

profile of the  $^{13}\text{CO}_2$  excretion rate. The parameters were estimated with Excel software (Microsoft Co., Ltd., Redmond, WA).

Statistical analysis

Categorical variables were compared using Fisher’s exact probability test. Other variables were expressed as median (range). Medians were compared using Mann–Whitney’s *U* test. All analyses were carried out on StatView statistical software, version 5.0 (Abacus Concepts, Inc., Berkeley, CA). A *P* value less than 0.05 was considered significant.

Results

Controls and early-stage, untreated PD patients (Figs. 1, 2)

The examinations were safely carried out in all PD patients and controls. The  $T_{\text{max}}$  was significantly delayed in patients with early-stage, untreated PD (median 1.17 h, range 0.67–1.83 h) as compared with the controls (median 0.83 h, range 0.67–1.00 h) ( $P < 0.001$ ). The HET was significantly delayed in patients with early-stage, untreated PD (median 2.04 h, range 1.75–3.07 h) as compared with the controls (median 1.44 h, range 1.30–1.64 h) ( $P < 0.001$ ). There was absolutely no overlap in the range of the HET between controls and early-stage, untreated PD patients.

Controls and advanced-stage, treated PD patients (Figs. 1, 2)

In all patients with advanced-stage, treated PD, the blood concentration of L-dopa was below 25 ng/ml (below the detection level) at start of examination. The  $T_{\text{max}}$  of GE

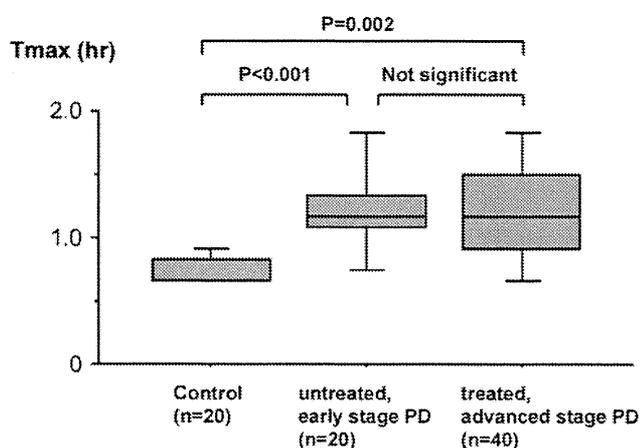


Fig. 1  $T_{\text{max}}$  in control, PD patients with untreated, early-stage and those with treated, advanced-stage

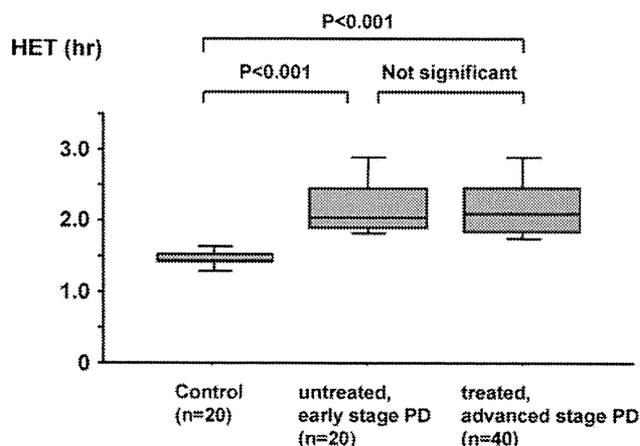


Fig. 2 HET in control, PD patients with untreated, early-stage and those with treated, advanced-stage

using the  $^{13}\text{C}$ -ABT was significantly delayed in patients with advanced-stage, treated PD (median 1.17 h, range 0.50–2.17 h) as compared with the controls (median 0.83 h, range 0.67–1.00 h) ( $P = 0.002$ ). The HET was significantly delayed in patients with advanced-stage, treated PD (median 2.09 h, range 1.60–3.30 h) as compared with the controls (median 1.44 h, range 1.30–1.64 h) ( $P < 0.001$ ). There was virtually no overlap between controls and advanced-stage, treated PD patients.

PD patients with untreated early-stage and treated advanced-stage (Figs. 1, 2; Table 1)

The  $T_{\text{max}}$  was not significantly delayed in PD patients with early-stage (median 1.17 h, range 0.67–1.83 h) as compared with those with advanced-stage (median 1.17 h, range 0.50–2.17 h) ( $P = 0.58$ ). The HET was not significantly delayed in PD patients with early-stage (median 2.04 h, range 1.75–3.07 h) as compared with those with advanced-stage (median 2.09 h, range 1.60–3.30 h) ( $P = 0.77$ ).

Discussion

In the present study we have shown two novel important points. Firstly, we demonstrated that gastric emptying is significantly delayed in early-stage, untreated PD patients. Secondly, delayed gastric emptying does not differ between patients with early-stage, untreated PD as compared to those with advanced-stage, treated PD.

PD patients sometimes complain of upper GI symptoms such as heartburn, nausea, vomiting, and full abdomen sensation [1–3]. With respect to the pathological background of PD, during the pre-symptomatic stage of PD, the idiopathic PD related abnormal synuclein immunostaining

**Table 1** Clinical characteristics of PD patients with untreated, early-stage and those with treated, advanced-stage

	Untreated, early-stage PD ( <i>n</i> = 20)	Treated, advanced-stage PD ( <i>n</i> = 40)	<i>P</i> value
Age (years)	70.5 (54–82)	67.0 (42–86)	0.49
Gender (male/female)	8/12	14/26	0.78*
BMI (kg m <sup>-2</sup> )	20.5 (17.8–27.9)	20.3 (16.8–27.1)	0.56
Duration (years)	0.9 (0.3–2.5)	6.0 (3.0–31.0)	<0.001
Duration with L-dopa (years)	0	5.5 (2.5–30.0)	<0.001
L-dopa (mg)	0	400 (100–750)	<0.001
Upper GI symptoms	6	11	0.99*
OH	9	21	0.78*
CV <sub>R-R</sub>	1.70 (0.83–4.64)	1.71 (0.67–5.14)	0.43
MIBG (H/M ratio)	1.45 (1.13–2.56)	1.49 (1.27–1.95)	0.80

Variables expressed as median (range)

Medians were compared using Mann–Whitney's *U* test

PD Parkinson's disease, BMI body mass index, GI gastrointestinal tract, OH orthostatic hypotension, CV<sub>R-R</sub> coefficient of variation of R-R intervals, MIBG I-[123]-metaiodo-benzylguanidine scintigraphy, H/M ratio heart/mediastinum ratio

\* Categorical variables were compared using Fisher's exact probability test

is confined to the medulla oblongata and olfactory bulb, according to Braak [6]. Neuronal degeneration occurring in the dorsal nucleus of the vagus may be responsible for the degeneration of the gastrointestinal myenteric plexuses. In the study of the enteric nervous system of PD, it has reported that Lewy bodies were found in the Auerbach's and Meissner's plexuses of the lower esophagus and stomach [23, 24]. Cytoplasmic inclusions similar to Lewy bodies were present in the ganglion cells of the colonic myenteric plexuses in PD patients [25]. The most likely causes of GI tract symptoms are degenerations of the dorsal vagal nucleus and the intramural plexus of the whole intestine [25]. These degenerations are likely to develop prior to the degeneration of dopaminergic neurons of the substantia nigra [7].

On the other hand, in lower GI symptoms, constipation has been identified as a rather frequent symptom in patients with PD ever since the first description of the disease [26–32]. Today, constipation is considered the most common manifestation of autonomic dysfunction in this disease occurring at a prevalence in the range of 70–80% [27, 30, 33, 34]. Constipation may precede development of PD [35]. It has been reported a prospective study which followed the bowel habits of 7,000 men for 24 years and reported that those with initial constipation (<1 bowel movement/day) had a threefold risk of developing PD after a mean interval of 10 years from initial constipation [35]. Involvement of the dorsal vagal nucleus, as would occur in Braak stage 1 may explain the pre-motor appearance of constipation [36]. It has been reported that motility of gut was controlled both by extrinsic inputs from the dorsal motor nucleus of the vagus and paravertebral sympathetic ganglia and by local reflexes mediated by intrinsic neurons

of the enteric nervous system [37]. Both the enteric nervous system and the dorsal motor nucleus of the vagus are affected by Lewy body pathology at early stages of PD [37]. This early involvement provides insights into the pathophysiology of gastrointestinal dysmotility in GI disorder and may constitute an important step in the etio-pathogenesis of Lewy body disease [37].

In the previous study, it was reported that delayed gastric emptying is common in patients with early-stage, treated PD [1, 2, 4]. However, because L-dopa therapy itself may worsen the symptoms of delayed gastric emptying [8, 9], their interpretation of the results of their study is limited. Gastric emptying of patients with early-stage, treated PD may be affected by L-dopa therapy. There is evidence to suggest that these complications in PD patients may be attributed to peripheral, pharmacokinetic mechanisms, mainly delayed gastric emptying as a side-effect of L-dopa [2, 38–40]. Dopaminergic agents also activate the vomiting center in the medulla via the chemoreceptor trigger zone, resulting in nausea, abdominal bloating, and vomiting [38].

In the present study, we tested whether there is the delayed gastric emptying of untreated patients with early-stage PD using <sup>13</sup>C-ABT. All PD patients were divided into two groups: those with untreated early-stage and those with treated advanced-stage. The patients with advanced-stage, treated PD were off drugs, including L-dopa, and the blood concentration of L-dopa was below the detection level at the start of examination. The two groups did not have significantly different blood parameters of gastric function such as pepsinogen I/II, serum gastrin, immunoglobulin G (IgG) anti-Helicobacter pylori antibody, hemoglobin A1c (HbA1c) [17] and neither was age of significant difference between the two groups [41]. The positive ratio of OH [18]

and  $CV_{R-R}$  [19], H/M ratio of MIBG scintigraphy [20] were not significantly different between the PD groups. Under these conditions, there was not a difference in gastric emptying of PD patients with untreated, early-stage and those with treated, advanced-stage. And there is the delayed gastric emptying of untreated patients with early-stage PD. At the early-stage of PD, gastric emptying is already delayed similarly to the advanced-stage. Therefore, we speculate that delayed gastric emptying may be one of markers of the pre-clinical stage of PD.

In conclusion, our study demonstrated that gastric emptying is significantly delayed in untreated, early-stage PD patients as compared to controls, and that delayed gastric emptying does not differ between PD patients with untreated, early-stage and those with treated, advanced-stage. From a viewpoint of early diagnosis, it will be important to actually measure gastric emptying in PD patients in clinical settings. Our results demonstrated that from early-stage of PD, gastric emptying is already delayed similar to the advanced-stage. Delayed gastric emptying may be one of markers of the pre-clinical stage of PD.

**Conflict of interest** All authors report no disclosure.

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# Diffuse Skeletal Muscles Uptake of [<sup>18</sup>F] Fluorodeoxyglucose on Positron Emission Tomography in Primary Muscle Peripheral T-cell Lymphoma

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## Abstract

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A 40-year-old man presented with weakness of neck extensor muscles. Cervical magnetic resonance imaging showed high-intensity areas in muscles of the left lateral cervical region on T2-weighted images. Fluorodeoxyglucose-positron emission tomography scan demonstrated striking fluorodeoxyglucose uptake by multiple skeletal muscles of the neck, chest, and abdominal region. Muscle biopsy demonstrated peripheral T-cell lymphoma, unspecified. The diagnosis was primary skeletal muscle peripheral T-cell lymphoma. Primary skeletal muscle non-Hodgkin's lymphoma of T-cell immunophenotype is extremely rare and fluorodeoxyglucose-positron emission tomography demonstrated striking fluorodeoxyglucose uptake in multiple skeletal muscles and served as a quite useful modality for the diagnosis of this patient.

**Key words:** primary skeletal muscle lymphoma, non-Hodgkin lymphoma, peripheral T-cell lymphoma, fluorodeoxyglucose on positron emission tomography, skeletal muscle

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## Introduction

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Primary skeletal muscle non-Hodgkin's lymphoma is extremely uncommon and it is associated with a poor prognosis (1). Additionally, the majority of these lymphomas demonstrate B-cell immunophenotype (1). We report a case with primary skeletal muscle non-Hodgkin's lymphoma of T-cell immunophenotype, that showed slow progression, characterized by striking fluorodeoxyglucose uptake involving multiple skeletal muscles on positron emission tomography scans.

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## Case Presentation

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In January 1996, a 27-year-old man had swelling of the

right side of the labia and oral mucosa. In April 1996, the patient visited a hospital and was diagnosed with chronic lymphadenitis by a biopsy of the oral mucosa. Oral steroid therapy improved the symptoms. In July 1996, the patient had left ptosis, and left facial weakness. Steroid therapy again improved the symptoms. In September 1998, the patient had diplopia and bilateral facial weakness (manual muscle test (MMT): 1/1). The symptoms were not improved by steroid therapy and remained constant. In May 2000, the patient developed weakness of the neck extensor (MMT: 4/4). After several months, the symptoms disappeared spontaneously. Thereafter, the patient remained free of any new symptoms for the subsequent 9 years.

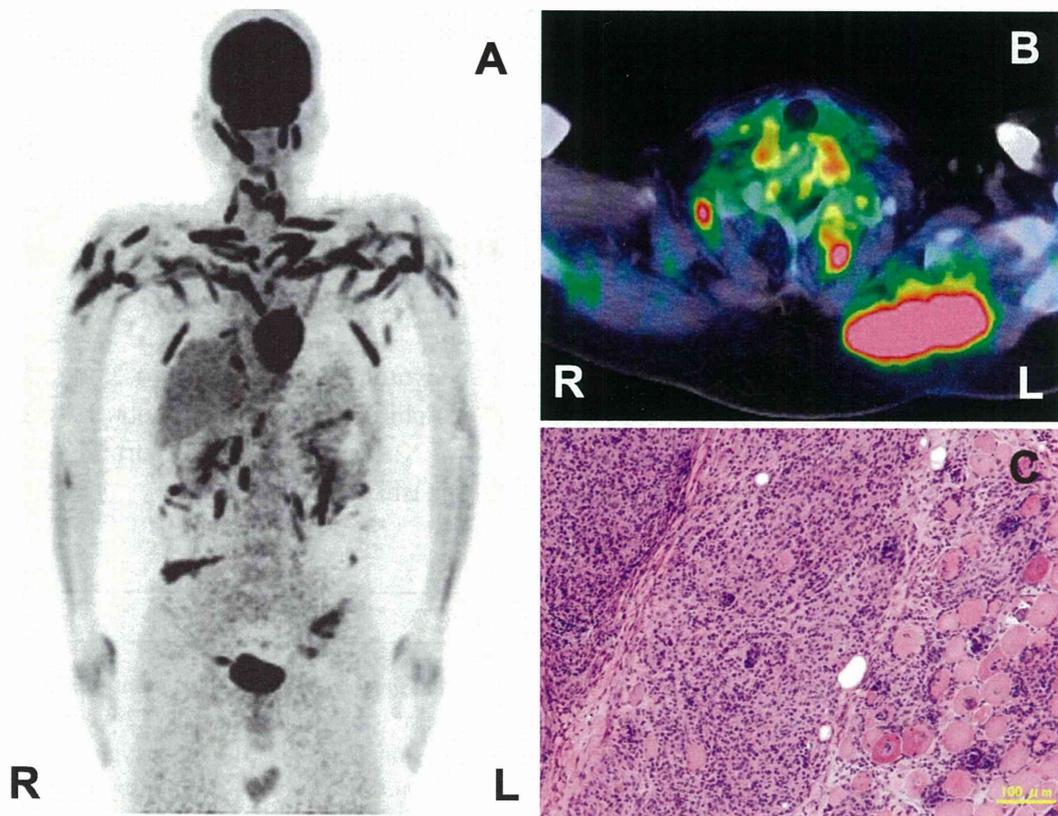
In May 2009, at the age of 40 years, weakness of neck extensor appeared again. The patient was admitted to our

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**Figure 1.** Fluorodeoxyglucose-positron emission tomography (PET) and biopsy of the muscle. (A) (B) Fluorodeoxyglucose-positron emission tomography (PET): Fluorodeoxyglucose-positron emission tomography (PET) demonstrated striking fluorodeoxyglucose uptake involving the skeletal muscles of the neck, chest, and abdominal region. The maximum standardized uptake value of the left trapezius muscle was 10.32. (C) Biopsy specimen of the muscle: Muscle biopsy of the left trapezius muscle demonstrated massive infiltration of atypical lymphocytes of varying sizes and clusters of epithelioid histiocytes destroying muscle bundles.

hospital. Neurologic evaluation showed weakness of the left pectoral girdle (MMT: 4), restricted bilateral eye movement, and facial weakness. However, no other muscle showed the weakness. The patient did not have myalgia. The patient did not appear to show a loss of weight, fever or sweating such as seen in B symptom. In the serum, creatine phosphokinase (CK) and soluble interleukin-2 receptor were high 311 IU/L (normal range, 40-200 IU/L) (CK-MM 95%) and 625 U/mL (normal range 145-519 U/mL), and continued to rise. Anti-human T-cell lymphotropic virus antibody and anti-human immunodeficiency virus antibody were negative. Cervical magnetic resonance imaging (MRI) showed high-intensity areas in the muscles of the left lateral cervical region on T2-weighted images. The muscles of other regions did not demonstrate abnormal findings on MRI. Fluorodeoxyglucose-positron emission tomography demonstrated striking fluorodeoxyglucose uptake by multiple skeletal muscles of the neck, chest, and abdominal region, but not of the lower extremities (Fig. 1A, 1B). Maximum standardized uptake value of the left trapezius muscle was 10.32. Ga scintigraphy showed no abnormal uptake. Computed tomography of the whole body revealed no evidence of lymphadenopathies, tumor infiltrations, or masses. There were no disease abnor-

malities involving the osseous structure. Bone marrow biopsy revealed a normocellular marrow with trilineage hemopoiesis and no evidence of lymphoma. Biopsy of the left trapezius muscle demonstrated massive infiltration of atypical lymphocytes which varied in size and clusters of epithelioid histiocytes destroying muscle bundles (Fig. 1C). Immunohistochemical staining revealed that the atypical lymphocytes were positive for CD3 and CD8, but negative for CD4, CD20, CD56, CD79a, and EBER. Histopathologic and immunocytochemical characteristics were consistent with those of peripheral T-cell lymphoma, unspecified (PTCL-U). The final diagnosis was established as primary skeletal muscle peripheral T-cell lymphoma. Chemotherapy with 6 cycles of cyclophosphamide, doxorubicin, vincristin and prednisone completely resolved the weakness of the left pectoral girdle, but it did not improve the eye movement or facial weakness. On serum analyses, CK and IL2R were decreased to the normal range. Fluorodeoxyglucose-positron emission tomography and magnetic resonance imaging improved after chemotherapy. The maximum standardized uptake value of the left trapezius muscle was reduced. After 6 months of chemotherapy, the symptoms remained the same.

## Discussion

A 40-year-old man was diagnosed with primary skeletal muscle peripheral T-cell lymphoma. Multiple neurologic deficits had appeared for 13 years from the initial symptoms. The patient had been diagnosed with idiopathic or unknown disease because there was no specific finding. Although not definite, if those symptoms were related to lymphoma, this case showed very slow progression. Fluorodeoxyglucose-positron emission tomography demonstrated striking fluorodeoxyglucose uptake by multiple skeletal muscles and was useful in detecting disseminated lymphoma in the skeletal muscles.

Primary skeletal muscle non-Hodgkin's lymphoma is a rare manifestation of non-Hodgkin's lymphoma, accounting only for 0.1% of all lymphomas (1). The frequency of primary skeletal muscle non-Hodgkin's lymphoma (7%) is increased in patients with Acquired Immune Deficiency Syndrome (AIDS)-associated lymphomas (2). It is difficult to differentiate between primary skeletal muscle lymphoma and secondary muscle involvement from bone lymphoma. The following criteria is used to diagnose primary muscular lymphoma: 1) histopathology proven non-Hodgkin's lymphoma of the muscle; 2) the absence of systemic/nodal disease at initial presentation; and 3) the presence of a soft tissue mass with normal adjacent marrow or marrow disease much less extensive than soft tissue (3). The present case was diagnosed as primary skeletal muscle non-Hodgkin's lymphoma because these three 3 criteria were fulfilled. In cases of primary skeletal muscle non-Hodgkin's lymphoma the lower extremities and pelvic region are most commonly affected (4). Additionally, the majority of reported cases had B-cell immunophenotypes and behaved in an aggressive biologic manner (5). T-cell neoplasms constitute 10% to 12% of all non-Hodgkin's lymphoma (6). PTCL-U represents the most common subtype of T-cell lymphomas, manifesting most often as a nodal disease (7). Extranodal PTCL-U is a rare presentation among neoplasms. Some cases of primary skeletal muscle T-cell lymphomas have been described in the literature (8-12). Furthermore, in a few reported cases fluorodeoxyglucose-positron emission tomography demonstrated striking fluorodeoxyglucose uptake in multiple skeletal muscles.

Fluorodeoxyglucose-positron emission tomography scans demonstrate multiple sites of hypermetabolic activity within musculature. A recent study suggests that fluorodeoxyglucose-positron emission tomography scans are sensitive to detect T-cell lymphomas in all regions except for the skin (13). Fluorodeoxyglucose-positron emission tomography is useful in aiding the diagnosis and staging of lymphomas of aggressive subtypes, including PTCL-U (14). However, other reasons for skeletal muscle fluorodeoxyglucose uptake may stem from a variety of factors such as physical exertion, talking, and chewing as normal physiological phenomena (15). As for pathological phenomena, elevated fluorode-

oxyglucose muscle may be associated with anxiety-induced neck muscle spasm (15), accessory muscle use in respiratory distress (16), intercostal and diaphragmatic activity in chronic obstructive pulmonary disease (17), abdominal muscle contraction in intractable vomiting (18), sepsis-induced shivering (19), hypoglycemia (20), infection, and inflammation of the muscle tissue that increases glycolytic activity (21, 22). In the present case, the patient did not satisfy these conditions. Skeletal muscle fluorodeoxyglucose uptake was induced by T-cell lymphoma, which histopathologically proved PTCL-U.

In conclusion, primary skeletal muscle non-Hodgkin's lymphoma of T-cell immunophenotype is extremely rare. Fluorodeoxyglucose-positron emission tomography demonstrated striking fluorodeoxyglucose uptake in multiple skeletal muscles, and served as a useful method for diagnosing this case.

Informed consent was obtained from this patient.

**The authors state that they have no Conflict of Interest (COI).**

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## Neuromyelitis Optica in Japanese Sisters

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### Abstract

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We report cases of Japanese sisters with neuromyelitis optica (NMO). The elder sister was 25, when she was diagnosed with right optic neuritis. After 3 months, she developed left optic neuritis and myelitis. At age 27, she had the second relapse, but she has been free from episodes thereafter. The younger sister was 26, when she was diagnosed with optic neuritis. Thus far, she has 9 relapses, comprising both myelitis and optic neuritis. Both sisters had normal brain MRI scans, longitudinally extensive transverse myelitis over 3 vertebral segments, and positive results for anti-aquaporin-4 antibody (AQAP4Ab). They fulfilled the Wingerchuk criteria for definite NMO. Both sisters shared some immunogenetic factors, but they were not exposed to the same environmental factors after their early twenties. The final disability status was almost the same in both cases, and both showed a very benign course. These data suggest that genetic factors affect the age at onset and environmental factors may affect the frequency of relapse.

**Key words:** neuromyelitis optica, familial, HLA, anti aquaporin-4 antibody, environmental factor

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### Introduction

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Neuromyelitis optica (NMO) is a demyelinating disorder of the central nervous system that generally affects the optic nerves and spinal cord but less often the brain (1, 2). Opticospinal multiple sclerosis (OSMS) in Asians has been suggested to be the same entity as NMO, because anti-aquaporin-4 antibody (AQP4Ab) has been reported to be present in 30% to 60% of OSMS patients (3). The incidence of human leukocyte antigen (HLA)-*DPB1\*0501* was significantly increased in anti-AQP4Ab-positive Japanese patients as compared with healthy controls, but not in anti-AQP4Ab-negative OSMS patients (4). Furthermore, cases of familial NMO have been reported suggesting genetic influence (5-9). The mechanism of NMO is speculated to be associated with humoral immunity, but still remains to be fully elucidated (4, 10, 11). Here, we report cases of Japanese sisters with NMO, in which the onset age was similar, but the frequency of relapse was different.

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### Case Report

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#### **Patient 1: Elder sister**

Patient 1 was a 54-year-old woman. She did not have any family history of immunological disorders. When she was 25, right-sided blindness developed and she was diagnosed as having optic neuritis. Steroid therapy completely improved her vision. After 3 months, she lost her left sight and had paraparesis and dysuria. The neurologic evaluation revealed blindness in the left eye, weakness of bilateral lower extremities, positive bilateral Babinski reflexes, and bilateral loss of sensation below the Th10 level. Steroid therapy improved her symptoms within several months. At age 27, she had paraparesis and dysuria. Steroid therapy improved her symptoms again. Thereafter, she has been free from episodes of neurological dysfunction. At age 50, a test for AQP4Ab was found to be positive (12, 13). Her HLA type was *A\*31, B\*61, \*51, DRB1\*0802, and DPB1\*0501*. At age

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**Figure 1.** Spinal magnetic resonance imaging (MRI) of patient 2: T2-weighted image (1.5 T, repetition time [TR] 3000 ms, echo time [TE] 90 ms) and T1-weighted image with gadolinium enhancement (1.5 T, TR 400 ms, TE 9 ms). When patient 2 had her last relapse at age 49, the spinal MRI scan showed high-intensity areas at Th2-4 on T2-weighted images with swelling and gadolinium enhancement.

53, spinal magnetic resonance imaging (MRI) did not demonstrate apparent abnormality in parenchymal signal intensity, and her brain MRI was normal. There has been no medication for the prevention of relapse. The final Expanded Disability Status Scale of Kurtzke score was 1.0 (14) and the final visual function of both eyes was normal.

#### **Patient 2: Younger sister**

Patient 2 was a 53-year-old woman. When she was 26, right-sided blindness developed and she was diagnosed as having optic neuritis. Steroid therapy improved her vision completely. At age 27, she had paraparesis, loss of sensation below the L1 level and dysuria. The neurologic evaluation revealed weakness of bilateral lower extremities, positive bilateral Babinski reflexes, and bilateral loss of sensation below the L1 level. Steroid therapy improved her symptoms completely within several months. At age 29, she had loss of sensation below the Th5 level and steroid therapy improved her symptoms again. To date she has had 9 relapses, comprising of both myelitis and optic neuritis. At age 49, a test for AQP4Ab was found to be positive. Her HLA type was A\*31, B\*61, \*51, DRB1\*0802, and DPB1\*0501, which is the same as that of her elder sister. At the last relapse, when she was 49, the MRI showed on T2-weighted images, and enlarged spinal cord lesions with gadolinium enhancement (Fig. 1). Her brain MRI was normal. Low-dose steroid treatment and immunosuppressant (azathioprine) treatment have been continued for the prevention of relapse. The final

Expanded Disability Status Scale of Kurtzke score was 2.0 (14) and the final visual function of both eyes was normal.

Both patients fulfilled the criteria of NMO of Wingerchuk et al (15). The sisters had lived in the same area until the elder sister got married at age 23. The younger sister married at age 22 and moved to a northern rural area of Japan. Thus, they shared some immunogenetic factors but were not exposed to the same environmental factors after their early twenties.

Informed consents was obtained from both patients.

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### **Discussion**

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These 2 cases of Japanese sisters met the criteria of NMO. Both sisters had positive results for AQP4Ab and shared some immunogenetic factors. Their onset ages of NMO were similar. The frequency of relapse was different. The final disability status was almost the same and both sisters showed a very benign course of the disease. These patients were the third cases of familial NMO in Japan (including that discussed in a Japanese language abstract) (7).

According to a literature search, several familial cases of NMO have been reported (5-9). Those cases included 7 cases of sisters, 2 mother-daughter pairs, 2 aunt-niece pairs, 2 brother-sister pairs, 1 father-daughter pair, 1 mother-son pair, and 1 family with 3 patients (mother-daughter-aunt). Ninety-three percent of these familial cases were fe-

male (11). The first report of familial NMO in 1938 described that identical twin women developed NMO at ages 24 and 26 years, respectively, but their HLA type was unknown (5). In the first report, 1 woman had 1 recurrence for 18 months; the other had 2 recurrences for 26 months (5). The second case of sisters in 1982 described 2 younger sisters who developed NMO at ages 3 years and 2 years 9 months. The HLA type of the elder sister was *A1, 2, BW35, W40, and BW622* and that of the younger sister was *A1, XBW35, and YBW622* (6). In another case of sisters, the women developed NMO at ages 62 and 59 years (7). The HLA type of the elder sister was *A2/33, B39/44, Cw7/-, DR4/6, and DQ1/3* and that of the younger sister was *A26/33, B44/62, Cw3/-, DR6/12, DQ1/-, and DP1/-* (7). The onset ages in the fourth case of sisters were 24 and 28 years (8). The HLA type of the elder sister was *A\*24, B\*07, \*15, DRB1\*01, and \*16 (DR2 positive)* and that of the younger sister was *A\*02, 24, B\*07, \*40, DRB1\*04, and \*08* (8). In the fifth report of 4 sibling cases, the onset ages were 29.2 and 28.1 years, 28.4 and 26.5 years, 33.9 and 32.1 years, and 40.7 and 25.1 years (9). There is little data regarding the long-term prognosis after onset. The fifth report described 4 sibling cases with recurrent episodes of optic neuritis and myelitis (1 and 6 times, 7 and 2 times, 5 and 13 times, and 3 and 7 times, respectively) (9). Interestingly, the initial episodes of NMO occurred at similar ages in these reports, except for the last pair of sisters. In the present cases, the onset ages of NMO were also similar (within 1 year). Such observation may suggest that there is a common trigger for the onset of NMO related to the immunogenetic background of NMO in sister cases.

In the present cases, although the frequency of relapse was different between the 2 sisters, the final disability status was almost the same and both sisters showed a very benign course of the disease. However, in previous reports on sisters with NMO, little data was provided on the final disability status. It would be interesting to study this aspect in the future. It is suggested that the clinical feature might be related to the immunogenetic background of NMO in sister cases.

The genetic contributions were supported by recent observations that have linked NMO to specific HLA loci, such as *DPB1\*0501* in Japanese patients (10). The *HLA-DPB1\*0501* allele was more frequent in 38 Japanese patients who were seropositive for NMO-IgG as compared to 52 patients with multiple sclerosis (MS) (4). Forty-five French Caucasian patients were compared to healthy controls and patients with MS for HLA class II A and B alleles; no association was found for *DRB1\*1501* with NMO. *HLA-DRB1\*03* was associated with NMO-IgG-seropositive NMO (16). The HLA type has been reported to be similar in sisters with NMO in 2 families (6-8). In the present cases, the sisters shared some immunogenetic factors because their HLA type was similar.

With regard to environmental factors, certain infections or cancers have been reported to complicate NMO (17, 18).

However, there is no report on environmental or disease triggers that have a firm association with NMO (11). In the present cases, the sisters had lived in the same area just before the first episode of NMO, but 1 sister moved to a northern rural area and was then exposed to a different environment. The difference in environmental factors may have affected the frequency of relapse, although the precise factors are unknown.

These observations suggest that genetic factors affect the ages at onset and that environmental factors may affect the frequency of relapse in familial NMO.

**The authors state that they have no Conflict of Interest (COI).**

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# Elevated Anti-Heat Shock Protein 60 Antibody Titer is Related to White Matter Hyperintensities

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*Background:* There are many reports that the antibody against heat shock protein 60 (Hsp60) is present in most patients with coronary artery disease and atherosclerosis, and that its titer correlates with disease severity. However, few reports have described the association between anti-Hsp60 antibody and cerebrovascular disease. *Methods:* We determined the anti-Hsp60 antibody titer in patients with neurologic diseases and healthy subjects using enzyme-linked immunosorbent assay (ELISA) and evaluated their findings of brain magnetic resonance imaging (MRI) of the white matter. White matter hyperintensities (WMHs) on T2-weighted and fluid-attenuated inversion recovery (FLAIR) images were classified into 2 categories: periventricular hyperintensity (PVH) and deep white matter hyperintensity (DWMH). The lesions in each category were then divided into 4 grades (grades 0-3) according to the Fazekas rating scale. *Results:* There were no significant differences in the titer between patients with neurologic diseases and healthy subjects. The mean grade of DWMHs (mean  $\pm$  SD,  $1.56 \pm 0.70$ ) was significantly higher in 18 subjects in the high-titer group ( $\geq 39.8$  ng/mL; mean titer + 2 SD in sera from 23 healthy subjects) than in 86 subjects (mean  $\pm$  SD,  $0.09 \pm 0.76$ ) in the normal-titer group ( $< 39.8$  ng/mL;  $P < .003$ ). The mean grade of PVHs (mean  $\pm$  SD,  $1.50 \pm 0.71$ ) was also significantly higher in the high-titer group than in the normal-titer group (mean  $\pm$  SD,  $1.17 \pm 0.62$ ;  $P < .02$ ). *Conclusions:* A significant correlation was noted between anti-Hsp60 antibody titer and the severity of WMHs on brain MR images. We suggest that an elevated titer of the anti-Hsp60 antibody could be a risk factor for cerebral small-vessel disease. **Key Words:** Autoantibody—endothelial cell—enzyme-linked immunosorbent assay—heat shock protein 60—white matter hyperintensity.  
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Human heat shock protein 60 (Hsp60) is a molecular chaperone that participates in the folding of mitochondrial proteins and facilitates the proteolytic degradation of misfolded or denatured proteins.<sup>1</sup> It has been recently reported that Hsp60 was detected on the membrane surface of stressed human endothelial cells<sup>2,3</sup> and that the antibody against human Hsp60 induces endothelial cell cytotoxicity.<sup>4,5</sup> Moreover, it was also reported that Hsp60 is an important target of anti-endothelial cell antibodies (AECAs) and that such an interaction contributes to pathogenic effects, particularly in vasculitis-associated systemic autoimmune diseases.<sup>6</sup> The anti-Hsp60 antibody is present in most patients with coronary artery disease<sup>7,8</sup>

and atherosclerosis,<sup>9-11</sup> and its titer correlates with disease severity.<sup>8</sup> However, few reports have described the association between anti-Hsp60 antibody and cerebrovascular disease.<sup>12,13</sup> Patients with systemic autoimmune diseases frequently show the abnormal findings of white matter lesions on brain magnetic resonance imaging (MRI) scans.<sup>14-16</sup> Previously, we reported the case of a patient with neuropsychiatric systemic lupus erythematosus (NPSLE) whose brain MRI scan revealed extended white matter hyperintensities (WMHs) on T2-weighted and fluid-attenuated inversion recovery (FLAIR) images that appeared to represent cerebral small-vessel disease (SVD).<sup>17</sup> The titer of the anti-Hsp60 antibody in serum from this patient was markedly higher than those from other NPSLE patients without WMHs and healthy controls. In this study, we aimed to determine whether an elevated titer of the anti-Hsp60 antibody is associated with cerebral SVD. We then determined the titer of the anti-Hsp60 antibodies in patients with neurologic diseases and evaluated the MRI findings of their white matter.

## Methods

### *Study Population*

Serum samples were obtained from 180 patients with neurologic diseases and 23 healthy subjects. Among the patients with neurologic diseases, 18 had multiple cerebral infarctions (MCIs), 69 had neurodegenerative diseases, 61 had neuroinflammatory diseases, and 32 had systemic autoimmune diseases complicated by neurologic symptoms. Among the patients with neurodegenerative diseases, 16 had Alzheimer disease (AD), 13 had amyotrophic lateral sclerosis (ALS), 22 had Parkinson disease (PD), and 18 had spinocerebellar degeneration (SCD). Among the patients with neuroinflammatory diseases, 11 had Guillain-Barré syndrome (GBS), 13 had meningitis, 26 had multiple sclerosis (MS), and 11 had neuromyelitis optica (NMO). Among the patients with systemic autoimmune diseases complicated by neurologic symptoms, 15 had systemic lupus erythematosus (SLE) and 17 had Sjögren syndrome (SS). The serum samples from the patients with neuroinflammatory diseases and patients with systemic autoimmune diseases complicated by neurologic symptoms were obtained in active phase, and the other samples were obtained in inactive phase. We evaluated the atherosclerotic risk factors (i.e., hypertension, diabetes mellitus, and hyperlipidemia) of these patients. The criteria for hypertension were a systolic blood pressure  $\geq 140$  mm Hg and/or a diastolic blood pressure  $\geq 90$  mm Hg, or current treatment with antihypertensives. The diabetes mellitus group included subjects who were diagnosed as having diabetes by the investigators on the basis of standard criteria or who were already treated with antidiabetic drugs. Hypercholesterolemia was defined as having a total serum cholesterol level  $\geq 220$  mg/dL (5.67 mmol/L) or current

treatment with cholesterol-lowering drugs. This study was approved by the Institutional Review Board of the Gifu University Graduate School of Medicine, Gifu City, Japan.

### *Determination of Anti-Hsp60 Antibody Titer*

Using enzyme-linked immunosorbent assay (ELISA), we determined the titer of anti-Hsp60 antibody in patients with neurologic diseases and healthy subjects. We carried out ELISA to analyze the reactivities of autoantibodies against human Hsp60, which were measured using an ELISA kit (Stressgen; Ann Arbor, MI). Sera diluted at 1:1000 in a dilution buffer were added to a pre-coated ready to use recombinant human Hsp60 immunoassay plate and then incubated for 2 hours at room temperature. After 4 washes, peroxidase-conjugated anti-human immunoglobulins G, A, or M were added to each well and then incubated for 1 hour at room temperature. After 4 washes, a stabilized tetramethylbenzidine substrate was added to each well and then incubated for 15 minutes at room temperature. The reaction was stopped by adding an acid stop solution, and the plate was read at 450 nm on a microplate reader. The Optical Density (OD) of control wells without Hsp60 was subtracted from the OD of Hsp60-coated wells. Serial dilutions of serum samples from healthy blood donors having high titers of the antibody against the tested Hsp60 were used as standards.

### *Assessment of MRI Scans*

Ninety of the 180 patients with neurologic disease and 14 of 23 healthy subjects who showed no risk factors were investigated for the MRI findings of the white matter. MRI scans were analyzed by 2 experienced neurologists for white matter abnormalities. WMHs on T2-weighted and FLAIR images were classified into 2 categories: periventricular hyperintensity (PVH) and deep white matter hyperintensity (DWMH). The lesions in each category were then divided into 4 grades (grades 0-3) according to the Fazekas rating scale.<sup>18,19</sup> PVHs were rated as follows: grade 0, no PVHs; grade 1, "caps" or pencil-thin lining; grade 2, smooth "halo"; or grade 3, irregular PVH extending into the deep white matter. DWMHs were rated as follows: grade 0, no DWMHs; grade 1, punctate foci; grade 2, initial confluence of foci; or grade 3, large confluent areas.

### *Statistical Analysis*

The Mann-Whitney *U* test and nonparametric analysis of variance (Kruskal-Wallis test) were used to assess the significance of differences in continuous variables between groups.  $P < .05$  was considered statistically significant.

## Results

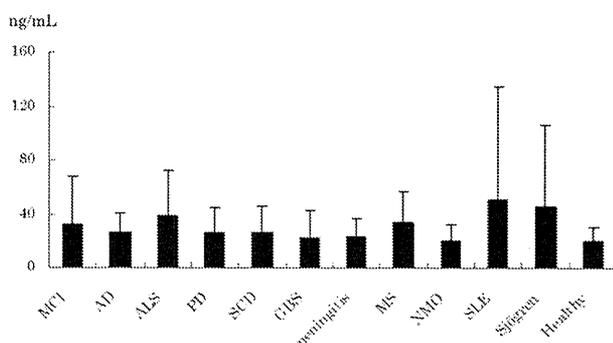
### Determination of Anti-Hsp60 Antibody Titer in Patients with Neurologic Diseases and Healthy Subjects

We determined the titer of the anti-Hsp60 antibody in sera from all the patients with neurologic disease and healthy subjects. The mean titers of the anti-Hsp60 antibody were  $31.9 (\pm 36.0 \text{ SD})$  ng/mL in the MCI patients,  $25.9 (\pm 14.6 \text{ SD})$  ng/mL in the AD patients,  $38.9 (\pm 33.5 \text{ SD})$  ng/mL in the ALS patients,  $26.2 (\pm 18.1 \text{ SD})$  ng/mL in the PD patients,  $25.9 (\pm 19.8 \text{ SD})$  ng/mL in the SCD patients,  $22.1 (\pm 20.1 \text{ SD})$  ng/mL in the GBS patients,  $23.6 (\pm 13.2 \text{ SD})$  ng/mL in the meningitis patients,  $33.2 (\pm 23.2 \text{ SD})$  ng/mL in the MS patients,  $20.7 (\pm 12.1 \text{ SD})$  ng/mL in the NMO patients,  $51.1 (\pm 83.8 \text{ SD})$  ng/mL in the SLE patients, and  $46.0 (\pm 60.4 \text{ SD})$  ng/mL in the SS patients (Fig 1). The mean titer of anti-Hsp60 antibodies in healthy subjects was  $20.0 (\pm 9.86 \text{ SD})$  ng/mL. The titers of anti-Hsp60 antibodies in patients with systemic autoimmune diseases (SLE and SS) complicated by neurologic symptoms tended to be higher than those in patients with other neurologic diseases and in healthy subjects, but this difference did not reach statistical significance. There were no significant differences in the titer between patients with neurologic diseases and healthy subjects (Fig 1).

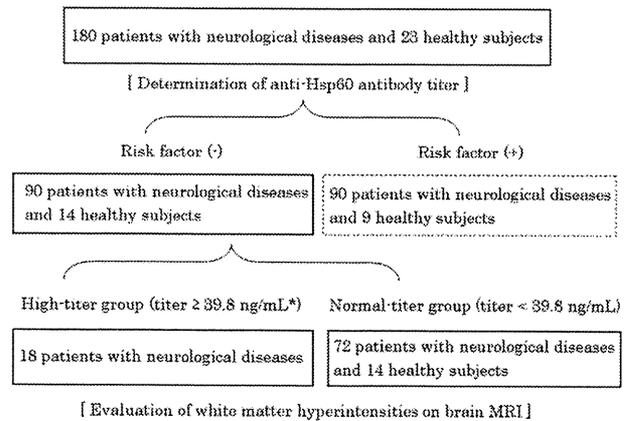
To evaluate the association between anti-Hsp60 antibody titer and the findings of white matter on brain MRI scans, we defined that the mean titer +2 SD in the sera from 23 healthy subjects was the cut-off value (39.8 ng/mL). We divided 90 patients and 14 healthy subjects who showed no risk factors and underwent brain MRI scans in 2 groups. Eighteen patients whose anti-Hsp60 antibody titers were above the cut-off value were classified into the high-titer group, and the remaining 72 patients with neurologic diseases and 14 healthy subjects whose anti-Hsp60 antibody titers were below the cut-off value were classified into the normal-titer group (Fig 2).

### Characterization of the High-Titer and Normal-Titer Groups

Among the 18 patients of the high-titer group, 1 had AD, 3 had ALS, 2 had PD, 1 had SCD, 1 had meningitis, 5 had MS, 3



**Figure 1.** Titers of anti-heat shock protein 60 antibody in patients with neurologic diseases and healthy subjects.



**Figure 2.** Study design. The asterisk indicates the cut-off value (mean titer + 2 standard deviations in sera from 23 healthy subjects).

had SLE, and 2 had SS. Among the 86 patients in the normal-titer group, 2 had MCI, 9 had AD, 2 had ALS, 9 had PD, 8 had SCD, 2 had GBS, 5 had meningitis, 13 had MS, 6 had NMO, 7 had SLE, and 9 had SS. There were 14 healthy subjects in the normal-titer group. The high-titer group had 9 females and 9 males, and the normal-titer group had 54 females and 32 males. The mean age of the high-titer group was 58.0 years ( $\pm 13.9 \text{ SD}$ ), and that of the normal-titer group was 57.9 years ( $\pm 14.2 \text{ SD}$ ). The mean titer of the anti-Hsp60 antibody of the high-titer group was  $89.2 (\pm 78.4 \text{ SD})$  ng/mL, and that of the normal-titer group was  $19.6 (\pm 8.56 \text{ SD})$  ng/mL (Table 1).

### MRI Findings of Patients and Healthy Subjects

According to the Fazekas rating scale, DWMHs of 90 patients and 14 healthy subjects were categorized as grade 0 ( $n = 24$ ), grade 1 ( $n = 50$ ), grade 2 ( $n = 27$ ), or grade 3 ( $n = 3$ ). In the high-titer group, DWMHs were rated as grade 3 in 1 patient, grade 2 in 9 patients, grade 1 in 7 patients, and grade 0 in 1 patient. In the normal-titer group, DWMHs were rated as grade 3 in 2 patients, grade 2 in 18 patients, grade 1 in 43 patients, and grade 0 in 23 patients. PVHs of 90 patients and 14 healthy subjects were categorized as grade 0 ( $n = 8$ ), grade 1 ( $n = 68$ ), grade 2 ( $n = 24$ ), or grade 3 ( $n = 4$ ). In the high-titer group, PVHs were rated as grade 3 in 1 patient, grade 2 in 8 patients, grade 1 in 8 patients, and grade 0 in 1 patient. In the normal-titer group, PVHs were rated as grade 3 in 3 patients, grade 2 in 16 patients, grade 1 in 60 patients, and grade 0 in 7 patients.

### Relationship Between Anti-Hsp60 Antibody Titer and WMHs on Brain MRI

The mean grade of DWMHs ( $1.56 \pm 0.70 \text{ SD}$ ) was significantly higher in the high-titer group than in the normal-titer group ( $0.09 \pm 0.76 \text{ SD}$ ;  $P < .003$ ). The mean grade of PVHs ( $1.50 \pm 0.71 \text{ SD}$ ) was also significantly

**Table 1.** Characteristics of the high- and normal-titer groups

Characteristic	High-titer group (n = 18)	Normal-titer group (n = 86)
Sex (female:male)	9:9	54:32
Age, y (mean $\pm$ SD)	58.0 $\pm$ 13.9	57.9 $\pm$ 14.2
Anti-Hsp60 antibody titer (mean $\pm$ SD, ng/mL)	89.2 $\pm$ 78.4	19.6 $\pm$ 8.56
Grade of DWMH (mean $\pm$ SD)	1.56 $\pm$ 0.70 <sup>†</sup>	0.99 $\pm$ 0.76
Grade of PVH (mean $\pm$ SD)	1.50 $\pm$ 0.71 <sup>‡</sup>	1.17 $\pm$ 0.62

Abbreviations: DWMH, deep white matter hyperintensity; Hsp60, heat shock protein 60; PVH, periventricular hyperintensity; SD, standard deviation.

<sup>†</sup> $P < .003$ , Comparison between the high- and normal-titer groups.

<sup>‡</sup> $P < .02$ , Comparison between the high- and normal-titer groups.

higher in the high-titer group than in the normal-titer group (1.17  $\pm$  0.62 SD;  $P < .02$ ; Table 1).

## Discussion

In this study, we determined the titer of the anti-Hsp60 antibody using ELISA and analyzed the brain MRI findings of the patients with high titers of this antibody. Our findings suggest a significant relationship between anti-Hsp60 antibody titer and the severity of WMHs on brain MRI scans. There is a previous report describing the association between anti-Hsp60 antibody and cerebrovascular disease.<sup>12</sup> However, our study showed the association between anti-Hsp60 antibody titer and WMHs on brain MRI scans for the first time. There is neuropathologic evidence that confluent MRI white matter lesions in the elderly reflect ischemic brain damage related to microangiopathy of cerebral small vessels.<sup>20-22</sup> Endothelial activation and dysfunction may play a causal role in the pathogenesis of cerebral SVD.<sup>23</sup> The abnormal WMHs on brain MRI scans may be related to the microangiopathy associated with vascular endothelial cell dysfunction of cerebral small vessels. Several risk factors have been identified for cerebral SVD.<sup>24,25</sup> Hypertension and age were the major risk factors for SVD.<sup>26-28</sup> It has been proposed that other risk factors are required to evaluate SVD. We suggest that an elevated anti-Hsp60 antibody titer could be a risk factor for cerebral SVD. Further studies using a large series of patients and controls are required to clarify the relation between anti-Hsp60 antibody titer and cerebral SVD.<sup>5</sup>

The pathogenicity of the anti-Hsp60 antibody remains unclear. Human Hsp60 is a molecular chaperone that participates in the folding of mitochondrial proteins and facilitates the proteolytic degradation of misfolded or denatured proteins.<sup>1</sup> However, it was reported that Hsp60 was detected on the membrane surface of stressed human endothelial cells,<sup>2,3</sup> and the antibody against human Hsp60 induces endothelial cell cytotoxicity.<sup>4,5</sup> Hsp60 is ubiquitous and structurally highly conserved molecules. Because of the strong homology between microbial and human Hsp60, an immune reaction against infectious

microbes may lead to a cross-reactive autoimmune response against the native human Hsp60, which is increasingly produced and expressed cell membranes in stressed endothelial cells.<sup>3,29,30</sup> Recently, one of the antigens that reacted with AECAs in sera from SLE patients has been identified as human Hsp60.<sup>2</sup> The anti-Hsp60 antibody is present in most patients with coronary artery disease,<sup>7,8</sup> and its titer correlates with disease severity.<sup>8</sup> Moreover, the anti-Hsp60 antibody could contribute to the initiation or amplification of vascular endothelial cell damage, a crucial event in atherosclerosis<sup>9-11</sup> and Kawasaki disease.<sup>31</sup> As determined from these reports, it is possible that the anti-Hsp60 antibody could react with Hsp60 expressed on the surface membrane of stressed endothelial cells of cerebral small vessels and might be related to their dysfunction corresponding to cerebral WMHs on brain MRI scans. However, more studies are required to clarify whether the anti-Hsp60 antibody indeed play a role in the dysfunction of endothelial cells of cerebral small vessels.

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# Antibodies Against the Tom40 Subunit of the Translocase of the Outer Mitochondrial Membrane Complex and Cognitive Impairment in Alzheimer's Disease

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**Abstract.** Recent studies suggest that microvascular abnormalities are involved in pathology and progression of Alzheimer's disease. The purpose of this study was to examine the presence of antibodies against cerebral microvascular endothelial cells specific for Alzheimer's disease, and to evaluate the association of these antibodies with cognitive impairment. The study included patients with Alzheimer's disease (age  $\geq 60$  years; 24 patients), control subjects without neurological diseases (age  $\geq 60$  years; 19 subjects), patients with multiple sclerosis (all ages; 17 patients), and healthy control subjects (age  $< 40$  years; 18 subjects). Serum was analyzed with 2-dimensional electrophoresis and Western blot, with cultured human brain microvascular endothelial cells as the antigen source. The anti-Tom40 antibody was identified significantly more frequently in patients with Alzheimer's disease than control subjects or patients with multiple sclerosis. In patients with Alzheimer's disease, the mean scores for the Mini-Mental State Examination were significantly lower for patients who were positive for anti-Tom40 antibody than those who were negative for anti-Tom40 antibody. In summary, the anti-Tom40 antibody is significantly associated with cognitive impairment in patients with Alzheimer's disease.

**Keywords:** Antigen, cognition, dementia, endothelial cell, mitochondrion, pathogenesis

## INTRODUCTION

Regional microvascular abnormalities have been identified around blood vessels in the brain in autopsies of patients with Alzheimer's disease (AD), and these abnormalities may precede cognitive and neurodegenerative changes in AD and other cerebrovascular diseases with or without amyloid deposition [1, 2].

Inflammation of microvascular endothelial cells of the brain may contribute to disease processes in AD [3, 4]. Patients with AD have a high titer of anti-brain autoantibodies [5, 6]. Autoantibodies to ecto-F1-ATPase, which could exert a pathogenetic role in AD, may be found in serum and cerebrospinal fluid from patients with AD [7].

We initially hypothesized that the anti-endothelial cell antibodies may be a pathogenetic factor contributing to cognitive impairment in AD. We examined anti-endothelial cell antibodies in the serum from patients with AD and target antigens by 2-dimensional electrophoresis, followed by Western blotting and

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liquid chromatography with tandem mass spectrometry. The target antigen of our detected antibody in the serum from patients with AD was the translocase of the outer mitochondrial membrane 40 kDa subunit (Tom40), which was present in not only endothelial cells but also other tissues including neurons. Interestingly, a recent study showed that the gene polymorphism encoding this protein predicts the age of onset of late-onset AD [8]. Therefore we hypothesized that this antibody may be associated with cognitive impairment in AD, and we evaluated the relation between the presence of this antibody and cognitive dysfunction in patients with AD.

## MATERIALS AND METHODS

### *Patients*

From May 2006 to April 2010, there were 155 consecutive patients >60 years evaluated as outpatients at the Department of Neurology and Geriatrics, Gifu University Graduate School of Medicine because of memory loss. Of these patients, we randomly selected 24 patients with AD and 19 control subjects without neurological diseases for inclusion in the study. All subjects with AD had physical and neurological examinations, neuropsychological testing, laboratory studies, and brain magnetic resonance imaging to exclude other causes of dementia. All patients with AD met International Classification of Diseases (ICD)-10 criteria for dementia.

We also selected 17 consecutive patients with multiple sclerosis (all ages) who were evaluated at our institution and 18 other healthy volunteers (age <40 years). The study was approved by the institutional review board of the Gifu University Graduate School of Medicine.

### *Serum samples*

Serum samples from all patients and control subjects were collected and stored at  $-30^{\circ}\text{C}$ . All protein spots corresponding to target antigens that reacted with antiendothelial cell antibodies in the serum samples were detected with 2-dimensional electrophoresis after immunoblotting.

### *Primary human brain microvascular endothelial cell proteins*

Primary cultured human brain microvascular endothelial cells (Applied Cell Biology Research

Institute, Kirkland, WA, USA) were used as the antigen source for 2-dimensional immunoblotting. The cultured cells were homogenized in lysis buffer (5 M urea; 2 M thiourea; 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate [CHAPS], 2%; 0.1 M dithiothreitol [DTT]; Pharmalyte [pH, 3 to 10], 2%) containing proteinase inhibitor mixtures (Complete Mini, Roche Diagnostics GmbH, Mannheim, Germany) and centrifuged at  $18\,800 \times g$  for 40 min, and the supernatant was used in all experiments. The protein concentration was determined by a protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA).

### *Electrophoresis, mass spectrometry, and immunoblotting*

The primary cultured human brain microvascular endothelial cell sample was loaded onto an immobilized and rehydrated dry strip (pH, 3 to 10; length, 13 cm) (GE Healthcare, Buckinghamshire, UK). Extracted protein (35 to 50  $\mu\text{g}$ ) was applied to the dry strip for Western blotting. In the slip loading method, isoelectric focusing in the first dimension was performed according to a previously reported method [9]. The electrophoresis in the second dimension was done vertically, using an electrophoresis apparatus (ERICA-S, DRC, Tokyo, Japan) at a constant voltage of 300 V for 2 h. After the electrophoresis, the sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels were stained (SYPRO Ruby, Bio-Rad Laboratories) or used for protein transfer onto polyvinylidene difluoride membranes. Separated proteins were electrophoretically transferred to a polyvinylidene difluoride membrane at a constant voltage of 32 V for 3 h using a buffer transfer tank with cool equipment (ERICA-S, DRC). Subsequently, this membrane was incubated in a blocking solution (5% skim milk in  $1 \times$  TBST;  $1 \times$  Tris-buffered saline containing 0.1% Tween 20) for 1.5 h at room temperature, and then reacted with a patient's serum diluted (1 : 2000), or commercially available rabbit anti-Tom40 polyclonal antibodies (Abnova, Taiwan) diluted (1 : 500), with 1% skim milk in  $1 \times$  TBST overnight in a cold room. The polyvinylidene difluoride membrane was washed 5 times with  $1 \times$  TBST and reacted with peroxidase-conjugated goat anti-human immunoglobulin (IgA, IgG, and IgM) antibodies (Life Technologies, Carlsbad, CA, USA) that had been diluted (1 : 2000), or peroxidase-conjugated goat anti-rabbit IgG antibodies (Santa Cruz Biotechnology, CA, USA) that had been diluted (1 : 8000), with 1% skim

milk in  $1 \times$  TBST for 1 h at room temperature. After 6 washes, the membrane was incubated with the Western blot detection reagent (ECL-Plus, GE Healthcare) for 5 min and then scanned using a luminescent image analyzer (LAS-3000 mini, Fujifilm, Kanagawa, Japan). The antibody-reactive protein spots were matched with the stained protein spots (SyproRuby, Bio-Rad Laboratories) using image analysis software (Adobe Photoshop 6.0, Adobe Systems, San Jose, CA, USA). We selected a protein spot that reacted with antibody that frequently appeared in the serum samples from AD patients, and analyzed the target antigen of this selected antibody using nanoscale capillary liquid chromatography (LC-VP, Shimadzu Corp., Kyoto, Japan) and an ion trap tandem mass spectrometer (LCQ Advantage MAX, Thermo Fisher Scientific, Waltham, MA, USA).

### Cognitive evaluation

The cognitive status in patients with AD was evaluated using the Mini-Mental State Examination.

### Data analysis

Fisher exact test was used for analysis of frequency data, and the Mann-Whitney test was used for continuous variable data. Statistical significance was defined by  $p < 0.05$ .

## RESULTS

We identified a single spot that reacted with antiendothelial cell antibody, and the antibody was detected significantly more frequently in patients with AD than control subjects or patients with multiple sclerosis (Fig. 1, Table 1). This immunoreactive spot was identified as translocase of the outer mitochondrial membrane 40 kDa subunit (Tom40) (accession number, O96008; Mascot score, 54; theoretical MW/pI, 37893/6.79). The Mascot score is defined as  $S = -10 * \text{Log}(P)$ , where P is the probability that the observed match is a random event; Mascot scores greater than 37 are significant. The 2-dimensional immunoblotting, with anti-Tom40 antibody, of serum from patients with AD and polyclonal anti-Tom40 antibody identified the spot corresponding to Tom40 (Fig. 1).

Cognitive evaluation of patients with AD showed that mean scores for the Mini-Mental State Examination were significantly lower for patients who were positive for anti-Tom40 antibody than those who were negative for anti-Tom40 antibody (Table 2).

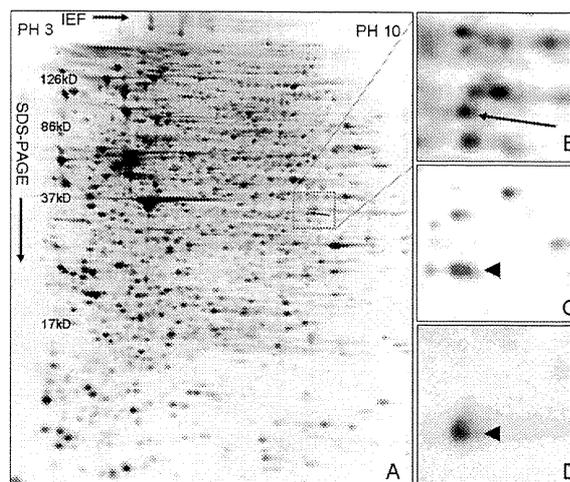


Fig. 1. Electrophoresis and immunoblotting gels of serum from a patient with AD. A, B) Total protein extracts of homogenized primary cultured human brain microvascular endothelial cells were separated by 2-dimensional (2D) electrophoresis and stained. Arrows indicate the spot that corresponded to the translocase of the outer mitochondrial membrane 40 kDa subunit Tom40. C) Polyvinylidene difluoride membrane after 2D immunoblotting of serum (dilution, 1 : 2000) of a patient with AD. D) Polyvinylidene difluoride membrane after 2D immunoblotting of polyclonal anti-Tom40 antibody (dilution, 1 : 500). The arrowhead indicates the protein spot corresponding to Tom40. IEF, isoelectric focusing. SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

Table 1

Characteristics of patients and presence of antibodies against the mitochondrial outer membrane antigen Tom40

	Alzheimer's disease	Control subjects with no neurological disease	Multiple sclerosis
Age group	$\geq 60$ y	$\geq 60$ y	$< 40$ y
No. patients	24	19	18
Gender, male <sup>a</sup>	10 (42)	10 (53)	10 (56)
Age (y) <sup>b</sup>	78	72	27
	(60 to 88)	(60 to 83)	(16 to 35)
Anti-Tom40 <sup>c</sup>			
positive <sup>a</sup>	10 (42)	1 (5.3)	2 (11)
$p <^d$	—	0.001	0.04

<sup>a</sup>Reported as number (%) patients; <sup>b</sup>Reported as mean (range); <sup>c</sup>anti-Tom40, antibodies against translocase of the outer mitochondrial membrane 40 kDa subunit Tom40; <sup>d</sup>Comparison with patients with Alzheimer's disease.

Table 2

Relation between anti-Tom40 antibody and cognitive scores in patients with Alzheimer's disease

	Anti-Tom40 antibody		$p <$
	Present	Absent	
Number of patients	10	10 <sup>b</sup>	
Mini-Mental State Examination <sup>a</sup>	$16 \pm 4$	$19 \pm 5$	0.05

<sup>a</sup>Reported as mean  $\pm$  SD; <sup>b</sup>The Mini-Mental State Examination was available only in 10 of the 14 patients who did not have anti-Tom40 antibody.