Multiple system atrophy (MSA) is a sporadic neurodegenerative disease manifested clinically by progressive ataxia, parkinsonism, and autonomic dysfunction. Its cause is unknown, and there is no curative therapy. \( \alpha \)-synuclein is an important protein forming aggregations called glial cytoplasmic inclusions (GCIs) in oligodendroglia; these aggregations are considered important in MSA pathogenesis. Overexpression of the human \( \alpha \)-synuclein gene in mice induces the formation of GCI-like aggregations in oligodendrocytes, leading mice to exhibit neurological signs similar to those in MSA patients. However, previous studies have excluded mutations within the coding region of the \( \alpha \)-synuclein gene in MSA patients. To determine whether alteration in the expression level of the \( \alpha \)-synuclein gene is associated with MSA pathogenesis, we used TaqMan quantitative PCR assay to analyze the \( \alpha \)-synuclein gene copy number in patients’ genomes. We also used quantitative RT-PCR and \textit{in situ} hybridization to analyze \( \alpha \)-synuclein mRNA expression in MSA patients’ brain tissues. We found no alteration in the \( \alpha \)-synuclein gene copy number in the patients’ genomes (n = 50). Quantitative analysis for \( \alpha \)-synuclein mRNA by the TaqMan method showed that \( \alpha \)-synuclein mRNA levels were comparable between control (n = 3) and MSA (n = 3) cerebella. On \textit{in situ} hybridization, the number of neurons with \( \alpha \)-synuclein mRNA expression was no greater in the cerebella of MSA patients (n = 3) than in the controls (n = 3). However, GCIs were seen in these MSA specimens on immunohistochemistry for \( \alpha \)-synuclein. These results suggest that \( \alpha \)-synuclein gene expression is not the fundamental cause of MSA.

Keywords: \( \alpha \)-synuclein, multiple system atrophy (MSA), quantitative PCR, \textit{in situ} hybridization, brain

Introduction

Multiple system atrophy (MSA) is a devastating adult-onset neurodegenerative disorder classified as a sporadic form of spinocerebellar degeneration. According to a nationwide survey of degenerative ataxic disease coordinated by the Ministry of Health, Labour, and Welfare of Japan, the incidence of spinocerebellar degeneration in Japan is estimated at 20 per 100,000 people. MSA accounts for approximately 34% of all spinocerebellar degeneration and is the most common form.

Clinically, MSA is a unified category of three diseases formerly called olivopontocerebellar atrophy (OPCA), striatonigral degeneration (SND), and Shy-Drager syndrome (SDS).\(^1\) Accordingly, MSA patients clinically show varying degrees of progressive ataxia (due to OPCA), parkinsonism (due to SND), and autonomic dysfunction (due to SDS). The average age of onset of MSA is 55 years, and both men and women are
affected. There are no effective therapies. Disease usually progresses relentlessly, and progression of ataxia, parkinsonism, or orthostatic hypotension may finally make patients bedridden, with urinary dysfunction leading to difficulty in voiding. Death often ensues approximately 10 years after onset. Patients often succumb to systemic infections such as aspiration pneumonia or urinary tract infection leading to sepsis, or to choking due to vocal cord paralysis.

Although the precise cause of MSA has not been discovered, formation of argentophilic “glial cytoplasmic inclusions” (GCIs) in the oligodendroglia is the pathological hallmark of MSA. GCI is seen mainly in the white matter throughout the central nervous system. GCIs are more frequently seen in structures with relatively intense degeneration than in structures with a lesser degree of degeneration. Therefore, GCIs are seen more frequently in the cerebellum, pons, inferior olives, striatum, substantia nigra, and pyramidal tracts than in other parts of the brain. Besides GCIs, other abnormal aggregations may be seen in the cell body and the nucleus of neurons, although there are far fewer of these neuronal inclusions than GCIs. Among the many constituents of GCIs, \(\alpha\)-synuclein has been discovered as the most important protein.\(^5\) \(\alpha\)-synuclein-positive GCIs have been documented immunohistochemically in all MSA patients, including a patient with very early disease whose condition was discovered only at autopsy.\(^6\) Although a number of neurodegenerations show aggregation of mutated proteins, mutation has been found at least in the coding region of the \(\alpha\)-synuclein gene in MSA patients’ genomes.

Transgenic (\(Tg\)) mice overexpressing human wild-type \(\alpha\)-synuclein in oligodendrocytes under the control of the 2,3-cyclic nucleotide 3’-phosphodiesterase (CNP) promoter show features of MSA, including the accumulation of filamentous human \(\alpha\)-synuclein aggregates in oligodendrocytes and autophagocytosis of myelin.\(^5\) Therefore, it is possible that the copy number of the \(\alpha\)-synuclein gene may be altered in the genomes of MSA patients. Interestingly, an increase in gene copy number is associated with some neurodegenerative diseases. For example, the copy number of the “normal” amyloid precursor protein gene is increased in several families affected with Alzheimer’s disease.\(^7\) Duplication of the normal peripheral myelin protein 22 (\(PMP22\)) gene has also been shown to cause a form of peripheral neuropathy, Charcot-Marie-Tooth disease type 1A.\(^8\) In light of these observations, we attempted to determine whether the \(\alpha\)-synuclein gene copy number was altered in the genomes of MSA patients. In addition to performing this genomic analysis, we also used TaqMan real-time reverse-transcription — polymerase chain reaction (RT-PCR) and \(in\ situ\) hybridization to analyze whether \(\alpha\)-synuclein gene expression was increased in MSA patients’ brain tissues.

### Materials and Methods

1. Patients and Samples

For the genome copy number analysis we studied 50 MSA patients. These patients were all typical MSA patients clinically diagnosed in our department. As a control, one healthy volunteer with no neurological disorders was enrolled. The genomic DNA of this control subject was used to assess the standard copy number, and the copy number of exon 4 of the core-binding factor beta subunit (\(CBFB\)) gene was analyzed, as described later.

For the TaqMan real-time RT-PCR analysis, three controls and three MSA subjects were studied. The control patients had died of amyotrophic lateral sclerosis (n = 2) or a non-neurological disorder (n = 1). Three MSA and three control cerebella were used for \(in\ situ\) hybridization.

2. Analysis of copy number of \(\alpha\)-synuclein gene

After informed consent had been obtained, peripheral blood was drawn and genomic DNA was extracted from whole blood cells by standard phenol-chloroform extraction.\(^9\) Quantitative PCR of genomic DNA was carried out by the TaqMan expression chemistry protocol (Applied Biosystems, USA) (Figure 1). In humans the \(\alpha\)-synuclein gene lies on chromosome 4q21. The copy number of the \(\alpha\)-synuclein gene was assessed relative to the copy number of exon 4 of \(CBFB\), which lies on chromosome 16q22.1. The copy number of each selected exon of the \(\alpha\)-synuclein gene was assessed against that of exon 4 of the \(CBFB\) gene in each of the 50 MSA individuals and then averaged over all MSA individuals.

Primers and probes for all six exons of the \(\alpha\)-synuclein gene and the \(CBFB\) gene exon 4 were designed by the manufacturer (Applied Biosystems) (Table 1). For each exon, 0.4 \(\mu\)L of genomic DNA was amplified with 9.8 \(\mu\)L TaqMan 2X Universal PCR Master Mix in an Applied Biosystems 7700 Sequence Detection System. The thermal cycle conditions were 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles at 95
Fig. 1. Validation of our TaqMan real-time PCR system.
A. Measuring threshold cycles in solutions with different concentrations.
We used the ABI Prism 7700 Sequence Detection System to capture the intensity of fluorescence with each amplification cycle of DNA solutions from a neurologically normal subject. The amplification plots were traced. The horizontal axis indicates the PCR cycle number and the vertical axis indicates arbitrary fluorescence intensity. Red plots represent 3125 diameters of a control DNA sample, green plots represent 625 diameters, yellow plots represent 125 diameters, light blue plots represent 25 diameters, pink plots represent 5 diameters and navy plots represent 1 diameter. When the amplification plots are shown on a logarithmic scale there is a straight-line relationship between the amount of DNA and cycle number when PCR amplification is in the exponential phase. During this phase, PCR cycle number is inversely correlated with the starting quantity of DNA solution. An arbitrary cycle threshold (Ct) is obtained in this exponential phase. In this case, the signal value used to determine Ct was 3 × 10⁻².
B. Standard curve for quantifying α-synuclein gene copy number.
Horizontal axis indicates the starting quantity of DNA solution and vertical axis indicates Ct. The seven circles show standard DNA solutions from a neurologically normal subject, with different starting quantities and no template control. The standard curve was prepared by using these standard solutions. The relative amounts of test samples (red circles: n = 12) were determined by plotting on this standard curve.
First, we checked whether our use of the ABI Prism 7700 Sequence Detection System was accurate. A DNA sample extracted from peripheral blood leukocytes drawn from a neurologically normal subject was sequentially diluted to 1:1, 1:5, 1:25, 1:125, 1:625, and 1:3125. These six samples were processed by the ABI Prism 7700 Sequence Detection System (Figure 1A). For each diluted solution, we analyzed the threshold cycle, which was the number of cycle reactions at which the amount of PCR product measured by TaqMan system reached $3 \times 10^{-2}$ (Figure 1A); then we plotted the starting quantity of DNA solution against the threshold cycle (Figure 1B). The relationship between starting quantity and threshold cycle was linear, indicating that our TaqMan System was accurate enough to perform real-time PCR analysis.

We then studied the genomic DNA obtained from 50 consenting MSA patients (26 males, 24 females). The average age at examination was 59 ± 14 years. These 50 MSA patients consisted of 34 OPCA patients, 12 SND patients, and 4 SDS patients. All of the patients were molecularly excluded from having spinocerebellar ataxia types 1, 2, 3, and 6 or dentatorubral-pallidoluysian atrophy. The ratio of a given $\alpha$-synuclein gene copy number against $CBFB$ exon 4 copy number was then calculated. Finally, the Mann-Whitney U-test was used to statistically assess the copy number differences between every single $\alpha$-synuclein gene exon and the $CBFB$ gene exon 4.

3. Real-time RT-PCR analysis

3-1) Isolation of RNA from frozen cerebellar tissues

From frozen cerebellar tissue (three controls and three MSA individuals), total RNA was first extracted as previously described. RT was then performed in a reaction volume of 20 μL containing 1 μg of total RNA, 1 μL of random hexamers, and dNTPs by using the SuperScript Preamplification System (Gibco BRL USA), in accordance with the manufacturer's instructions.

3-2) TaqMan real-time RT-PCR procedure

To determine the expression levels of the endogenous control, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) detection reagents (Perkin-Elmer) were used for the quantitation of GAPDH mRNA. The ABI Prism 7700 Sequence Detection System was applied in the same way as for genomic DNA by using $\alpha$-synuclein primer and an exon 6 probe. Since only a small number of samples were available for examination (three each for control and MSA subjects), statistical comparison of the control and MSA groups was not performed.

4. In situ hybridization

4-1) Cloning of human $\alpha$-synuclein cDNA and in vitro transcription

To clone $\alpha$-synuclein cDNA we used human frozen brain tissue obtained at autopsy. Total RNA was extracted from 0.86 g of cerebellum as previously described. RT was performed in a reaction volume of 20 μL containing 1 μg of total RNA, 1 μL of random hexamers, and dNTPs using the SuperScript Preamplification System (Gibco BRL USA) in accordance with the manufacturer's instructions. PCR was undertaken in a final volume of 20 μL, containing 1 μL of first-strand cDNA, 2.5 pmol each of forward and reverse primers ($\alpha$-synuclein forward primer: 5'-CTTCCTCAGTCCTCCTACATCA-3'; $\alpha$-synuclein reverse primer: 5'-GGCTGCTCCCTGCTGCTGGTT-3'), 2.5 mM of each dNTP, and 0.1 μL of Taq polymerase (Takara, Japan). The thermal conditions were initial denaturation
at 95 °C for 12 min; 30 cycles of denaturation (95 °C for 1 min), annealing (60 °C for 30 s), and extension (72 °C for 45 s); and a final extension at 72 °C for 5 min, performed with an ABI GeneAmp PCR system 9700 (PE Applied Biosystems). With this primer pair and conditions, the α-synuclein mRNA was clearly amplified, yielding a major amplicon 976 base pairs (bp) long. This PCR product was sub-cloned into a vector, pCR-TOPO (Invitrogen, Carlsbad, CA, USA). After checking the sequence of insertion, we obtained two α-synuclein cDNA clones with either sense- or antisense-direction.

Then, the α-synuclein cDNA in the pCR-TOPO clones was linearized by digestion with KpnI restriction enzyme. The digested clones were purified after agarose gel electrophoresis. Then in vitro transcription was performed by incubation with T7 RNA polymerase (Roche, Germany) as indicated by the manufacturer’s protocol. This allowed us to generate sense and anti-sense RNA probes, depending on the direction of the PCR product against the vector. To generate the cRNA probe, digoxigenin-labeled UTP (DIG RNA Labeling Kit, Roche, Germany) was incorporated as indicated by the manufacturer.

After in vitro transcription, 2 μL of DNase I was added and the mixture further incubated for 15 min at 37 °C; 1 μL of 0.5 M EDTA (pH 8.0) was then added. After the addition of lithium chloride and ethanol, the labeled probes were then precipitated by centrifugation (14000×g), dried under vacuum, and resuspended in 50 μL of RNase-free water. To fragment the 976-bp cRNA probe into a 150-bp cRNA probe, 10 μL each of 0.4 M NaHCO₃ (sodium hydrogen carbonate) and 0.6 M Na₂CO₃ (sodium carbonate) were added to a 50 μL RNA probe solution and incubated for 30 min at 60 °C. This alkaline hydrolysis reaction was terminated by the addition of 1 μL of a solution of 0.5 M EDTA (pH 8.0).

4-2) In situ hybridization procedure

Both frozen sections and formalin-fixed paraffin-embedded specimens were used. The cerebellum and pons of three controls and three MSA patients were studied.

Frozen brain tissues obtained at autopsy and stored at -80 °C until use were dissected in a cold chamber at -20 °C, and cryostat frozen sections were made at 6 μm thickness. Then the sections were air-dried, fixed with 4% paraformaldehyde for 20 min, and then rinsed in RNase-free water. Formalin-fixed paraffin-embedded sections were also used, since tissues are more suitable than frozen sections for histological analysis. In this case, sections were first deparaffinized in xylene and put through gradually decreasing concentrations of ethanol solutions.

Both frozen or formalin-fixed tissue sections were digested at 37 °C for 30 min with proteinase K at a final concentration of 8 μg/mL; the sections were then washed with 1 × PBS (phosphate-buffered saline) for 1 min. Sections were post-fixed in 4% paraformaldehyde in 0.1 M PBS for 10 min and then washed with 1 × PBS for 1 min. Sections were next incubated in 0.2 N HCl at room temperature for 10 min and then washed with 1 × PBS for 1 min. Then the sections were rinsed in 0.1 M triethanolamine and 0.25% acetic anhydride for 10 min. All sections were dehydrated in graded ethanol and air-dried.

For pre-hybridization, a 1 mL pre-hybridization mixture (containing 50 μL of 20 mg/mL yeast tRNA, 20 μL of 1 M Tris-HCl (pH 8.0), 5 μL of 0.5 M EDTA (pH 8.0), 60 μL of 20 × Denhardt’s solution, 50 μL of 5 M NaCl, and 500 μL of formamide) was incubated at 85 °C for 10 min. A hybridization solution was made by adding a cRNA probe solution to a 1 mL pre-hybridization mixture to give a final concentration of 2 ng/mL. This hybridization solution was heat denatured at 85 °C for 3 min, immediately quenched in ice, and put on air-dried sections for overnight incubation at 55 °C. The next day, sections were washed successively in solutions of 1) 50% formamide in SSC (3 M NaCl, 0.3 M sodium citrate) at 50 °C for 30 min, 2) TNE buffer (5 M NaCl, 1 M Tris-HCl (pH 7.5), 0.5 M EDTA (pH 8.0) at 37 °C for 10 min, 3) TNE buffer containing 1 μg/mL RNase A at 37 °C for 30 min, 4) TNE buffer at 37 °C for 10 min, 5) 2× SSC at 50 °C for 30 min, and twice in 6) 0.2× SSC at 50 °C for 20 min. Then the hybridized digoxigenin-labeled probe was detected by incubation with an alkaline-phosphatase-conjugated anti-digoxigenin Fab fragment antibody raised in sheep (Roche, Germany). The section was finally developed in nitroblue tetrazolium chloride (Roche, Germany) and 5-bromo-4-chloro-3-indolyl phosphate, toluidine salt (Roche, Germany) at room temperature for 4 to 7 h.

5. Immunohistochemistry for α-synuclein

Formalin-fixed paraffin-embedded sections 6 μm thick were used as previously described. Endogenous peroxidase activity was quenched by immersing sections in 0.3% hydrogen peroxide for 30 min; then the sections were incubated with normal horse serum for 20 min for blocking of non-specific reactions. LB509, a mouse monoclonal antibody for α-synuclein, was used as the primary antibody at a
dilution (in PBS) of 1:200. The sections were incubated with diluted LB509 overnight at 4 °C. Serial detection of primary antibody was performed with biotinylated horse anti-mouse IgG (Vector Laboratories, Burlingame, CA), and ABC complex (Vector), and the reaction was finally developed with diaminobenzidine tetrahydrochloride containing 0.001% H2O2.

6. Informed consent
For genomic analysis, informed consent was obtained from all MSA patients and control subjects. Written materials for obtaining informed consent conformed to the guidelines for genetic analysis of human materials, Ministry of Health, Labour and Welfare, Japan. All human tissue samples were obtained at autopsy after informed consent had been obtained. All patients’ families were informed of, and agreed to, the potential use of brain samples for research aims. This study was approved by the Ethical Committee of Tokyo Medical and Dental University.

Results

1) α-synuclein gene copy number in MSA patients’ genomes

After validating our real-time PCR system, we analyzed the copy number of the α-synuclein gene against that of the CBFB gene in 50 MSA patients (Figure 2). The relative ratio of the copy number of each α-synuclein exon against that of the CBFB gene exon 4 was 0.92±0.05 (mean±SEM) for exon 1; 0.89±0.05 for exon 2; 0.90±0.06 for exon 3; 0.91±0.05 for exon 4; 0.83±0.05 for exon 5; and 0.90±0.05 for exon 6. None of these values was statistically significant. Mann-Whitney’s U-test was used to statistically assess the copy number differences between every single α-synuclein gene exon and the CBFB gene exon 4. To validate this result, CBFB exon 4 was measured against the same exon. This ratio was 0.92±0.05. These results suggest that the α-synuclein gene copy number in the genomes of MSA patients was not significantly different from the copy number of the CBFB gene.

2) Real-time RT-PCR

We compared the level of expression of the mRNA of exon 6 of α-synuclein with that of GAPDH mRNA (Figure 3). In control cerebella (n = 3), the ratio of α-synuclein mRNA to GAPDH mRNA ranged from 0.84 to 1.41, with a mean value of 1.11±0.17. In MSA cere-

bella (n = 3), the ratio of α-synuclein mRNA to GAPDH mRNA ranged from 0.41 to 1.14, with a mean value of 0.84±0.22 (mean ±SEM) and that in controls was 1.11±0.17, showing no obvious difference between the two groups.

3) In situ hybridization

In situ hybridization of α-synuclein mRNA was analyzed in control and MSA brain tissues. In control brains, in situ hybridization using the sense probe did not detect any significant signal in brain sections. In contrast, in situ hybridization using the anti-sense probe detected several small signals in granule cells
**Fig. 4. In situ hybridization in control and MSA cerebella**

In situ hybridization analyses of α-synuclein mRNA expression in the brains of normal subjects (A, B, E, and F) and MSA patients (C, D, G, and H) using α-synuclein-specific antisense and sense probes. Granule cells reacted with the antisense probes in both the MSA and control specimen, but not with the sense probes. No hybridization signals were detected in Purkinje cells in the MSA or control specimen. A, C, E, and G were reacted with antisense probes and B, D, F, and H with the corresponding sense probes. A, B, C, and D Scale-bar=200µm E, F, G, and H Scale-bar=40µm.
(Figure 4). Purkinje cells showed no notable signals, indicating that the α-synuclein gene may not be highly expressed in these neurons. Glial cells, including oligodendroglia, did not show notable signals.

In sections from MSA patients’ brains, the specificity of our in situ hybridization technique was again confirmed: notable signals were detected only with anti-sense probes (Figure 4). Although these patients showed GCIs, the signals detected in in situ hybridization with the anti-sense probe did not appear different from those detected in control brain tissues. Again, oligodendroglia in the cerebellar white matter did not show notable signals, indicating that α-synuclein mRNA expression was not increased in the cerebella of MSA patients compared with that of the controls.

4) Immunohistochemistry for α-synuclein protein in control and MSA cerebella

On specimens examined for in situ hybridization we undertook immunohistochemistry for α-synuclein protein (Figure 5). In the control sample there was only a faint immunoreactivity for α-synuclein (Figure 5A). In contrast, strongly immunoreactive GCIs were scattered in the MSA cerebellum (Figure 5B). This indicates that MSA specimens were abnormally immunoreactive against LB509, a monoclonal antibody for α-synuclein.

Discussion

To our knowledge, this is the first study to have analyzed the α-synuclein gene copy number in MSA patients’s genomic DNA. Our results on TaqMan real-time PCR showed that the α-synuclein gene copy number was not altered in MSA patients. Previous studies have shown that mutations are not present, at least in the coding region of the α-synuclein gene, and single-nucleotide polymorphisms have not been shown to be associated with MSA patients.11 These facts would suggest that the α-synuclein gene itself is not associated with MSA pathogenesis.

We also analyzed mRNA levels in the MSA cerebellum in two ways. The first was by quantitative real-time RT-PCR and the second by in situ hybridization. There has been only one quantitative RT-PCR analysis of α-synuclein mRNA in MSA brains.12 The researchers probed exon 5 of α-synuclein mRNA but, like us, they did not detect any increase in the expression of α-synuclein mRNA. Although the number of samples we were able to use was small (n = 3), our result did not indicate any increase in α-synuclein mRNA levels in MSA.

Although the expression of α-synuclein mRNA did not increase on real-time RT-PCR, increased expression could still have been apparent in MSA in specific cell types, such as oligodendroglia. This possibility was clarifiable by in situ hybridization. Our results showed that α-synuclein mRNA was predominantly expressed in...
in normal neurons. This is in accordance with the results of a previous study,\textsuperscript{14} and also with the fact that the gene product, \( \alpha \)-synuclein protein, is concentrated in presynaptic nerve terminals.\textsuperscript{14}

From our \textit{in situ} hybridization study of the MSA cerebellum, we can draw two plausible conclusions. First, it is highly probable that alteration in the expression level of \( \alpha \)-synuclein mRNA is not the fundamental phenomenon in the MSA brain, since no increase in positive mRNA signals was detected in MSA brains. This notion was not restricted to neurons. Glial cells, including oligodendroglia, in which formation of GCIs predominates, were not found to have a strong hybridization signal. Given that accumulation of aggregated \( \alpha \)-synuclein protein in both oligodendroglia and neurons is the fundamental finding in MSA, it is probable that abnormal intracellular metabolism of \( \alpha \)-synuclein protein, rather than alteration of its mRNA expression, is associated with MSA pathogenesis. In line with this hypothesis, the important molecular chaperones Hsc70 and Hsp 70 are both localized not only in GCIs, but also in glial nuclear inclusions, neuronal cytoplasmic inclusions (NCIs) and neuronal nuclear inclusions (NNIs), whereas such immunoreactivities are not seen in control brains.\textsuperscript{15}

The second important conclusion drawn from our study is that expression of \( \alpha \)-synuclein mRNA did not appear to be correlated with the severity of neuronal loss. For example, the Purkinje cell, one of the neurons most intensely affected in MSA,\textsuperscript{16} lacked a signal of \( \alpha \)-synuclein mRNA expression. This is in accord with the fact that NCLs and NNIs are seldom seen in Purkinje cells.\textsuperscript{4} Considering that the incidence of GCIs is correlated with the severity of neuronal losses in two important neuronal systems, the olivoponto-cerebellar and striato-nigral systems,\textsuperscript{17} and that NCLs and NNIs tend to be restricted to some neuronal populations,\textsuperscript{18} neuronal degeneration might, at least in part, be caused secondarily by some as-yet-clarified mechanisms, such as myelin destruction due to oligodendroglial degeneration.

In conclusion, our results would suggest that the \( \alpha \)-synuclein gene copy number is not altered in MSA subjects. Although the numbers of MSA brains analyzed were small to draw a conclusion, TaqMan RT-PCR and \textit{in situ} hybridization did not appear to support the hypothesis that \( \alpha \)-synuclein mRNA expression level is the primary determinant of the pathogenesis of MSA. Investigations of the abnormal metabolism or behavior of the \( \alpha \)-synuclein protein may be important to identify the mechanism of pathogenesis of MSA.

\textbf{References}