fig. 2B). There was no significant difference in the UPDRS scores between the two groups.

DISCUSSION

In the cross-sectional part of this study, we confirmed our previous results in an increased number of patients, while in the longitudinal part of the study, we observed significantly reduced perfusion in the same patients over time. In PD without dementia patients, significantly reduced perfusion was observed in frontal areas, whereas in PDD patients, significantly reduced perfusion was observed in parietal areas.

Although this is the first study to show a differential progression of hypoperfusion in PD without dementia and in PDD, our study could be biased by its relatively small sample size. Given the nature of the longitudinal study using paired *t*-tests, we did not perform a direct subtraction comparison between them. Not only disease progression rates but also the development of dementia varies from one case to another. The age of disease onset can influence both of these parameters.[16] In concordance with this observation, of the patients in the longitudinal study, although there was no statistical difference, the PD without dementia group tended to have a lower age of onset and a longer disease duration than the PDD group. Other limitations may be that no detailed neuropsychological examinations were conducted, and may be that not all PD without dementia patients took a second SPECT scan, as would have been the case in a fully organized study.

Firbank et al. reported the results of two longitudinal cerebral blood flow SPECT studies conducted over a one-year period in PD[17] and in PDD and DLB patients.[18] In PD, there was a significant decrease in perfusion with clusters in the frontal lobe,[17] whereas there was no reduction in perfusion in either PDD or DLB.[18] In a cross-sectional study, Firbank et al. also reported that the precuneus and the inferior parietal regions showed perfusion deficits in PDD compared with the healthy controls, similar to the pattern observed in DLB.[19] On the other hand, in the current study, we compared two consecutive SPECT data sets, with a longer observation interval focusing on the time during which patients developed dementia.

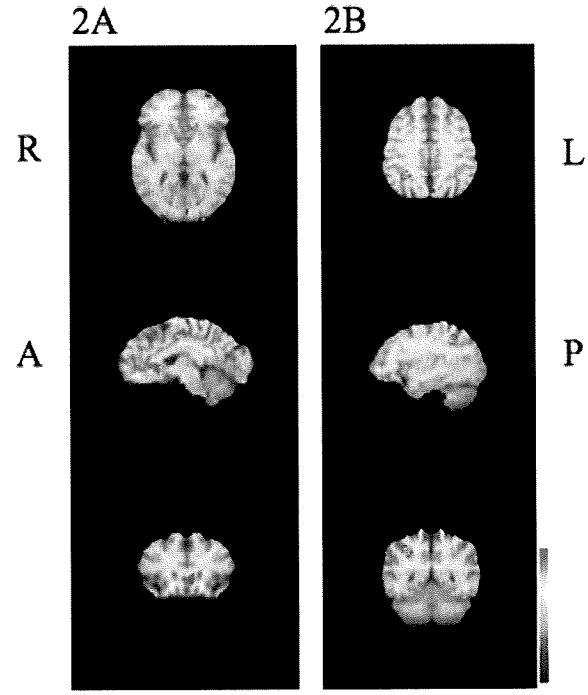

FIG. 2. Areas of reduced perfusion during the course of Parkinson's disease. Results of three-dimensional stereotactic surface projection SPECT maps, where areas of significantly reduced perfusion during the disease course are shown in pseudocolors. Axial (*top*), sagittal (*middle*), and coronal (*bottom*) views are shown. (**A**) Parkinson's disease without dementia patients (n = 9). A reduced perfusion area was observed in the left anterior cingulate gyrus (BA 25, Z: 4.85). (**B**) Parkinson's disease with dementia patients (n = 12). A reduced perfusion area was observed in the right inferior parietal lobule (BA 40, Z: 4.45). R: right, L: left, A: anterior, P: posterior.

In voxel-based morphometry (VBM) studies, Burton et al. reported that PD patients have significant reductions in grey matter in the frontal and temporal lobes compared to normal subjects, that there was significant grey matter atrophy bilaterally in the occipital lobe of PDD patients compared with PD patients, and that the pattern of grey matter volume loss in PDD resembles that observed in DLB.[20] Beyer et al. observed more pronounced cortical atrophy in DLB than in PDD in the temporal, parietal, and occipital lobes.[21] Hence, based on the perfusion SPECT and VBM studies, we hypothesize that loss of activity in the parietal area, for instance the inferior parietal lobule or the posterior cingulate gyrus, in addition to that in the frontal area, parallels the development of dementia in PDD.

Clinicopathological studies suggest that Lewy body-type pathology is more strongly associated with PDD than is Alzheimer's disease-type pathology.[22] Brain areas such as the inferior parietal lobule, the posterior cingulate gyrus, the precuneus or the medial frontal gyrus are included in corticocortical connections. Neurochemically, not only dopaminergic pathways, but also cholinergic[23] and monoaminergic pathways[24] are involved in cognitive impairment in PD.

Apparently, the onset of dementia in PDD is insidious.[6] Although the PDD patients in our study took more than 9 years to develop dementia, it is occasionally difficult to clearly define when a PD patient develops dementia. In this study, the overall diagnosis of dementia was made only after a patient's cognitive decline was evident for at least several months. Hence, we needed a longer observation interval than other studies.[17,18]

We applied the revised criteria for the diagnosis of DLB to diagnose PDD,[5] therefore, our results may not be fully generalized to the entire population of patients with PDD. Alternatively, DSM-IV criteria for dementia[25] could have been applied. However, this also might not capture all PDD patients as they are biased toward amnestic-cortical dementia. Current clinical diagnostic criteria for dementia associated with PD emphasize cognitive impairments in attention, executive and visuospatial functions, as well as in memory, with relatively preserved core language functions.[6]

In terms of statistical analysis, 3D-SSP in NEUROSTAT has several advantages. The normalized activity of each patient is compared with the reference control database by means of a *Z*-score. It enables one to visualize the medial surface of the brain, so hypoperfusion areas, such as the anterior and posterior cingulate gyri or medial frontal gyrus, are easily visualized. In addition, 3D-SSP maps can be observed in axial, sagittal and coronal planes with multiple slices.

In conclusion, we compared SPECT data sets from PD without dementia and PDD patients in both cross-sectional and longitudinal analyses. In the cross-sectional study, PDD patients showed reduced perfusion in the posterior parietal areas compared with PD without dementia patients. In the longitudinal study, significantly reduced perfusion was observed in the left anterior frontal gyrus in PD without dementia, and in the right inferior parietal lobule in those that developed PDD. From the result, we speculate that dementia in PDD may be related to loss of activity in corticocortical connections to the posterior parietal area.

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