一型認知症患者の剖検脳ではニューロンに免疫グロブリンの沈着が有意に多くみられることが報告されている（Levin et al., Brain research. 2010）。

今回、晩期発症型アルツハイマー型認知症患者の血清中に特異的に存在する抗血管内皮細胞抗体の認識抗原蛋白の一つとして Mitochondrial import receptor subunit TOM40 homolog (Tom40)を同定した。Tom40 はミトコンドリア外膜に存在し、ミトコンドリア内への蛋白の選択輸送に関与するチャンネルを形成していることが知られている。Tom40 をコードする遺伝子 TOMM40 は 19 番染色体上の APOE 遺伝子近傍に位置し、最近のゲノムワイド関連解析の結果、TOMM40 のリスクアリルはコントロールと比較し晩期発症型アルツハイマー型認知症の発症が２倍になる（Potkin et al., PLoS ONE, 2009）といった報告や TOMM40 のイントロン変異（poly-T insertion）を持つ患者は、晩期発症型アルツハイマー型認知症の発症が約 7 年早くなる（Rosese et al., Pharmacogenomics J, 2010）といった報告もある。一方、アルツハイマー型認知症患者の脳内においては、エネルギー代謝障害と低還流状態がおきているとされている。ミトコンドリア機能障害及び血管障害はアルツハイマー型認知症の病態において極めて重要な役割を担っているものの、その原因に関しては十分解明されていない。健常者にはなくアルツハイマー型認知症患者の脳内のみで、アミロイド前駆体蛋白がミトコンドリアの蛋白輸送に関連するチャンネルに沈着しているといった報告や（Devi L et al., J Neurosci. 2006）。アミロイド前駆体蛋白が、Tom40 と結合し 480kDa の安定した複合体を形成する、もしくはアミロイド前駆体蛋白が Tom40 とミトコンドリア内膜の蛋白である Tim23 と結合し、620kDa のさらに大きな複合体を形成することによりミトコンドリア内への蛋白輸送が障害されミトコンドリア機能障害が生じるといった報告もある（Anandatheerthavarada HK et al., J Cell Biol. 2003）。一方、Aβ のミトコンドリア内への輸送が Tom40 を介して行われ、神経毒性を生じるといった報告もある（Hansson Petersen CA et al., Proc Natl Acad Sci. USA, 2008）。これらの報告が示すように Tom40 はアルツハイマー型認知症の病態機序、特にミトコンドリア機能障害の原因を考える上で極めて重要な分子であることが予想される。

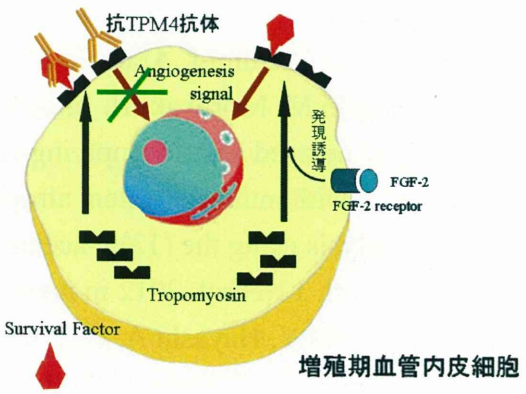
今回、抗 Tom40 抗体はアルツハイマー型認知症患者の認知機能障害と有意な関連性が認められた。抗 Tom40 抗体の病的意義に関しては不明であるが、Tom40 の局在性を考慮すると、抗体が病態に直接的な影響を及ぼすのは困難と思われる。しかし、自己抗体が細胞内に選択的に取り込まれ、細胞内抗原と結合することを指摘した報告もあり（Deng SX et al., Int Immunol. 2000; Seddiki N et al., J Immunol. 2001）、今後は抗 Tom40 抗体の病態に及ぼす影響につき、培養細胞や免疫動物などを用いて検討する必要があると考えられた。

また同じく今回アルツハイマー形認知症患者より新たに同定した Protein

disulfide isomerase（PDI）に関しては、蛋白のジスルフィド結合を触媒することにより蛋白のフォールディングを促進することが知られている。最近アルツハイマー型認知症患者の神経原線維変化中に、このPDI陽性の封入体が存在することが報告された（Honjo et al., Brain Res, 2010）。今後、これら自己抗体とアルツハイマー型認知症の病態との関連性を検討することにより、同疾患の病態機序の解明ならびに新たな診断マーカーの確立と治療法の開発につながる可能性も考えられた。

大脳微小血管内皮細胞障害は、認知機能障害と歩行障害などの運動機能障害をきたす原因となりうる大脳白質病変と密接に関連し、その血清学的診断マーカーの確立や治療法の開発は極めて重要と考えられる。今回同定した抗血管内皮細胞抗体以外にも、新たなバイオマーカーとなり得る抗血管内皮細胞抗体が存在する可能性があり、今後は、今回用いた二次元免疫ブロット法に変わる新たな自己抗体の検出法の開発も重要と考える。

図９）抗TPM4抗体の血管新生阻害作用機序の仮説



（Donate F, et al. Current Cancer Drug Targets 2004より一部改変）

E. 結論

1. 高齢者は若年者に比較し、多くの抗血管内皮細胞抗体を有する
2. 広範な大脳白質病変と関連することが予想される抗血管内皮細胞抗体が存在する。
3. 抗TPM4抗体高値は、大脳深部白質の癒合性病変の出現に関連する独立した危険因子である。
4. TPM4は大脳微小血管内皮細胞の細胞膜表面にも発現している。
5. 抗TPM4抗体は、大脳微小血管内皮細胞のangiogenesisを抑制することにより大脳白質病変の進展に関っている可能性がある。
6. 抗Tom40抗体は、アルツハイマー型認知症患者の認知機能障害と関連する可能性がある。

F. 健康危険情報

なし

G. 研究発表

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H. 知的財産権の出願・登録状況

1. 特許取得
   なし
2. 実用新案登録
   なし
3. その他
   なし

Ⅱ　研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Sakurai T, Kimura A, Yamada M, Koumura A, Hayashi Y, Tanaka Y, Hozumi I, Inuzuka T	Identification of antibodies as biological markers in serum from multiple sclerosis patients by immunoproteomic approach	Journal of Neuroimmunology	233	175-180	2011
Hozumi I, Hasegawa T, Honda A, Ozawa K, Hayashi Y, Hashimoto K, Yamada M, Koumura A, Sakurai T, Kimura A, Tanaka Y, Satoh M, Inuzuka T	Patterns of levels of biological metals in CSF differ among neurodegenerative diseases	Journal of the Neurological Sciences	303	95-99	2011
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Yamaguchi Y, Hayashi A, Campagnoni CW, Kimura A, Inuzuka T, Baba H	L-MPZ, a novel isoform of myelin P0, is produced by stop codon readthrough	Journal of Biological Chemistry		In press	2012

Ⅲ　研究成果の刊行物・別刷り

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Identification of antibodies as biological markers in serum from multiple sclerosis patients by immunoproteomic approach

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ABSTRACT

We identified the antibody against mitochondrial heat shock protein 70 (mtHSP70) in serum from multiple sclerosis (MS) patients by proteomics-based analysis. The prevalence of the anti-mtHSP70 antibody is significantly higher in serum from MS patients than in serum from Parkinson disease patients, multiple cerebral infarction patients, infectious meningoencephalitis patients, and healthy controls (HCs) (68% sensitivity; 74% specificity). We studied the clinical features and magnetic resonance imaging findings of MS patients with the anti-mtHSP70 antibody. As a result, there were no significant differences between the anti-mtHSP70-antibody-positive and -negative MS patients. Additionally, in our comprehensive analysis of the prevalence of both the anti-mtHSP70 antibody and the anti-phosphoglycerate mutase 1 (PGAM1) antibody, which was previously reported by us to also show a higher prevalence in serum from MS patients, the positivity rates of both these antibodies were significantly higher in serum from MS patients than in serum from patients with other neurological diseases and from HCs; moreover, the specificity of this combination assay was higher than that of the assay of only one antibody (57% sensitivity; 93% specificity). Results of our study suggest that not only the anti-PGAM1 antibody but also the anti-mtHSP70 antibody is good diagnostic markers of MS and the combination of both these antibodies is useful for a more specific diagnosis of MS.

1. Introduction

The mechanism underlying the pathogenesis of multiple sclerosis (MS) is considered to mainly involve cell-mediated immunity; however, recently, humoral immunity has also been noted. The lesions of MS are pathologically found to show four fundamentally different patterns of demyelination: T cell-mediated or T cell-plus antibody-mediated autoimmune encephalomyelitis (patterns I and II) and a primary oligodendrocyte dystrophy, similar to virus- or toxin-induced demyelination (patterns III and IV) (Luchinetti et al., 2000). MS patients with the pattern II pathology are more likely to respond favorably to therapeutic plasma exchange (Keegan et al., 2005). Recently, B cell targeting therapy in a group of MS patients has shown encouraging preliminary results (Hauser et al., 2008). These findings suggest that humoral immunity in part plays a role in the pathophysiology of MS. A previous study showed that several antibodies against proteins, such as myelin oligodendrocyte glycoprotein (MOG), myelin basic protein (MBP), proteolipid protein peptide (PLP), Nogo-A, and heat shock protein 60 (HSP60), are present in MS patients (Reindl et al., 2006). However, no autoantibodies that would enable the discrimination between MS patients and healthy controls have been identified to date.

Recently, an immunoproteomic approach has been used in various methods for searching autoantibodies associated with MS and other autoimmune diseases such as neuropsychiatric systemic lupus erythematosus, and Hashimoto's encephalitis (Lefranc et al., 2007; Gini et al., 2008; Mathey et al., 2007; Lovato et al., 2008; Kimura et al., 2010).

In this study, we used proteomics-based analysis to screen for antibodies specifically found in MS patients. As a result, we identified the antibody against mitochondrial heat shock protein 70 (mtHSP70) in serum from MS patients. To evaluate the specificity of this antibody, we assessed its prevalence in serum from MS patients, Parkinson disease (PD) patients, multiple cerebral infarction (MCI) patients, infectious meningoencephalitis (IME) patients, and healthy controls (HCs). We also studied the clinical features and magnetic resonance imaging (MRI) findings of MS patients with this antibody. Additionally, we comprehensively analyzed the prevalence of both the anti-mtHSP70 antibody and the anti-phosphoglycerate mutase 1 (PGAM1) antibody, which was previously reported by us to also show a higher prevalence in serum from MS patients than in serum from patients with other neurological diseases and from HCs (Kimura et al., 2010).

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2. Materials and methods

2.1. Patients and serum samples

Serum samples were collected from 25 MS patients [male:female = 13:12; age range, 27–75; mean age, 47]; 21 PD patients [male: female = 11:10; age range, 50–85; mean age, 68]; 19 MCI patients [male:female = 9:10; age range, 57–83; mean age, 72]; 20 IME patients [male:female = 15:5; age range, 15–74; mean age, 47]; and 27 HCs [male:female = 14:13; age range, 16–80; mean age, 48]. All the MS patients were diagnosed as having clinically definite MS in accordance with the criteria of Poser and colleagues (Poser et al., 1983). Among the 25 MS patients, serum samples were collected from 16 patients in relapse and the remaining patients in remission. We examined all the MS patients' clinical data, and brain and spinal cord MR images obtained at the same time we collected the patients' serum samples. Age, gender, disease course and duration, expanded disability status scale (EDSS), complication of optic neuritis, and number of relapses were recorded. We examined the number of cerebral hyperintense lesions, and the presence of hyperintense spinal cord, cerebellar, and brain stem lesions on T2-weighted images (WIs) obtained by brain and spinal cord MRI. We also examined the presence of cerebral atrophy in T1 WIs obtained by brain MRI.

For screening antibodies specifically found in MS patients, we investigated all the target spots corresponding to proteins that reacted with antibodies in the serum from 12 MS patients randomly selected from among the 25 and 12 HCs by two-dimensional (2-D) electrophoresis using the total proteins of rat cerebrums as samples, followed by Western blotting. All the target spots that reacted with antibodies in the serum from the 12 HCs were subtracted from the spots that reacted with antibodies in the serum from the 12 MS patients. After subtraction, a distinctive spot of the remaining target spots was analyzed by mass spectrometry. This study was approved by the institutional review board of the Gifu University Graduate School of Medicine, Gifu City, Japan.

2.2. Preparation of tissue proteins

Under ether anesthesia, adult Wister rats were killed. Their cerebrums were immediately removed and frozen in dry-ice powder. The frozen brain tissue was homogenized using a tissue homogenizer, and protein concentration was determined by Bio-Rad protein assay based on the Bradford method [Life Science (Research, Education, Process Separations, Food/Animal/Environment Testing), Hercules, CA, USA].

2.3. 2-D electrophoresis and immunoblotting

The samples were dissolved in DeStreak rehydration solution (GE Healthcare Bio-Sciences, Piscataway, NJ, USA) and loaded onto an immobilized rehydrated dry strip (pHs 4–7, 13 cm long, GE Healthcare). Up to 100 μg of the proteins was applied to a dry strip for Western blotting. Isoelectric focusing was conducted at 20 °C for 85 000 Vh at a maximum of 8000 V, using a horizontal electrophoresis system (Multiphor III, GE Healthcare). Before separation in the second dimension, isoelectric polyacrylamide gel (IPG) strips were equilibrated for 15 min in a buffer containing 2% sodium dodecyl sulfate (SDS), 6 M urea, 30% volume by volume (v/v) glycerol, 0.001% BPB, and 50 mM Tris–HCl (pH 8.8) under reducing conditions, with 65 mM DTT, followed by further incubation for 15 min in the same buffer under alkylating conditions with 140 mM iodoacetamide. Equilibrated IPG strips were transferred to a 12.5% polyacrylamide gel.

The run in the second dimension was carried out vertically, using an electrophoresis apparatus (ERICA-S, DRC) at a constant voltage of 300 V for 2 h. After the electrophoresis, the SDS-polyacrylamide gel electrophoresis (PAGE) gels were stained with Coomassie Brilliant Blue (CBB) (GelCode Blue Stain Reagent, Pierce) or used for protein transfer onto polyvinylidene difluoride (PVDF) membranes. The separated proteins were electrophoretically transferred to a PVDF membrane at 0.8 mA/cm^2 for 1 h, using a semidry blotting apparatus (TE77 PWR Semi-Dry Transfer Unit, GE Healthcare). The PVDF membrane was stained with a fluorescent total protein stain (Deep Purple Total Protein Stain, GE Healthcare) and scanned using a variable mode imager (Typhoon 9400, GE Healthcare). Subsequently, this membrane was incubated in a blocking solution [5% skim milk in 1× Tris-buffered saline Tween-20 (TBST); 1× Tris-buffered saline (TBS) containing 0.1% Tween 20] overnight in a cold room and after three washes reacted with patient serum, diluted to 1:1500 with 1% skim milk in 1× TBST, for 1 h at room temperature. The PVDF membrane was washed five times with 1× TBST and reacted with peroxidase-conjugated goat anti-human Ig (A + G + M) antibodies (P.A.R.I.S.), diluted to 1:2000 with 1% skim milk in 1× TBST, for 1 h at room temperature. After six washes, the membrane was incubated with the WB detection reagent (ECL Plus, GE Healthcare) for 5 min and scanned using Typhoon 9400. The immunoreactive protein spots were matched with the fluorescent stained total protein spots using an image analysis software (Adobe Photoshop 6.0, Adobe Systems).

2.4. In-gel digestion and mass spectrometric identification of proteins

In-gel digestion and mass spectrometric identification of proteins were performed in accordance with a standard protocol (Yamagata et al., 2002). Briefly, the identified protein spots were excised from the 2-D gels using clean scalpels, and the excised gels were washed twice with Milli-Q water and dehydrated in 100% acetonitrile (ACN) until they turned opaque white. The spots were then dried in a vacuum centrifuge and subsequently rehydrated in 10 μl of digestion solution consisting of 50 mM NH_4HCO_3, 5 mM $CaCl_2$, and 0.01 μg/μl modified sequence-grade trypsin (Promega). After incubation for 16 h at 37 °C the digestion was terminated by adding 10 μl of 5% trifluoroacetic acid (TFA). Peptides were extracted three times for 20 min with 5% TFA in 50% ACN, and the extracts were pooled and dried in a vacuum centrifuge. The dried materials were resuspended with 10 μl of 0.1% TFA. To remove excess salts from the extracts, solid phase extraction was performed using ZipTip C18 (Millipore) in accordance with the manufacturer's instructions. Peptides were eluted from ZipTip using 0.1% TFA in 50% ACN, and 1 μl of the eluants was spotted onto a target plate. Then, the spots on the target plate were immediately mixed with 0.5 μl of a matrix solution containing 0.3 mg/ml α-cyano-hydroxycinnamic acid, 33% acetone, and 66% ethanol, and were completely air-dried at room temperature. Mass spectrometry and tandem mass spectrometry (MS/MS) spectra were obtained using an ultraflex time-of-flight (TOF)/TOF mass spectrometer (Bruker Daltonics). An external peptide mixture was used to calibrate the instrument. Proteins were identified using the MASCOT software (Matrix Science) with the NCBInr database.

2.5. One-dimensional electrophoresis and immunoblotting using human recombinant mtHSP70

For one-dimensional (1-D) immunoblotting analysis, the commercially available, full-length, human recombinant mtHSP70 (Abnova, molecular weight: 100.76 kDa with its N-terminal GST-tag), produced by a method based on the wheat-germ-cell-free expression system, was separated by 4–20% SDS-PAGE. Immunoblotting was carried out as described in Section 2.3 except for blocking for 1 h at room temperature and reaction with patient serum diluted to 1:2000 overnight in a cold room. We tested the serum samples from 25 MS patients, 21 PD patients, 19 MCI patients, 20 IME patients, and 27 HCs.

The MS patients were divided into two groups on the basis of the presence or absence of the anti-mtHSP70 antibody. The group positive for the anti-mtHSP70 antibody was compared with that negative for the antibody to identify specific patterns of clinical features and MRI findings.

Additionally, we analyzed the prevalence of both the anti-mtHSP70 antibody and the anti-PGAM1 antibody in the serum from MS patients, PD patients, MCI patients, IME patients, and HCs. We have already examined the anti-PGAM1 antibody in serum from 17 of 25 MS patients, 21 PD patients, 19 MCI patients, 17 of 20 IME patients, and 17 of 27 HCs (Kimura et al., 2010).

2.6. Statistical analyses

Fisher's exact probability test or the Chi-square test with Yates' continuity correction was used for the analysis of frequency data, and Student's t-test was used for continuous variable data. P values <0.05 were considered significant.

3. Results

3.1. Screening and identification of target antigen of antibodies in serum from MS patients

We detected by 2-D immunoblotting 66 spots that reacted with antibodies in serum from 12 MS patients and 57 spots that reacted with antibodies in serum from 12 HCs. The latter 57 target spots were subtracted from the former 66 spots. After subtraction, there were 35 remaining spots that reacted with antibodies in serum from the 12 MS patients. Among these spots specific for MS patients, we investigated one spot [observed molecular weight (MW)/pI: 67(kDa)/5.8] that reacted with the serum antibodies most commonly observed in MS patients (5 of 12 patients). This spot that corresponded to the protein on the 2-D electrophoresis gels was analyzed by mass spectrometry. This immunoreactive spot was identified as mtHSP70 [accession number, P48721; score/coverage identification (%), 202/11; number of matched peptides, 7; theoretical MW/pI: 74(kDa)/5.9].

Fig. 1 shows the PVDF membrane to which separated proteins were transferred and stained with the fluorescent total protein stain reagent (A) and 2-D immunoblotting using serum from MS patient with the anti-mtHSP70 antibody (B). The spot indicated by arrows reacted with the serum antibodies most commonly observed in MS patients (5 of 12 patients). We analyzed this spot and obtained MS/MS spectra of seven peptides. We show these seven peptides in Fig. 2(A–G). Subsequently, this spot was identified as mtHSP70 using a protein identification software (H).

3.2. Immunoreactivity of serum from MS patients, PD patients, MCI patients, IME patients, and HCs against full-length human recombinant mtHSP70

To evaluate the specificity of the anti-mtHSP70 antibody, we assessed the prevalence of this antibody in serum from MS patients, PD patients, MCI patients, IME patients, and HCs by 1-D immunoblotting using the human mtHSP70 full-length recombinant protein with GST as the antigen (Fig. 3). As a result, the positivity rates were 68% (17 of 25) in MS patients, 28.6% (6 of 21) in PD patients, 26.3% (5 of 19) in MCI patients, 20% (4 of 20) in IME patients, and 29.6% (8 of 27) in HCs. The prevalence of the anti-mtHSP70 antibody was statistically significantly higher in serum from MS patients than in serum from PD patients ($P<0.02$), MCI patients ($P<0.02$), IME patients ($P<0.004$), and HCs ($P<0.02$) (68% sensitivity; 74% specificity).

3.3. Comparison of clinical features and MRI findings between anti-mtHSP70-antibody-positive and -negative MS patients

The comparison between the anti-mtHSP70-antibody-positive and -negative MS patients is shown in Tables 1 and 2. The gender ratio and age were similar between the two groups. Regarding the relapsing–remitting MS (RRMS) state of patients when their serum samples were collected, there was no significant difference between the antibody-positive and -negative MS patients. In addition, no significant differences were found in disease course and duration, EDSS in the relapse state, complication of optic neuritis, or number of

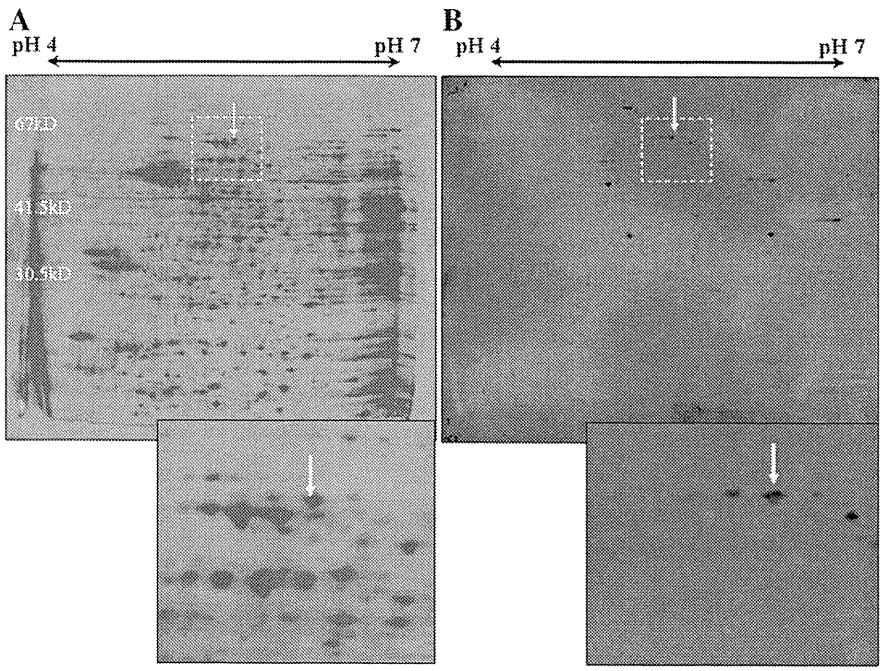

Fig. 1. Polyvinylidene difluoride membrane on to which separated proteins were transferred and stained with fluorescent total protein stain reagent, and two-dimensional immunoblotting result for multiple sclerosis patient with anti-mitochondrial heat shock protein 70 antibody. PVDF membrane on to which separated proteins were transferred and stained with fluorescent total protein stain reagent (A). PVDF membrane reacted with 1:1500-diluted serum from MS patients with anti-mtHSP70 antibody (B). Arrows indicate the spot that we analyzed by mass spectrometry. Abbreviations: MS, multiple sclerosis; mtHSP70, mitochondrial heat shock protein 70; PVDF, polyvinylidene difluoride.

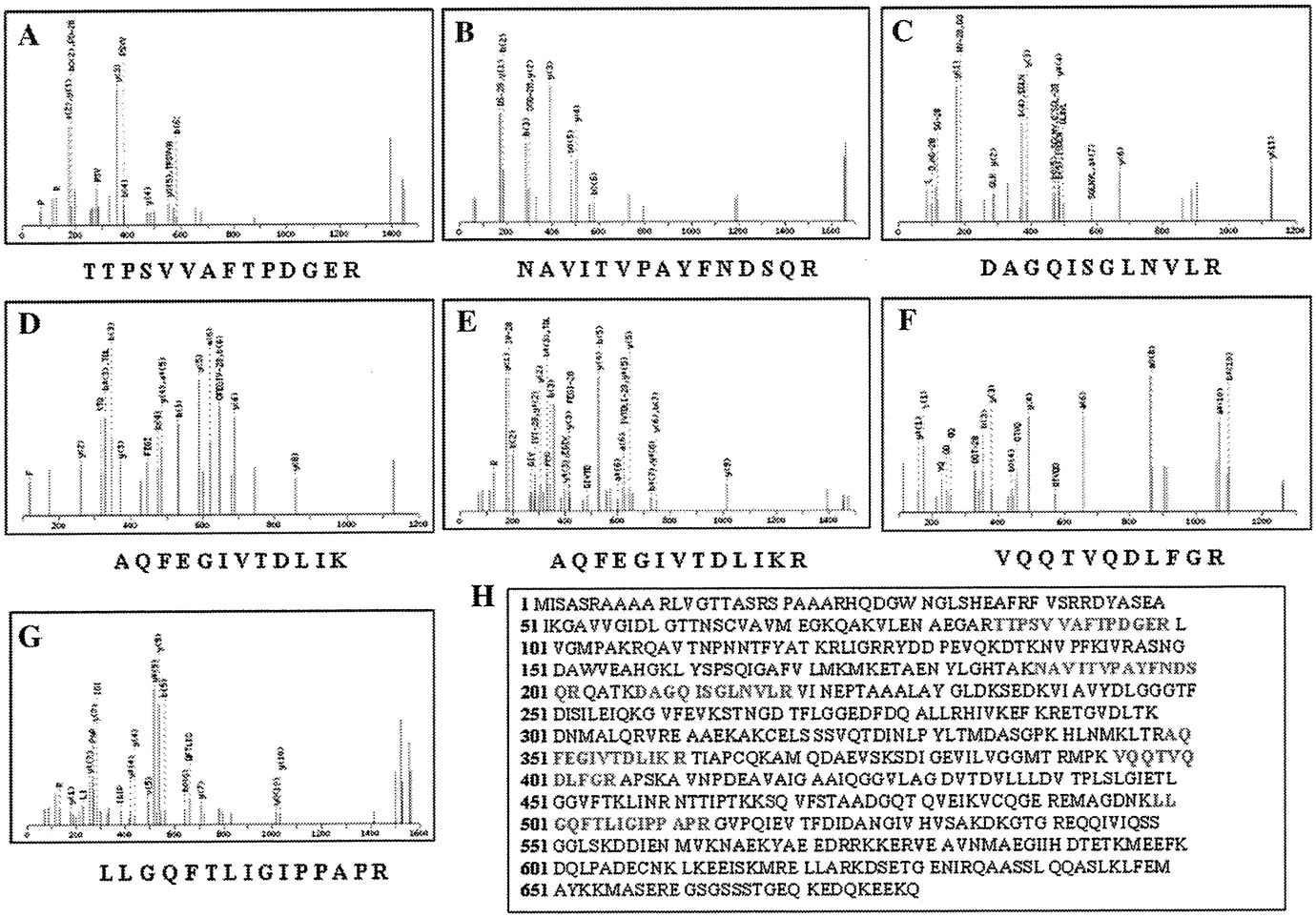

Fig. 2. Identification of mitochondrial heat shock protein 70 by mass spectrometry. MS/MS spectra of seven peptides of mtHSP70 (A–G) and total amino acid sequences of mtHSP70 (H). Sequences in bold red letters indicate the matched sequences of seven peptides. Abbreviations: MS/MS, tandem mass spectrometry; mtHSP70, mitochondrial heat shock protein 70.

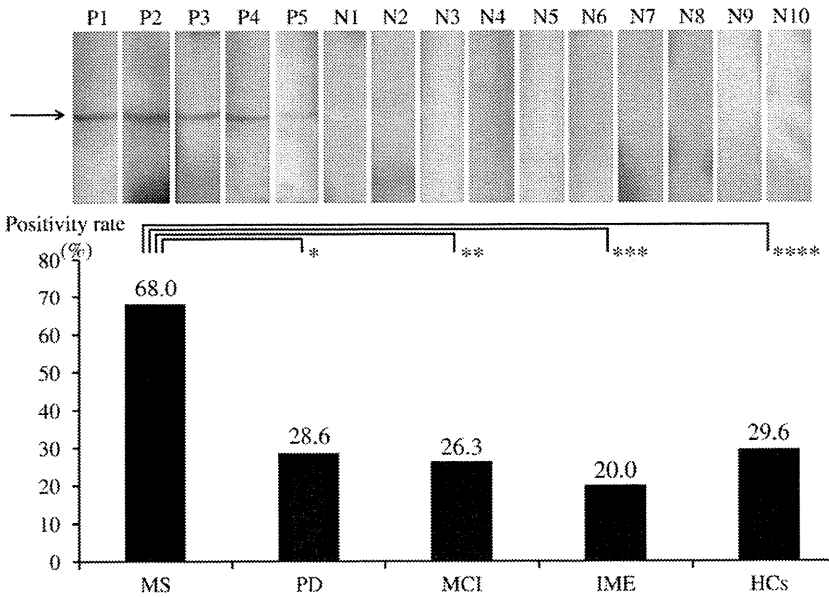

Fig. 3. Immunoblotting of human, mitochondrial heat shock protein 70, full-length, recombinant protein and prevalence of the anti-mitochondrial heat shock protein 70 antibody. Arrows indicate positive bands that immunoreacted with the anti-mtHSP70 antibody. P1–5, 1:2000-diluted serum from MS patients without anti-mtHSP70 antibody; N1–2, 1:2000-diluted serum from PD patients without anti-mtHSP70 antibody; N3–4, 1:2000-diluted serum from MCI patients without anti-mtHSP70 antibody; N5–6, 1:2000-diluted serum from IME patients without anti-mtHSP70 antibody; N7–8, 1:2000-diluted serum from HCs without anti-mtHSP70 antibody; N9–10 *$P<0.02$, **$P<0.02$, ***$P<0.004$, ****$P<0.02$. Abbreviations: HCs, healthy controls; IME, infectious meningoencephalitis; MCI, multiple cerebral infarction; MS, multiple sclerosis; mtHSP70, mitochondrial heat shock protein 70; PD, Parkinson disease.

Table 1
Comparison of clinical features according to anti-mitochondrial heat shock protein 70 antibody status.

	Anti-mtHSP70 antibody positive (n = 17)	Anti-mtHSP70 antibody negative (n = 8)	*P*-value
% Female	47%	38%	0.92
Age, years	46.5 ± 12.7[a]	50.4 ± 14.1[a]	0.50
RRMS patients' state when serum was collected	Relapse: 7 (50%) Remission: 7 (50%)	Relapse: 5 (100%) Remission: 0 (0%)	0.17
Disease course	RRMS: 14 (82%) SPMS: 3 (18%)	RRMS: 5 (63%) SPMS: 3 (38%)	0.34
EDSS in relapse state	3.6 ± 2.2[a]	5.1 ± 2.7[a]	0.25
Disease duration, years	8.3 ± 8.8[a]	6.6 ± 5.4[a]	0.64
Complication of optic neuritis	4 (24%)	3 (38%)	1.00
Number of relapses	3.6 ± 2.3[a]	4.5 ± 3.4[a]	0.54

Abbreviations: mtHSP70, mitochondrial heat shock protein 70; RRMS, relapsing–remitting multiple sclerosis; SPMS, secondary progressive multiple sclerosis; EDSS, expanded disability status scale.
[a] Mean ± S.D.

relapses between the two groups. No significant differences were found in the number of hyperintense cerebral lesions or in the frequency of the presence of hyperintense spinal cord, cerebellar, and brain stem lesions in T2 WIs obtained by MRI. The frequency of the presence of cerebral atrophy was not significantly different between the anti-mtHSP70-antibody-positive MS patients and the anti-mtHSP70-antibody-negative MS patients.

3.4. Analysis of prevalence of both anti-mtHSP70 antibody and anti-PGAM1 antibody in serum from MS patients, PD patients, MCI patients, IME patients, and HCs

We assessed the prevalence of both the anti-mtHSP70 antibody and the anti-PGAM1 antibody in serum from 21 MS patients, 21 PD patients, 19 MCI patients, 17 IME patients, and 17 HCs. As a result, the positivity rates of both these antibodies were 57% (12 of 21) in MS patients, 0% (0 of 21) in PD patients, 15.8% (3 of 19) in MCI patients, 0% (0 of 17) in IME patients, and 11.8% (2 of 17) in HCs. The positivity rates of both these antibodies in MS patients are significantly higher than those in PD patients ($P<0.0002$), MCI patients ($P<0.008$), IME patients ($P<0.0005$), and HCs ($P<0.006$). The specificity of this combination assay was higher (93%) than that of the assay of only one antibody (anti-mtHSP70 antibody, 74%; anti-PGAM1 antibody, 73%).

4. Discussion

We identified mtHSP70 as the target antigen of the antibody in serum from MS patients by proteomics-based analysis. Western blotting analysis using the human recombinant protein showed that the prevalence of the anti-mtHSP70 antibody is significantly higher in serum from MS patients than in serum from PD patients, MCI patients, IME patients, and HCs. Previously, we reported that the prevalence of the anti-PGAM1 antibody is much higher in serum from MS patients than in serum from patients with other neurological diseases and from HCs (Kimura et al., 2010). Moreover, to establish more specific biomarkers in serum from MS patients, we assayed the prevalence of both the anti-mtHSP70 antibody and the anti-PGAM1 antibody. As a result, the specificity of this combination assay was higher than that of the assay of only one antibody. We suggest that this combination assay is a useful diagnostic method to detect the markers in serum from MS patients. Further studies are required to assess the specificity of this combination assay in a large cohort of MS patients.

Table 2
Comparison of magnetic resonance imaging findings according to anti-mitochondrial heat shock protein 70 antibody status.

	Anti-mtHSP70 antibody positive (n = 17)	Anti-mtHSP70 antibody negative (n = 8)	*P*-value
T2 HI lesions			
Cerebral lesions			0.79
Number ≥ 9	11 (65%)	4 (50%)	
Number < 9	6 (35%)	4 (50%)	
Brainstem lesions	5 (29%)	5 (63%)	0.26
Cerebellar lesions	2 (12%)	2 (25%)	0.76
Spinal cord lesions	10 (59%)	2 (25%)	0.25
Cerebral atrophy	2 (12%)	3 (38%)	0.33

Abbreviations: HI, hyperintensity; mtHSP70, mitochondrial heat shock protein 70.

In recent years, the need for multiplex autoantibody profiling approaches has become evident in the research field of autoimmunity (Tozzoli, 2007; Plebani et al., 2009). For MS, the necessity for a panel of several markers is also explained by the enormous heterogeneity, which is a characteristic of this disease. In addition, because most of the low-affinity autoantibodies are also present in HCs (Lionel et al., 2005; Lefranc et al., 2004), multiplex analysis may be useful for detecting specific diagnostic markers of MS. Different multiplexing approaches have already been used for the identification of an MS-specific autoantibody fingerprint in MS serum and MS cerebrospinal fluid (CSF) (Somers et al., 2008). They reported the identification of a novel panel of 8 antigenic targets with 45% sensitivity and 86% specificity using a phage display library derived from MS brain plaques. The combination assay of two antibodies in our study showed higher sensitivities and specificities than the assay they developed.

HSPs are the most abundant among soluble intracellular proteins and are called stress proteins or molecular chaperones that assist cell rescue through the folding of synthesized or stress-denatured proteins. There are more than ten different families of human HSPs, such as HSP60, HSP70, HSP90, and small HSPs. The HSP70 family includes at least eight homologous chaperone proteins: HSP70-1a, HSP70-1b, HSP70-1t, HSP70-2, HSP70-5, HSP70-6, HSC70, and HSP70-9 (Daugaard et al., 2007). HSP70-9, an alternative name for mtHSP70, and 75 kDa glucose-regulated protein (GRP75) among others are localized to mitochondria (Daugaard et al., 2007). The functions of mtHSP70 are reported to be as follows: a specific 42-amino-acid-targeting signal delivers mtHSP70 to the mitochondrial lumen, where it interacts with incoming proteins and assists them in the correct folding after the transmembrane transport (Deocaris et al., 2006; Mizzen et al., 1989).

Concerning the relationship between the HSP70 family and MS, extracellular HSP70 family members have a significant adjuvant-like effect by associating with an immunodominant myelin basic protein (MBP)-derived peptide, and in vitro generated complexes of MBP 84–106 and HSP70 stimulate the proliferation of peptide-specific human T cell lines with normally suboptimal concentrations of antigens (Cwiklinska et al., 2003; Lund et al., 2006; Mycko et al., 2004). Another study demonstrated an increased immunoreactivity of mtHSP70 in MS lesions, particularly in astrocytes and axons, which induces decrements of reactive oxygen species, improvement of mitochondrial function, and protection of astrocytes (Witte et al., 2009). From these reports, the HSP70 family including mtHSP70 might play an important role in the etiology of MS.

The pathophysiological role of the anti-mtHSP70 antibody remains unclarified. A previous study showed that antigen microarrays identified the antibodies against HSP60 and HSP70 whose levels are higher in serum from RRMS patients than SPMS patients, PPMS patients, and HCs (Quintana et al., 2008). Another report showed that the levels of antibodies against HSP70 family proteins are significantly higher in CSF from MS patients than in CSF from patients with motor neuron disease, and that the levels of these antibodies in CSF from MS patients tend to increase as disease activity increases (Chiba et al., 2006). In addition, the anti-HSP70 antibody in CSF from MS patients may modify the HSP70-mediated antigen presentation and augment HSP70-induced proinflammatory cytokine production in monocytic

cells (Yokota et al., 2010). In this study, we demonstrated no correlation between the presence of the anti-mtHSP70 antibody in serum and disease activity or severity. We suggest that the anti-mtHSP70 antibody may be secondarily produced in immune responses by which mtHSP70 is expressed extracellularly in active MS lesions. However, we have to conduct more studies to clarify the role of the anti-mtHSP70 antibody in the pathogenesis of MS.

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Patterns of levels of biological metals in CSF differ among neurodegenerative diseases

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ABSTRACT

We measured the levels of some biological metals: copper (Cu), iron (Fe), magnesium (Mg), manganese (Mn), and zinc (Zn) in the cerebrospinal fluid (CSF) in patients with neurodegenerative diseases (52 patients with amyotrophic lateral sclerosis (ALS)), 21 patients with Alzheimer's disease (AD), and 20 patients with Parkinson's disease (PD) by inductively coupled plasma mass spectrometry (ICP-MS). The diagnoses were additionally supported by neuroimaging techniques for AD and PD. In ALS, the levels of Mg ($p<0.01$ significant difference), Fe, Cu ($p<0.05$), and Zn ($p<0.10$) in CSF were higher than those in controls. Some patients showed very high levels of Cu and Zn before the critical deterioration of the disease. In AD, the levels of Cu and Zn in CSF were significantly higher in patients with late-onset AD ($p<0.01$). In PD, we found significantly increased levels of especially Cu and Zn in particular ($p<0.01$) and Mn ($p<0.05$) in CSF. A multiple comparison test suggested that the increased level of Mg in ALS and that of Mn in PD were the pathognomonic features. These findings suggest that Cu and Zn in particular play important roles in the onset and/or progression of ALS, AD, and PD. Therefore, Cu-chelating agents and modulators of Cu and Zn such as metallothionein (MT) can be new candidates for the treatment of ALS, AD, and PD.

1. Introduction

Biological metals such as copper (Cu), iron (Fe), magnesium (Mg), manganese (Mn), and zinc (Zn) have been considered to play very important roles in the progression of some neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease (PD), and amyotrophic lateral sclerosis (ALS) [1–3]. However, the roles and the metabolisms of such metals remain to be elucidated. Not only the direct toxicity of metals but also the oxidative stress via metals, and metal-associated enzymes and transcription factors modify the progression and diversity of the neurodegenerative diseases. Recently, we have found significantly increased levels of Cu, Zn, Fe, and Mg in the cerebrospinal fluid (CSF) of three patients with 'Fahr's disease' (idiopathic bilateral striato-pallido-dentate calcinosis (IBSPDC), its nomenclature remains controversial) by highly sensitive inductively coupled plasma mass spectrometry (ICP-MS) [4].

Recently, the diagnoses for neurodegenerative diseases such as AD and PD have been more accurate than before using the neuroimage techniques such as magnetic resonance imaging (MRI) with a quantitative analytical method [5], positron emission tomography (PET) or ^{99m}Tc-ethyl cysteinate dimmer-(ECD)-single photon emission computed tomography (SPECT) with quantitative analyses [6], and metaiodobenzylguanidine (MIBG)-cardioscintigraphy with quantitative measurements [7].

Some metals have been thought to be associated with the onset and/or progress of neurodegenerative diseases; Cu, Zn, and Fe for AD, Fe for PD, and Cu and Zn for familial ALS [1]. The mutations of superoxide dismutase 1 (SOD 1) including Cu and Zn in mice cause ALS [8]. Recently, the development of methods of measuring metals has progressed such as ICP-MS [4,9]. With this development, it is possible to clarify the molecular mechanisms underlying the development of neurodegenerative diseases and identify implicated metalloproteins and enzymes. In this situation, it is important to measure accurately the levels of metals in CSF of patients with ALS, AD, and PD using ICP-MS. We speculate on the molecular mechanisms and the roles of metals in neurodegenerative diseases, and develop new therapeutic strategies on the basis of the metal metabolism.

We measured the levels of some biological metals including Cu, Fe, Mg, Mn, and Zn in the CSF of 52 patients with ALS using ICP-MS. We compared the measured values with other clinical data including the subtypes, duration, and the levels of the metals in the serum in the patients with ALS. In addition, we had examined the levels of the

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heavy metals in the CSF of patients with typical features of AD and PD using neuroimaging techniques, and the pathognomonic patterns of neurodegenerative diseases were analyzed by a multiple comparison test.

2. Methods

2.1. CSF sample collection

We obtained samples of the CSF from 52 patients with ALS, 21 patients with AD, and 20 patients with PD. All the patients with ALS fulfilled the revised El Escorial criteria [10] for clinically definite and probable ALS (mean age 65.1 ± 1.6, 28 cases, classical type, 22 cases, bulbar type; and 2 cases, familial type; 17 females and 35 males). We chose samples from patients with AD diagnosed on the basis of the Diagnostic and Statistical Manual for Mental Disorders (4th ed. DSM-IV) [11]. Patients were selected on the basis of both MRI and SPECT findings (n = 21; 7 early-onset type and 14 late-onset type; 8 females and 13 males) to rule out other dementia such as vascular dementia and frontotemporal dementia [5,6]. We excluded patients with abnormal MIBG findings from the AD group to rule out Lewy body disease. We chose 20 patients (11 females and 9 males) with PD diagnosed on the basis of the criteria of British Brain Bank [12] and supported by MRI, ECD-SPECT, and MIBG-cardioscintigraphy to rule out other types of parkinsonism, such as drug-induced parkinsonism and progressive supranuclear palsy. Fifteen patients (9 females and 6 males) with unspecific neurological diseases were used as controls in the study. The first CSF samples that were obtained after the diagnosis were analyzed in this study. The study was approved by the Ethics Committee of the Gifu University Graduate School of Medicine.

2.2. Metals in CSF analysis

The CSF samples were moistly powdered to ash with perhydroxyl-nitrate, and the levels of metals (Cu, Fe, Mg, Mn, and Zn) were measured at least twice using ICP-MS (HP4500, Agilent Technologies, Japan) as previously described [4].

2.3. Statistical analyses

Data were statistically analyzed between disease groups and the control group using the Student's *t*-test. The correlations between the levels of metals in the CSF and other clinical data were analyzed using Pearson Product Moment correlation. Clinical data include age, time between the CSF examination and the disease onset, serum Cu and Zn levels, severity (mini-mental state examination (MMSE) in AD), and the clinical disease subtypes. Correlation coefficients >0.70 were considered significant. Multiple comparisons among disease groups were analyzed using Tukey's honestly significant difference (HSD) test. A significant level of 0.05 was used for all statistical tests (two-tailed). Statistical analyses were performed using IBM SPSS Statistics Base 18.

3. Results

The levels of Cu, Zn, Fe, and Mg in the CSF in patients with ALS, AD, and PD, and controls are shown in Table 1.

In ALS patients, the levels of Cu, Fe ($p<0.05$), and particularly Mg ($p<0.01$) were significantly higher in the CSF of the patients with ALS, and those of Zn were slightly elevated ($p<0.10$) than those in the controls. The data on Cu and Zn in ALS patients, were very widely scattered, because 2 patients had very high levels of Cu (>49.1 ng/ml: > the mean level in ALS + 2 SD) and 3 patients had very high levels of Zn (> 33.5 ng/ml: > the mean level in ALS + 2 SD) in the study. Interestingly these 5 patients with very high levels of Cu and Zn had underwent gastrostomy or tracheostomy within 6 months after the spinal tap in the clinical follow-up research, although all the patients who underwent gastrostomy or tracheostomy within 6 months after the spinal tap did not necessarily show high levels of Cu or Zn. A follow-up study revealed that the patients showed transiently very high levels of Cu or Zinc in CSF (data not shown). Then, we classified 50 ALS patients (exclusion of 2 patients with the familial type) according to the clinical subtypes: classical type (n = 28) and bulbar type (n = 22), and rapidly progressive types (n = 25) and slowly progressive types (n = 25) (data not shown). The patients with the rapid progressive type are those who underwent gastrostomy or tracheostomy, or who died within 2 years of the onset of the disease. No significant correlation was detected between two types. The analyses using Pearson's chi-square test supports the notion that the bulbar type is also generally the rapidly-progressive type ($p<0.01$). We show the levels of biological metals in the CSF in ALS patients in Fig. 1.

Table 1
Levels of metals in the CSF of patients with ALS, AD, and PD. The levels of Cu, Fe, Mg, Mn, and Zn in CSF of patients and controls (n = 15). Fifty patients with ALS (except familial type (n = 2)) are divided into classical type (n = 28) and bulbar type (n = 22). The patients with AD are divided into two groups: early-onset type (the onset is below 65 years) (n = 7), and late-onset type (the onset is at 65 and over 65 years) (n = 14). Statistical analysis was performed using the Student's *t*-test. Significant difference, ** $p<0.01$, * $p<0.05$, + $p<0.10$.

Cont and Pt		Age	Cu	Fe	Mg	Mn	Zn
		years	ng/ml	ng/ml	μg/ml	ng/ml	ng/ml
Cont	Av	48.4	10.2	238.0	29.6	1.9	5.3
(n = 15)	S.D	22.2	2.1	54.7	6.5	1.0	3.3
ALS	Av	65	19.5*	282.5*	35.9**	2.2	11.1 + 11.2
(n = 52)	S.D	11.7	14.8	74.9	4.8	1.5	
Classical	Av	64.6	18.3	276.8	35.2	2.2	12.7
(n = 28)	S.D	10.6	9.3	74.7	5.1	1.4	13.0
Bulbar	Av	67.7	21.0	285.9	36.6	2.3	9.3
(n = 22)	S.D	10.7	19.8	78.9	4.7	1.6	8.7
AD	Av	65.4	17.4*	238.6	31.8	1.8	8.4
(n = 21)	S.D	13.1	10.4	38.7	4.0	0.9	6.4
Early-onset AD	Av	49.6	10.3	221.6	33.8	1.2	3.9
(n = 7)	S.D	8.1	5.4	16.5	4.8	0.3	3.3
Late-onset AD	Av	73.3	20.9**	247.2	30.8	2.1	10.7**
(n = 14)	S.D	5.6	10.7	44.1	3.3	1.0	6.5
PD	Av	68.7	18.8**	263.9	31.6	3.3*	14.5**
(n = 20)	S.D	5.8	6.9	112.9	3.6	2.1	7.6

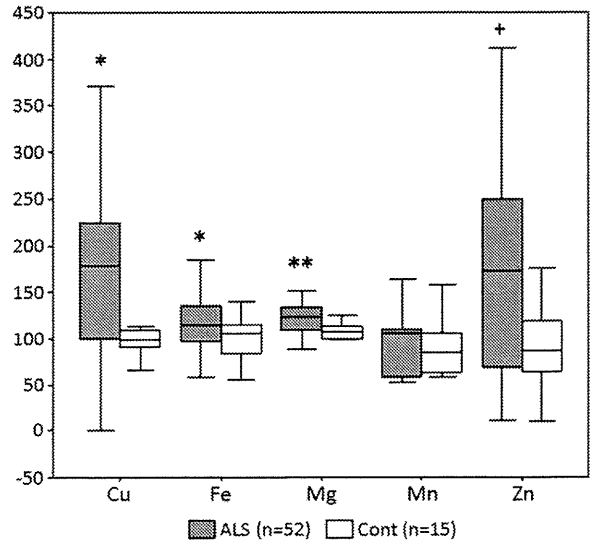

Fig. 1. The levels of the biological metals in the CSF of patients with ALS. The levels of Cu, Fe, Mg, Mn, and Zn in the CSF in patients and controls are shown in the box-and whisker type figure using SPSS. The levels of Mg (** significant difference: $p<0.01$), Fe, Cu (* $P<0.05$) and Zn (+ $p<0.10$), were higher in ALS patients than in controls.

In AD patients, we found significantly increased levels of Cu in the CSF ($p<0.05$). Then, we classified the AD patients according to the clinical subtype; early-onset AD (Alzheimer's disease with the onset under 65 years) ($n=7$) and late-onset AD (senile dementia of Alzheimer type (SDAT), onset at 65 and over 65 years) ($n=14$) (Table 1). The levels of Cu and Zn in the CSF were significantly higher in the patients with late-onset AD than in the controls. Correlation between the levels of Cu and Zn in the CSF was clearly recognized in patients with AD ($\sigma=0.812$) as well as in the controls ($\sigma=0.725$), but not in patients with ALS or PD. Although the ages of AD patients were significantly higher than those of the controls, the level of each metal did not correlate with the ages of the controls and AD patients. No other significant correlations could be observed between the levels of metals in the CSF and clinical manifestations such as MMSE, and serum Cu and Zn levels in this study. We show the levels of the biological metals in the CSF only in late-onset AD in Fig. 2.

In PD patients, we found significantly increased levels of Cu and Zn in particular ($p<0.01$), and Mn ($p<0.05$) in CSF (Table 1). We show the levels of the biological metals in the CSF in PD in Fig. 3.

In addition, to clarify the pathognomonic features, we performed a multiple comparison using Tukey's HSD test. The level of Mg in ALS was significantly higher than those in AD and PD ($p<0.01$). The level of Mn in PD was significantly higher than those in ALS and AD ($p<0.01$) (Fig. 4).

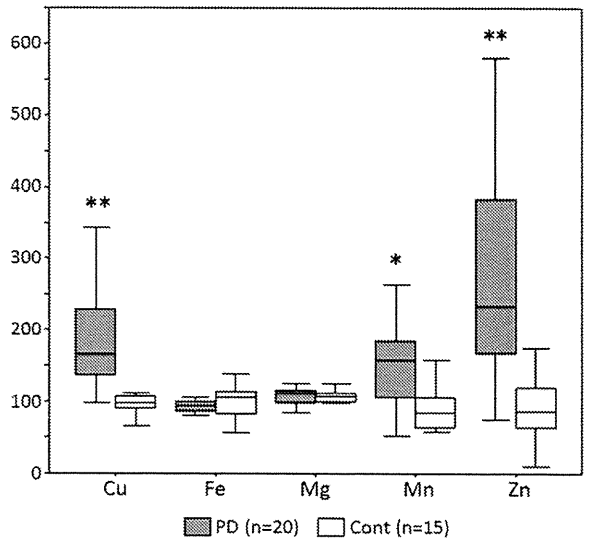

Fig. 3. The levels of biological metals in the CSF of patients with PD. The average levels of Cu, Fe, Mg, Mn, and Zn in the CSF in patients and controls are shown in the box-and whisker type figure using SPSS. The average levels of Mg, Fe, Cu, Zn, and Mn in the CSF in controls are shown to be set at 100 (%) in the figure. The levels of Cu and Zn (** $p<0.01$, respectively) and Mn (* $P<0.05$) in CSF were higher in PD patients than in controls.

4. Discussion

We measured the levels of some important metals (Cu, Fe, Mg, Mn, and Zn) in the CSF of patients with neurodegenerative diseases (AD, PD and ALS). We were able to find some pathognomonic patterns in the levels of the biological metals in the neurodegenerative diseases. Several remarkable studies on metals in the CSF of patients with neurodegenerative diseases have been published and we discuss some important metals for each disease.

In ALS, Kanias and Kapaki reported that the levels of Cu and Zn in CSF were higher in patients with ALS (age >40) than in older controls (age >40) as determined by atomic absorption spectrophotometry [13]. This is compatible with our finding. In particular, Cu and Zn are considered to play pivotal roles in the onset and/or progression of ALS. Studies on the spinal cord of G93A SOD-1 transgenic mice revealed high levels of Cu and labile Zn [14,15]. In this study, intriguingly, 5 patients showed very high levels of Cu and Zn before their critical deterioration. A researcher had observed that some patients with ALS showed transiently high levels of Zn in the urine during the course of the disease (personal communication). The mechanism remains to be elucidated and it remains to be clarified whether these phenomena are a harbinger or a result. In our study the levels of Mg were also significantly elevated ($P<0.01$) and the levels of Fe are also increased than those in the controls ($p<0.10$). Glutamate excitotoxity is suspected to cause motor neuron damage [16] and Mg ions inhibit the opening of NMDA receptors [17]. Taken together, the findings

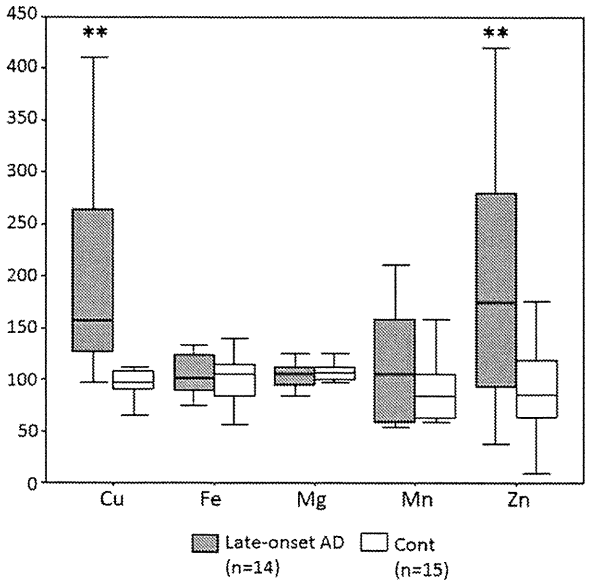

Fig. 2. The levels of biological metals in the CSF of patients with late-onset AD. The average levels of Cu, Fe, Mg, Mn, and Zn in the CSF in patients and controls are shown in the box-and whisker type figure using SPSS. The average levels of Mg, Fe, Cu, Zn, and Mn in the CSF in controls are shown to be set at 100 (%) in the figure. The levels of Cu and Zn (** $p<0.01$, respectively) in CSF were higher in late-onset AD than in controls.

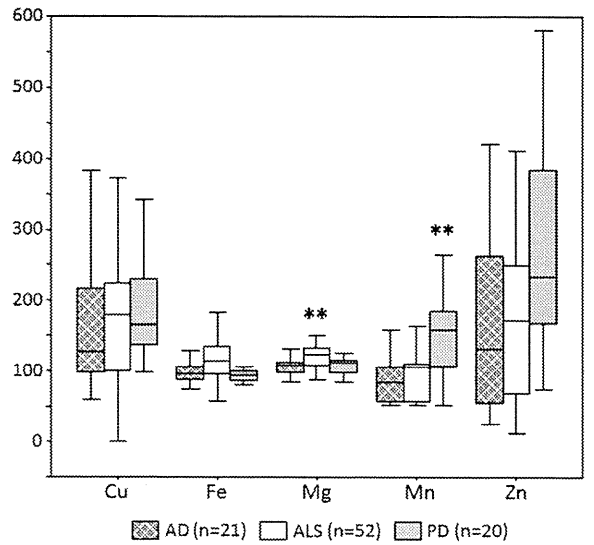

Fig. 4. The levels of the biological metals in the CSF among patients with ALS, AD and PD. The level of Mg in ALS patients was significantly higher than those in AD and PD patients (** $p<0.01$), and the levels of Mn were significantly higher than those in ALS and AD patients (** $p<0.01$) according to Tukey's HSD test.

suggest that multiple metals complexly contribute to the onset and/or progression of ALS.

We selected the patients with typical AD features using imaging studies, because the levels of Mg and Ca were reported to be increased in the CSF in patients with Levy body disease (LBD) than in those with AD [18]. Our study showed that the levels of Cu and Zn in CSF markedly higher in patients with late-onset AD. As similarly observed in ALS [13], markedly higher levels of Cu and Zn were also observed in late-onset AD patients in our study. However, no association of the levels of metals with age was found in both controls and patients with AD. A recent study showed that the serum copper level is associated with the MMSE score worsening in patients with AD [19]. Zn level was also reported to be increased in the human AD-affected cortex [20]. However, we found no association among the level of Cu in the CSF, the level of Cu in the serum, and the MMSE score in this study. A positive correlation between Cu and Zn levels in CSF was found in controls and patients with AD, although it is generally considered that there is a negative correlation between Cu and Zn levels in serum. However, the positive correlation between Cu and Zn levels in CSF in patients with AD was not observed in patients with ALS and PD. The mechanism underlying the correlation is unclear but some other pathognomonic factors may affect the levels of Cu or/and Zn in patients with ALS and PD. A study on Japanese American men suggested that Zn and Cu modulate Aβ-42 levels in CSF [21]. Therefore, both Cu and Zn are considered to be the main metals that are strongly associated with the onset and/or progression of AD, particularly late-onset AD.

In PD, our study showed that the levels of Cu and Zn in CSF were significantly ($p<0.01$) higher and the level of Mn was also higher ($p<0.05$) than those in the controls. Mn intoxication has been well known to cause parkinsonism. A survey suggested that chronic occupational exposure to Mn or Cu is associated with PD [22]. Low-level Mg intake over generations was shown to cause the degeneration of the substantial nigra in rats [23]. A study by ICP-AES showed lower Fe and Si levels in the CSF of 91 PD patients than in 18 controls in Italy and the levels of Mg concentration decreased in the CSF with the duration and severity of the disease [24]. The lower level of Fe and the decrease in the levels of Mg with time were not observed in our study. The reason is still unknown.

There are other studies on metals in the CSF of AD, PD and ALS patients. The important points are the methods of measurement of metals and the diagnosis of the diseases. ICP-MS is more sensitive and accurate than the conventional colorimetery and atomic absorption spectrophotometry methods for the simultaneous measurement of several biological metals such as Cu, Fe, Mg, Mn, and Zn [4,9]. We are able to accurately diagnose AD and PD by neuroimaging techniques [5–7]. However, there are some limitations in our study. The numbers of controls, and AD and PD patients were relatively small, and controls were significantly younger than the patients with ALS, AD, and PD ($p<0.01$). However, the levels of the metals in the CSF did not correlate with age. There may be several pathological factors that affect the levels of the metals in the CSF such as environmental factors including diet, drugs, life styles, the time of examination, and possibly races. We should examine the changes in the levels of metals in the CSF during the course of the diseases, particularly ALS. The levels of metals in the CSF only indicate the levels of metabolites similar to those in urine. We should examine the changes in the levels of metals and metal-transporting proteins in the causative parts for each disease to clarify the roles of metals in the brain and the spinal cord in the future.

Taken together, Cu and Zn are considered to play important roles in ALS, AD, and PD. Multiple metals seem to complexly contribute to the development of ALS and a surge of Cu or Zn level may be a harbinger of critical deterioration in ALS. The increased level of Cu and Zn in the CSF were prominent in the late-onset AD. The increased level of Mg in ALS and that of Mn in PD may be pathognomonic features. Cu and Zn may not be essential for the pathogenesis of neurodegenerative diseases but they probably promote the progression of the diseases through oxidative stress and conformational change of pivotal proteins. Cu-chelating agents [14], Zn-chelating agents [15], and, moreover, metallothioneins, which maintain Zn and Cu homeostasis [25,26], can be new candidates for the treatment of neurodegenerative diseases, based on the findings.

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ORIGINAL COMMUNICATION

Is there a delayed gastric emptying of patients with early-stage, untreated Parkinson's disease? An analysis using the ^{13}C-acetate breath test

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Abstract During the pre-symptomatic stage of Parkinson's disease (PD), the idiopathic PD related abnormal synuclein immunostaining is confined to the medulla oblongata and olfactory bulb, according to Braak. 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. These lesions may cause dysfunction of the gastrointestinal tract (GI) as pre-clinical symptoms of PD. However, because L-dopa therapy itself may worsen the symptoms of the digestive tract function, it is needed to evaluate the gastrointestinal tract function in patients with early-stage, untreated (de novo) PD. In the present study, using the ^{13}C-acetate breath test (^{13}C-ABT), we investigated gastric emptying in 20 untreated, early-stage PD patients and 40 treated, advanced-stage PD patients, and 20 healthy volunteers. Gastric emptying was examined by the ^{13}C-ABT [the half emptying time (HET), the peak time of the ^{13}C% dose-excess curve (T_{max})]. The T_{max} and HET of gastric emptying as assessed using the ^{13}C-ABT was significantly delayed in untreated, early-stage PD patients as compared to the controls ($P < 0.001$). The T_{max} and HET of gastric emptying were not significantly delayed in untreated, early-stage PD patients as compared to treated, advanced-stage PD patients. The results demonstrated that delay in gastric emptying did not differ between untreated, early-stage and treated, advanced-stage PD patients. Gastric emptying of untreated, early-stage PD is already delayed. Delayed gastric emptying may be one of markers of the pre-clinical stage of PD.

Keywords Parkinson's disease · Gastric emptying · Untreated (de novo) early-stage · ^{13}C-acetate breath test

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Introduction

Patients with Parkinson's disease (PD) often complain of gastrointestinal (GI) tract symptoms such as heartburn, nausea, vomiting, and full abdomen sensation [1–3]. Some studies have reported on the dysfunction of the GI tract in PD patients [1, 2, 4, 5].

During the pre-symptomatic stage of PD, the idiopathic PD related abnormal synuclein immunostaining is confined to the medulla oblongata and olfactory bulb, according to Braak [6]. The most likely causes of GI tract symptoms are degenerations of the dorsal vagal nucleus and the intramural plexus of the whole intestine [7]. These degenerations are likely to develop prior to the degeneration of dopaminergic neurons of the substantia nigra [7]. Therefore, in the previous study, it was reported that delayed gastric emptying was 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 treated PD may be affected by L-dopa therapy. It was not clear whether there is the delayed gastric emptying of patients with early-stage,