labelled with patients' antibodies, commercial $GABA_B$ antibodies, and Bassoon was quantified using a software macro (written by EH) in ImageJ.

Owing to the reactivity of patients' antibodies with rat tissue and hippocampal neuronal cultures, and the homology between human and rat $GABA_B$ receptor sequences (the B1 receptor subunit has 91·3% cDNA sequence identity and 98·6% amino acid sequence identity in the two species),[16] HEK293 cells were transfected with plasmids containing rodent $GABA_{B1}$ or $GABA_{B2}$ or plasmids without an insert (control), by use of a method previously reported.[7] In other experiments, cells were transfected with $GABA_{B1}$ and $GABA_{B2}$ in equimolar ratios. Cells were then grown for 24 h before assessment. Transfected cells were fixed in 4% paraformaldehyde, made permeable with 0·1% Triton X-100, and then incubated overnight at 4°C with patients' serum (1:200) or CSF (100%) and the guineapig polyclonal $GABA_{B1}$ receptor antibody (1:20 000) or a polyclonal $GABA_{B2}$ receptor antibody (1:10 000, generated by SJM), washed in PBS, and incubated with the appropriate Alexa Fluor secondary antibodies (1:2000). Results were photographed as before.

Antibody titres were obtained by use of HEK293 cells expressing $GABA_{B1/B2}$ incubated with serial dilutions of serum and CSF, starting at 1:1 dilution. Patients' antibody IgG subtypes in serum or CSF were identified by use of the HEK293 transfected cells and secondary anti-human

	MRI	CSF	Serum antibody titres*	CSF antibody titres*	Chronological list of treatments	Outcome (duration of follow-up)
Patient						
1	FLAIR/T2 increased signal in medial temporal lobes	9 WBC per μL; protein 350 mg/L; no OCBs	640	160	IVIg, corticosteroids, chemotherapy	Substantial improvement; mild residual short-term memory deficit; lives independently; seizure free (12 months)
2	Normal	Normal	1280	..	Corticosteroids, IVIg, chemotherapy	Substantial improvement; died of metastatic disease (15 months)
3	FLAIR/T2 increased signal in medial temporal lobes	..	160	..	Tumour removal (lobectomy), IVIg	Partial improvement after tumour removal and IVIg (4 months); lost to follow-up
4	Normal	..	2560	640	None	Died soon after presentation of rapidly progressive respiratory failure
5	FLAIR/T2 increased signal in medial temporal lobes	..	1280	..	Supportive	Died 6 months after symptom presentation; $GABA_B$ antibodies detected after patient's death in archived serum
6	FLAIR/T2 increased signal in small area of corpus callosum	95 WBC per μL; protein 1040 mg/L; increased IgG index	..	640	Corticosteroids, mycophenylate mofetil	Substantial improvement; lives independently; seizure free (9 months)
7	FLAIR/T2 increased signal in left medial temporal lobe	..	..	640	Corticosteroids, plasma exchange	Initial substantial response to corticosteroids; relapsed 1 month later; died after 5 months in ICU with refractory seizures, status epilepticus, and systemic complications; $GABA_B$ antibodies detected after patient's death in archived serum
8	FLAIR/T2 increased signal in medial temporal lobes	19 WBC per μL; protein 460 mg/L	5120	2560	Corticosteroids, plasma exchange	Substantial improvement; mild residual short-term memory deficit; seizure free (3 months)
9	FLAIR/T2 increased signal in medial temporal lobes	75 WBC per μL; protein 260 mg/L; OCBs present	Negative	4	Corticosteroids	Full recovery (41 months)
10	FLAIR/T2 increased signal in medial temporal lobes	81 WBC per μL; protein 300 mg/L	10 240	..	Corticosteroids	Substantial improvement. Residual short-term memory deficit. Lives independently. Seizure free (72 months)
11	Normal	20 WBC per μL; protein 220 mg/L	40	40	Corticosteroids	Full recovery (6 months)
12	FLAIR/T2 increased signal in left medial temporal lobe and insula	950 WBC per μL; OCBs present	Negative	10	Symptomatic	Temporal lobe biopsy 20 months after symptom presentation showing reactive astrocytosis, without inflammation; no follow-up available after biopsy
13	FLAIR/T2 increased signal in medial temporal lobes	4 WBC per μL; protein 1090 mg/L; 6 OCBs	Negative	4	Corticosteroids	Full recovery, except for infrequent brief episodes of visual hallucinations (10 months)
14	FLAIR/T2 increased signal in left medial temporal lobe	Traumatic; negative cytology	..	80	Chemotherapy	Residual short-term memory deficit; seizures controlled; died of sepsis (3 months)
15	Normal	0 WBC per μL; protein 950 mg/L	..	640	IVIg, corticosteroids, chemotherapy	Seizures responded to antiepileptics; memory deficit persisted; died of cancer-related treatment (2 months)
Control						
1	Normal	3 WBC per μL; protein 780 mg/L; 1 OCB	Negative	2	IVIg	No seizures or cognitive deficits; limited response of cerebellar ataxia to IVIg (12 months)
2	Normal	2 WBC per μL; protein 520 mg/L; OCBs present	Negative	2	IVIg, corticosteroids	No seizures or cognitive deficits; full recovery after steroids and IVIg (12 months)

*Titres defined as the reciprocal of the maximal dilution that gave positive immunostaining. FLAIR=fluid-attenuated inversion recovery. WBC=white blood cells (normal <4 per μL). OCB=oligoclonal band. IVIg=intravenous immunoglobulin. ICU=intensive care unit.

Table 2: **Diagnostic tests, treatment, and outcome**

antibodies specific for IgG1, IgG2, IgG3, or IgG4 (all 1:50; Sigma-Aldrich) as reported.[17]

Statistical analysis

The association between $GABA_B$ receptor antibodies and other autoantibodies (GAD65, N-type voltage-gated calcium channel, thyroid peroxidase, thyroglobulin, or SOX1) and that between neurological improvement and cancer treatment or immunotherapy were analysed with Fisher's two-sided exact test. The colocalisation of patients' antibodies with the polyclonal $GABA_B$ receptor antibodies or antibodies to the synaptic marker Bassoon was analysed with the Student's *t* test.

Role of the funding source

The study sponsor had no role in the study design, data collection, data analysis, data interpretation, or writing of the report. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.

Results

In May, 2008, a 60-year-old woman with a long history of smoking was admitted to hospital with confusion, memory problems, and new-onset generalised tonic-clonic and partial complex seizures refractory to treatment (index patient; patient 1). At examination, she was confused about the time and where she was and had poor concentration and short-term memory (table 1). Although she had saccadic pursuits with lateral gaze, no cranial nerve abnormalities were noted. Strength, sensation, reflexes, and coordination were normal. MRI of the brain showed increased fluid-attenuated inversion recovery (FLAIR) signal in the medial temporal lobe of both hemispheres, compatible with limbic encephalitis (table 2, figure 1A). Diffuse slowing and bilateral periodic lateralised epileptiform discharges were seen on encephalography (EEG). In the CSF there were nine white blood cells per µL, total protein concentration was 350 mg/L, and glucose concentration was 3·94 mmol/L; there were no oligoclonal bands and cytological findings were normal. PCR for herpes simplex virus, West Nile virus, and St Louis encephalitis were negative. The patient had hyponatraemia (119 mEq/L) caused by syndrome of inappropriate antidiuretic hormone secretion. Combined CT and fluorodeoxyglucose-PET showed mediastinal lymphadenopathy, which was proven by biopsy to be small-cell lung cancer. The patient was treated with antiepileptic drugs (levetiracetam, valproic acid, and phenytoin) and immunotherapy (intravenous immunoglobulins and corticosteroids), immediately followed by chemotherapy with cisplatin and etoposide. The patient's short-term memory and cognition improved, and seizures resolved. After chemotherapy the patient had standard prophylactic whole-brain radiation therapy. Brain MRI 1 month after symptom presentation showed improvement of the abnormal FLAIR signal (figure 1B); MRI at 3 months and 9 months were unchanged except for progressive general atrophy, probably secondary to radiation (figure 1C, D). 1 year after symptom presentation, the patient had only mild deficits in memory and cognition and lived independently.

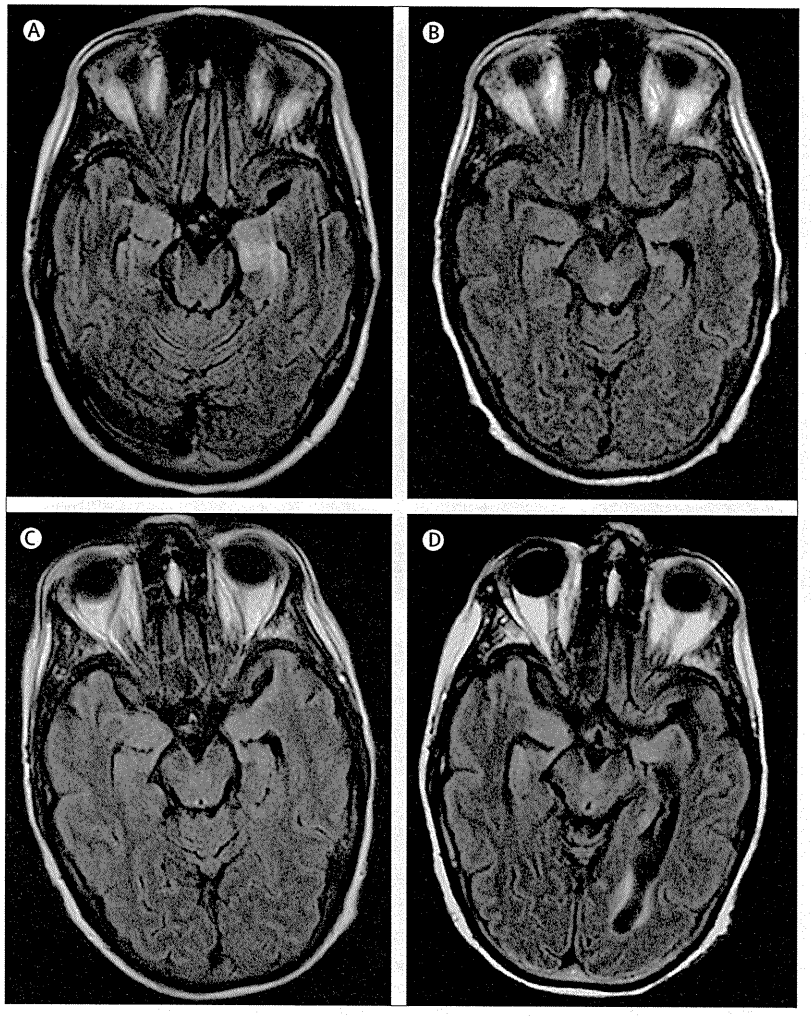

Figure 1: **MRI of a patient with $GABA_B$ receptor antibodies and limbic encephalitis**
Axial fluid-attenuated inversion recovery (FLAIR) MRI from patient 1 at presentation (A) showed increased signal in the medial temporal lobes, which was more pronounced on the left. Repeat study at 1 month (B) showed improvement of the FLAIR signal that remained stable at 3 months and 9 months (C, D), with development of mild generalised atrophy (the patient received standard whole-brain radiation therapy as prophylaxis for small-cell lung cancer metastases).

Sera and CSF from the index patient and the 14 other patients (patients 1–15) showed a pattern of reactivity with the neuropil of rat brain (figure 2) that was different from that reported with antibodies against NR1 subunits of the NMDA receptor, GluR1/2 subunits of the AMPA receptor, or voltage-gated potassium channels.[7,8,18] When non-fixed and non-permeabilised cultures of rat hippocampal neurons were incubated with patients' serum or CSF, intense reactivity with the cell surface was seen (figure 3A). Similar studies with serum or CSF from

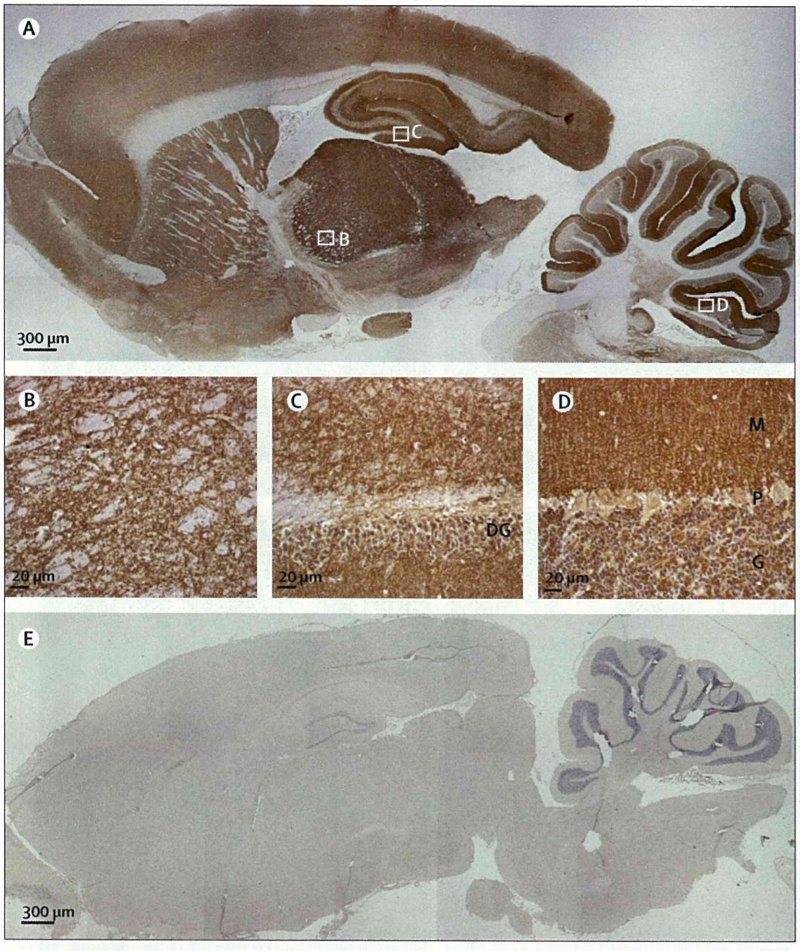

Figure 2: **Immunolabelling of rat brain with patients' antibodies**
Sagittal section of rat brain immunolabelled with CSF of a patient with limbic encephalitis (A) and a control individual (E). Note the extensive staining in A of the neuropil of thalamus (B), hippocampus (C), cerebellum (D), and cerebral cortex. DG=dentate gyrus. M=molecular layer. P=Purkinje cell layer. G=granular cell layer. Avidin–biotin–peroxidase method; sections counterstained with haematoxylin.

control individuals showed no reactivity with rat brain tissue (figure 2E) or cultures of neurons (figure 3B).

The $GABA_B$ receptor was identified as the target antigen by immunoprecipitation of the antigen with patients' serum samples and peptide sequence recognition ($GABA_{B1}$ and $GABA_{B2}$) by mass spectrometry (webappendix). Immunoprecipitates were obtained using the serum from four patients. Electrophoresis of the immunoprecipitates showed similar protein bands at about 90 kDa and 105 kDa (Figure 3C). The indicated protein bands contained sequences derived from $GABA_{B1}$ and $GABA_{B2}$ (protein scores for B1: 160, 225, 342, and 178; protein scores for B2: 1094, 1784, 1946, and 2653).

See Online for webappendix

The results were confirmed by immunoblotting the immunoprecipitates with antibodies specific for $GABA_{B1}$ and $GABA_{B2}$. Immunoblot analysis confirmed that the band at about 105 kDa was recognised by anti-$GABA_{B1}$ and anti-$GABA_{B2}$ antibodies, and the band at about 90 kDa was recognised by anti-$GABA_{B1}$ antibodies (figure 3D).

Colocalisation of patients' antibodies with the $GABA_B$ receptor and the synaptic and extrasynaptic location of the target receptors were noted on confocal microscopy. The colocalisation of patients' antibody clusters with $GABA_B$ receptor clusters (figure 4) was quantified for the dendrites of 23 neurons on four separate coverslips. 103% (SE 0·8%) of the clusters labelled with antibodies from patients colocalised with clusters labelled by the guineapig polyclonal $GABA_B$ receptor antibody, and 107% (SE 0·7%) of guineapig antibody-labelled clusters colocalised with those labelled by patients' antibodies (numbers slightly higher than 100% occur because of overlapping of a few clusters labelled by patient antibodies with two guineapig antibody-labelled clusters and vice versa). These results suggest that all patients' antineuronal cell-surface antibodies target the $GABA_B$ receptors and that almost all neuronal $GABA_B$ receptors are labelled by patients' antibodies. 62% (SE 1·3%) of $GABA_B$ receptor clusters labelled by patients' antibodies were also labelled by Bassoon, significantly fewer than those also labelled by guineapig $GABA_B$ receptor antibodies (Student's t test, $p<0·0001$), suggesting that patient antibodies bind both synaptic and extrasynaptic $GABA_B$ receptors.

The location of the epitope in $GABA_{B1}$ was identified with HEK293 cells transfected with $GABA_{B1}$, $GABA_{B2}$, or both $GABA_B$ receptor subunits. All 15 patients had serum or CSF antibodies that reacted with $GABA_{B1}$ (figure 5), and one had additional reactivity with the $GABA_{B2}$ subunit (data not shown). Similar studies with the 104 control individuals showed that two patients, both with syndromes attributed to GAD65 autoimmunity, had $GABA_{B1}$ receptor antibodies at low titres (CSF 1:2, serum negative), which did not bind at detectable levels to sections of rat brain (Fisher's exact test, $p<0·0001$, data not shown).

Samples from the six patients for whom sufficient serum or CSF was available were analysed for antibody IgG subtypes. All six patients had IgG1 $GABA_{B1}$ antibodies, two had additional IgG3, and one had IgG2 antibodies.

Table 1 shows demographic features and symptoms of the 15 patients and the two control individuals who had antibodies to GAD65. Among the 15 patients, median age was 62 years (range 24–75); eight were men. All patients had seizures, confusion, and memory deficits. In 13 patients the seizures were the presenting symptom; in two (patients 3 and 13) memory deficit and confusion were the presenting symptoms. After further clinical assessment most seizures appeared to have a temporal-lobe onset with secondary generalisation, and three patients had status epilepticus.

Ten patients had unilateral or bilateral increases in medial temporal lobe FLAIR/T2 signal consistent with limbic encephalitis, and one had a small area of increased FLAIR signal in the corpus callosum (table 2). Four patients had normal brain MRI.

CSF was abnormal in nine of ten patients for whom data were available. The most common CSF abnormality was lymphocytic pleocytosis in eight patients. EEG results were available from 12 patients: nine had temporal-lobe seizures, epileptiform discharges, or temporal-lobe slowing; two had generalised slowing; and one had no abnormalities. Several types of seizures were noted on EEG, including complex partial seizures (often of temporal-lobe onset), status epilepticus, and subclinical seizures.

The two control individuals with low titre $GABA_{B1}$ antibodies developed different syndromes in association with high titre GAD65 antibodies in serum and CSF. Neither of these two patients developed seizures or limbic dysfunction (table 1). One had progressive cerebellar ataxia, and the other had gait instability, muscle stiffness, rigidity, myoclonus, and dysarthria, categorised as progressive encephalomyelitis with rigidity and myoclonus.

In addition to $GABA_B$ antibodies, seven of 15 patients had antibodies to one or more of the following: GAD65 (3 patients), thyroid peroxidase (3 patients), N-type voltage-gated calcium channels (3 patients), and SOX1 (1 patient). Only one of the three patients with GAD65 antibodies had endocrinopathy, and one of the three patients with voltage-gated calcium channel antibodies had small-cell lung cancer (table 1). The patient with SOX1 antibodies had small-cell lung cancer.

Seven patients had tumours (table 1), detected at the time of neurological symptom presentation. Of these patients, five had small-cell lung cancer, one had a lung tumour of neuroendocrine origin, and one had mediastinal adenopathy. No other systemic tumours were identified. Because most lung tumours were diagnosed by use of needle biopsy, no tissue was available for analysis of $GABA_B$ receptor expression. However, three of four small-cell lung cancers from control individuals without antibodies or encephalitis (archived tissue from the Division of Anatomic Pathology, University of Pennsylvania) showed reactivity with a guineapig polyclonal antibody to $GABA_{B1}$ receptor and patients' biotinylated IgG, suggesting that these receptors are expressed by small-cell lung cancer (webappendix).

Five of the patients were young (median age 30 years, range 24–45), were non-smokers, and had negative cancer screening including CT/fluorodeoxyglucose-PET, and two of these patients had long-term follow-up (41 and 72 months), making the presence of cancer unlikely.

Nine of 15 patients had a neurological response to immunotherapy (six) or treatment of the tumour as well as immunotherapy (three). The median follow-up of these nine patients was 10 months (range 3–72 months). One patient (patient 2) later died of tumour progression (15 months) and one (patient 3) was lost to follow-up at 4 months. Six patients did not have sustained neurological improvement: three patients (patients 4, 14, and 15) died from tumour or chemotherapy-related complications soon after presentation of the disorder, two were diagnosed with $GABA_B$ receptor antibodies after death (patients 5 and 7), and one was lost to follow-up (patient 12). Of the latter three, only patient 7 was thought to have an autoimmune disorder, and therefore this patient received corticosteroids and plasma exchange; the other two patients did not receive immunotherapy. Overall, after excluding one non-assessable patient (patient 12) nine of the ten patients who received immunotherapy and cancer treatment (when a tumour was found) showed neurological improvement, while none of the four patients (patients 4, 5, 14, and 15) who did not receive immunotherapy or whose tumour treatment was not completed showed improvement (Fisher's exact test p=0·005).

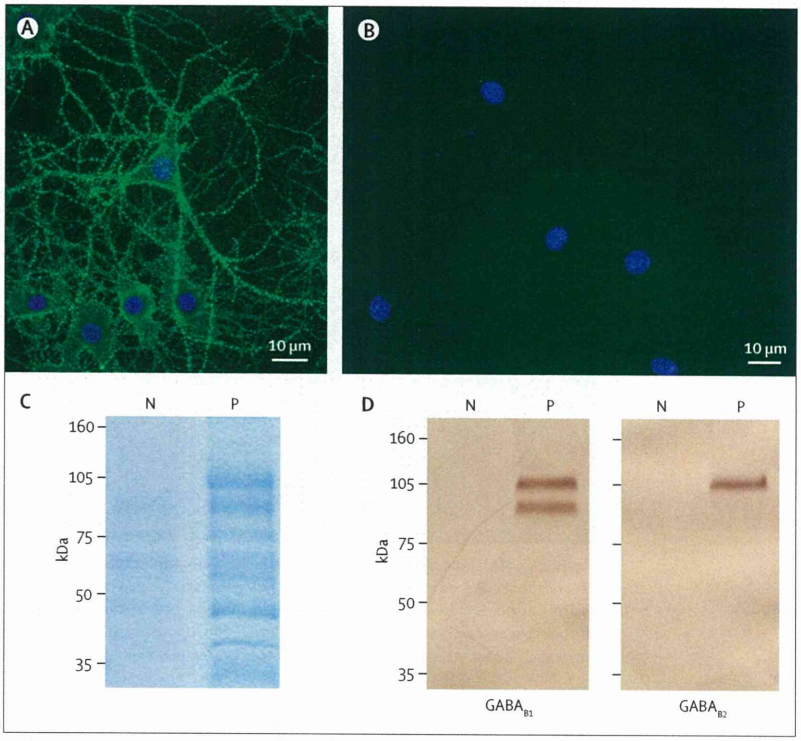

Figure 3: **Culture of rat hippocampal neurons incubated (live, non-permeabilised) with the CSF of a patient with limbic encephalitis and a control individual**
Note the intense punctate reactivity of patient's antibodies with cell surface antigens (A) and the absence of reactivity in the control (B); nuclei of neurons stained with 4',6-diamidino-2-phenylindole (DAPI). The surface antigens were precipitated using the antibodies within the patient's serum, and then electrophoretically separated and visualised with EZBlue (C). Patient's antibodies (P) precipitated two main protein bands at about 105 kDa and 90 kDa; these bands are not seen in the precipitate using serum from a control individual (N). Sequencing of the 105 kDa band by use of mass spectrometry showed it contained the B1 and B2 subunits of the $GABA_B$ receptor (webappendix). The 90 kDa and other smaller bands were proteolytic fragments and patient's IgG products. Subsequent transfer of the gel to nitrocellulose and immunoblotting with antibodies specific for each of the $GABA_B$ (D) subunits confirmed that patient's antibodies precipitated the B1 and B2 subunits (105 kDa) and that the 90 kDa band was a proteolytic fragment of B1.

Discussion

15 patients had autoimmune encephalitis associated with antibodies to extracellular epitopes of the $GABA_B$ receptor

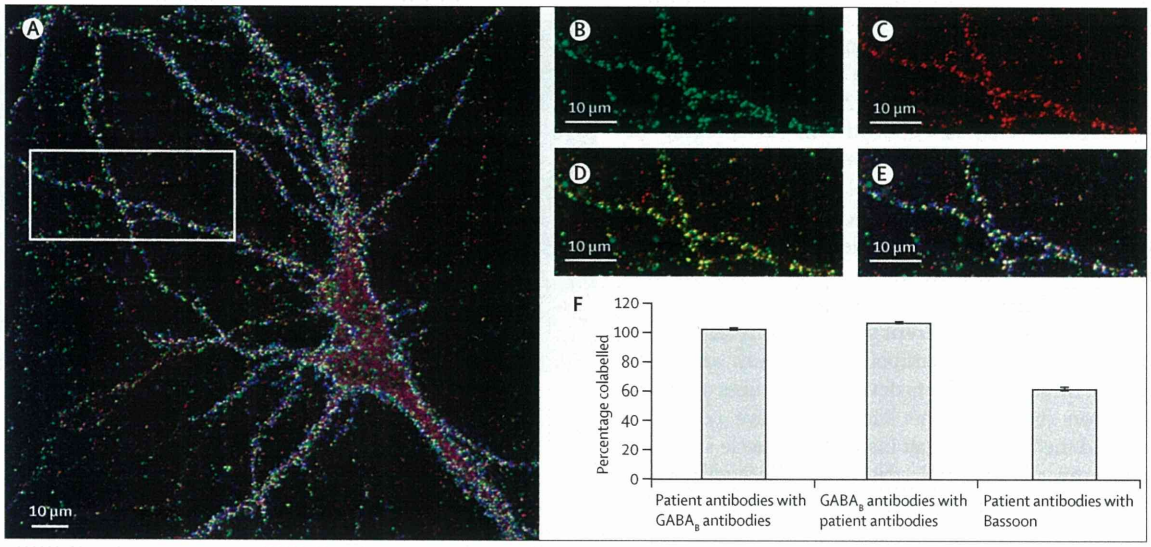

Figure 4: **Confocal image of a cultured triple labelled embryonic rat hippocampal neuron**
Patient's antibodies are in green, a guineapig polyclonal antibody against an intracellular epitope of the GABA$_{B1}$ receptor is in red, and an antibody to the presynaptic marker Bassoon is in blue (A). Area of dendrite from the same neuron showing patient's antibody staining (B), guineapig polyclonal GABA$_{B1}$ receptor antibody staining (C), both patient and guineapig antibody staining (D), and triple staining (E). The colocalisation of labelling of the dendrites of 23 neurons was quantified (F). This suggests that patients' antibodies bind both synaptic and extrasynaptic GABA$_B$ receptors.

and nine responded to treatment. On the basis of clinical, MRI, and EEG findings, the brain regions most affected are the hippocampi and temporal lobes. Thus, it is not surprising that the resulting syndrome is similar to other types of limbic encephalitis (eg, encephalitis associated with antibodies against AMPA receptors or voltage-gated potassium channels), although some clinical and immunological features might suggest GABA$_B$ receptor autoimmunity. We have reported development of seizures in all patients, the association with lung cancer in seven patients (five pathologically confirmed as small-cell lung cancer), and the presence of autoantibodies of unclear relation to this type of limbic encephalitis in seven patients. Disruption of GABA$_B$ receptors by patients' antibodies is a possible explanation for the symptoms because pharmacological[19–21] and genetic[3,4] changes to these receptors in rodents result in phenotypes similar to limbic encephalitis, including prominent seizures, memory deficits, increased anxiety, and mood dysregulation.[22] Moreover, in human beings, some GABA$_B$ receptor polymorphisms are associated with temporal-lobe epilepsy.[23]

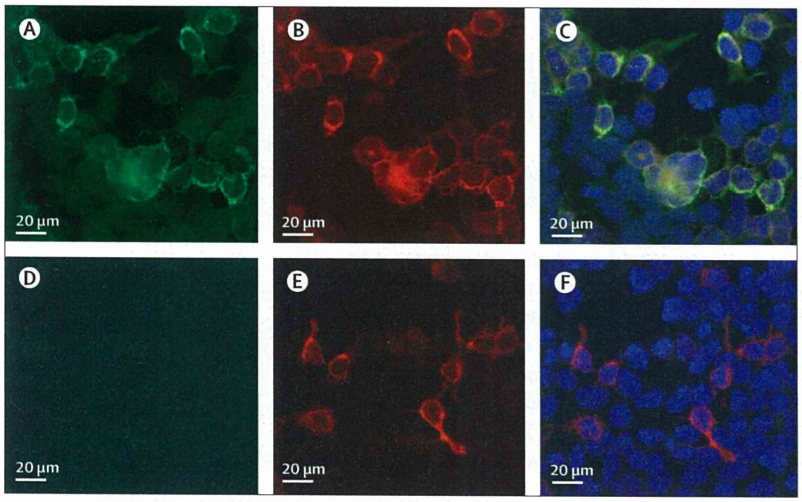

Figure 5: **Detection of antibodies to the GABA$_{B1}$ subunit using a HEK293 cell-based assay**
HEK293 cells transfected with the GABA$_{B1}$ receptor subunit show reactivity with CSF from a patient with limbic encephalitis (A) and a polyclonal antibody against the B1 subunit of the GABA$_B$ receptor (B); both reactivities are merged in C. Similarly transfected cells do not react with CSF from a control individual (D) but do show reactivity with a polyclonal antibody against the B1 subunit of the GABA$_B$ receptor (E); reactivities merged in F. Immunofluorescent method.

GABA$_B$ receptors are G-protein-coupled receptors composed of two subunits, GABA$_{B1}$ and GABA$_{B2}$.[19,24] GABA$_B$ receptors mediate presynaptic inhibition by at least two mechanisms: the activation of G-protein-coupled-inward rectifying potassium channels and the inhibition of calcium channels.[25] These receptors also attenuate presynaptic firing frequencies.[26] Postsynaptic GABA$_B$ receptors mediate inhibition by similar mechanisms[27] and by inducing a slow inhibitory postsynaptic potential.[28] GABA$_B$ receptors limit the duration of network high-activity states, preventing excessive neuronal synchronisation, and allowing new stimuli to break synchronous activity.[29,30] GABA$_B$ receptors are widely distributed in the brain and spinal cord, but the highest levels of GABA$_B$ receptors are found in the hippocampus, thalamus, and cerebellum.[31] In the current study, the corresponding areas of rat brain were more intensely immunolabelled by patients' antibodies. The main antigen recognised by the patients' antibodies, the GABA$_{B1}$ subunit, is necessary for GABA binding and receptor function, whereas the GABA$_{B2}$ subunit is

required for localisation of the receptor to appropriate areas of the cell membrane and G-protein coupling.[32,33]

By use of a HEK293 cell-based assay we showed that the sera or CSF of all 15 patients had antibodies that reacted with $GABA_{B1}$, with additional reactivity to $GABA_{B2}$ in one patient. These findings suggest that HEK293 cells expressing $GABA_{B1/B2}$ or $GABA_{B1}$ could be used as a diagnostic test.

A third of patients with encephalitis and $GABA_B$ receptor antibodies had pathologically confirmed small-cell lung cancer (age range 53–70 years, all smokers). The involvement of this type of tumour in paraneoplastic disorders and its ability to express synaptic proteins, including $GABA_B$ receptors, suggests that it might trigger the immune response against these receptors. In a subgroup of patients with limbic encephalitis and small-cell lung cancer previously thought to be without antibodies or attributed to antibodies against intracellular antigens, $GABA_B$ receptor autoimmunity is probably involved,[34] particularly in patients who improved after treatment of the tumour or immunotherapy.[35,36] Moreover, $GABA_B$ receptor autoimmune encephalitis also seems to develop without cancer association. In this respect, $GABA_B$ receptor autoimmune encephalitis is similar to other synaptic autoimmunities of the CNS (those involving antibodies to NMDA receptors or AMPA receptors)[8–10] or peripheral nervous system (those involving antibodies to acetylcholine receptors or P/Q-type voltage-gated calcium channels) that can develop with or without cancer.[37] As occurs in some of these disorders,[8] almost half of the patients with $GABA_B$ receptor autoimmune encephalitis (including five without tumours) had additional autoantibodies (to TPO, GAD65, SOX1, or N-type voltage-gated calcium channels), suggesting autoimmunity. The overlap with antibodies to GAD65 (an intracellular antigen) suggests that some patients with limbic encephalitis attributed to GAD65 autoimmunity might have $GABA_B$ receptor antibodies as a more likely cause of the symptoms.[38,39] As more relevant cell-surface or synaptic autoantigens are identified, subsets of disorders with unclear definitions, such as steroid-responsive encephalitis or Hashimoto's encephalitis without thyroid peroxidase antibodies in the CSF, will probably be reclassified.

The small number of patients with $GABA_B$ receptor antibodies and the retrospective identification of patients prevented us from assessing the contribution of cancer treatment, immunotherapy, or both, to neurological improvement. Moreover, we were unable to correlate antibody titres with clinical outcome because we did not have serial serum or CSF samples. As this disorder becomes more widely recognised, additional symptoms are likely to be identified. On the basis of the distribution of $GABA_B$ receptors in the brain, one would expect that some patients might develop encephalitis or seizure disorders with less focal limbic dysfunction. This could be tested using HEK293 cells that express $GABA_{B1/B2}$ or $GABA_{B1}$, as described in this paper. By the time antibodies are detected the serum titres can be very low, and we suggest examining both serum and CSF. Identification of these antibodies should prompt the search for a small-cell lung cancer. Recognition of this disorder is important because it is potentially responsive to immunotherapy and treatment of the tumour. The binding of patients' antibodies to the $GABA_B$ receptor in live rat neurons, and the similarity of the syndrome to experimental phenotypes in which this receptor does not function properly, suggest the antibodies are pathogenic. Although $GABA_{B1}$ receptor antibodies are mainly IgG1 and thus able to activate complement, the role of complement-mediated cytotoxicity is questionable in this potentially reversible disorder in which neurons are the main targets. Future studies should focus on the disease mechanism and effects of the antibodies.

Contributors

RC, JR, DF, MBS, WG, AK, KO, TI, MG, FG, and JD designed the study and clinically assessed the patients. EL, ML, XP, EH, and MG did the laboratory studies and prepared the figures. EL, SJM, RB-G, and JD were involved in study design, data analysis, and writing of the report.

Conflicts of interest

RC has received honoraria from Boehringer-Ingelheim and Orion Pharma for projects unrelated to the current study. SJM has received reimbursement for travel and accommodation expenses as well as funding support from Pfizer. JD has received royalties from a patent related to Ma2 autoantibody test and has filed patent applications for NMDA and $GABA_B$ receptor autoantibody tests. JD has received funding from Euroimmun for projects unrelated to the current study. All other authors have no conflicts of interest.

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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

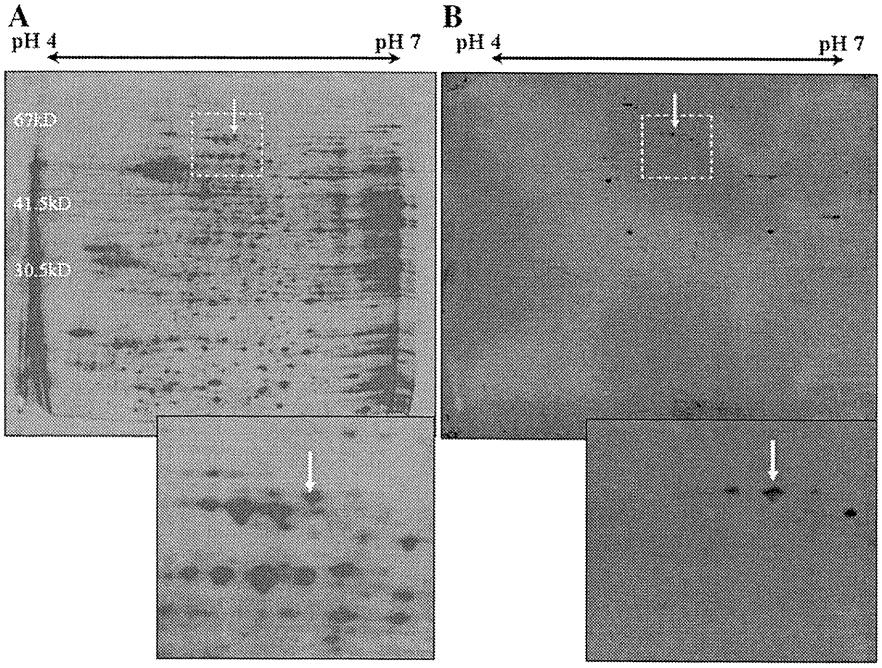

Fig. 1. Polyvinylidene difluoride membrane on to which separated proteins were transferred and stained with fluorescent total protein stain reagent, and two-dimensional immunoblotting result for multiple sclerosis patient with anti-mitochondrial heat shock protein 70 antibody. PVDF membrane on to which 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.

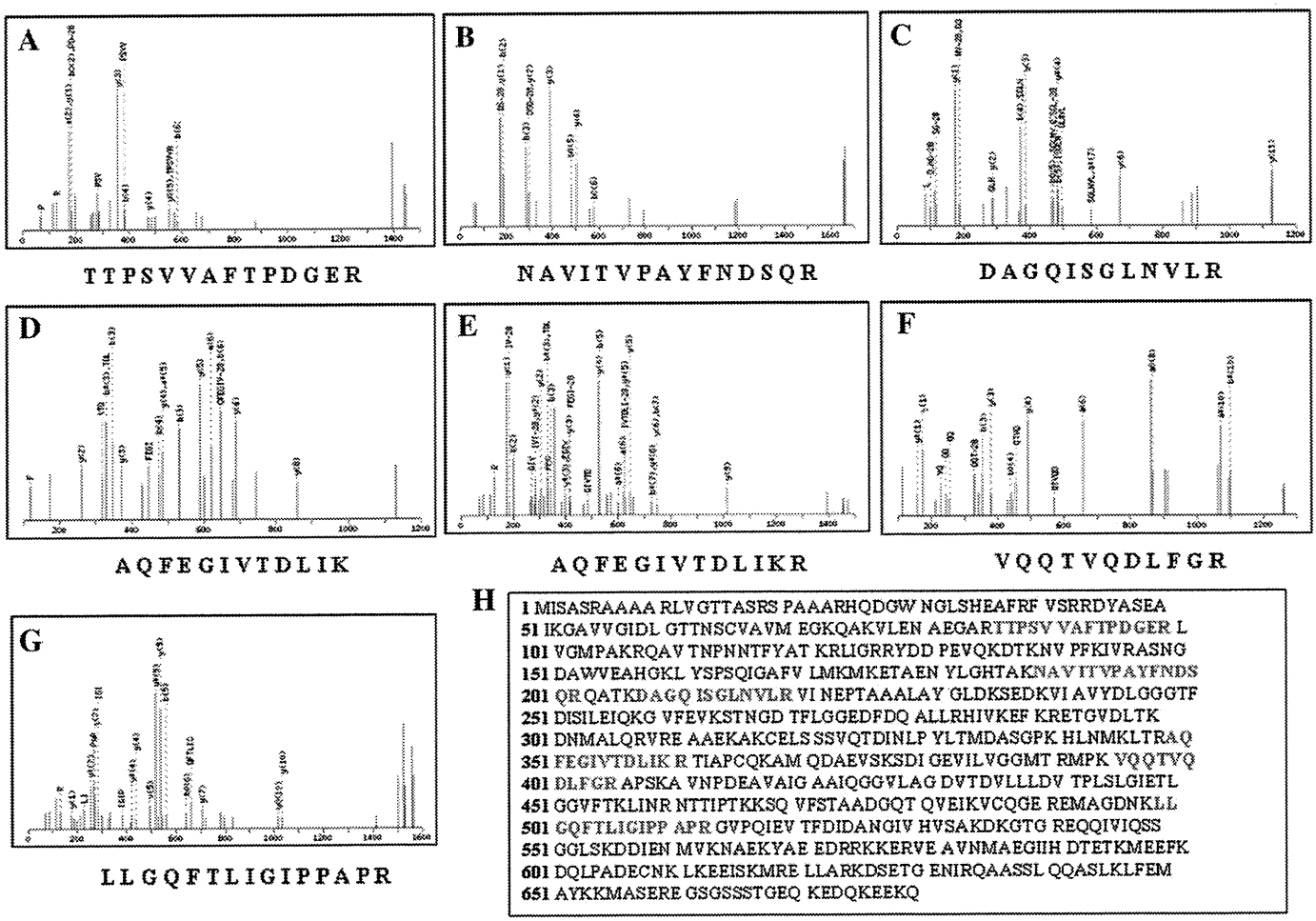

Fig. 2. Identification of mitochondrial heat shock protein 70 by mass spectrometry. MS/MS spectra of seven peptides of mtHSP70 (A–G) and total amino acid sequences of mtHSP70 (H). Sequences in bold red letters indicate the matched sequences of seven peptides. Abbreviations: MS/MS, tandem mass spectrometry;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

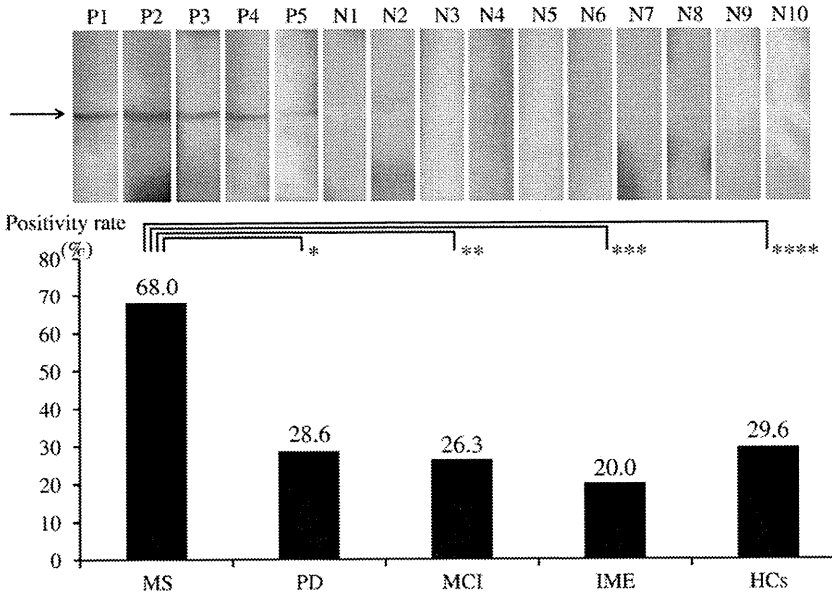

Fig. 3. Immunoblotting of human, mitochondrial heat shock protein 70, full-length, recombinant protein and prevalence of the anti-mitochondrial heat shock protein 70 antibody. Arrows indicate positive bands that immunoreacted with the anti-mtHSP70 antibody. P1–5, 1:2000-diluted serum from MS patients 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

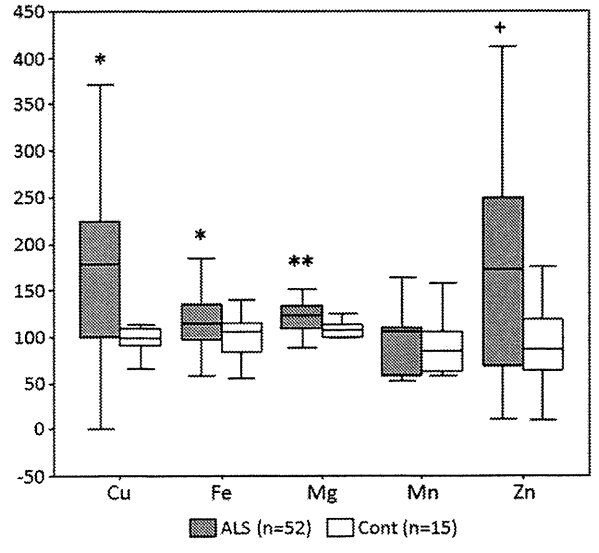

Fig. 1. The levels of the biological metals in the CSF of patients with ALS. The levels of Cu, Fe, Mg, Mn, and Zn in the CSF in patients and controls are shown in the box-and whisker type figure using SPSS. The levels of Mg (** significant difference: $p<0.01$), Fe, Cu (* $P<0.05$) and Zn (+ $p<0.10$),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

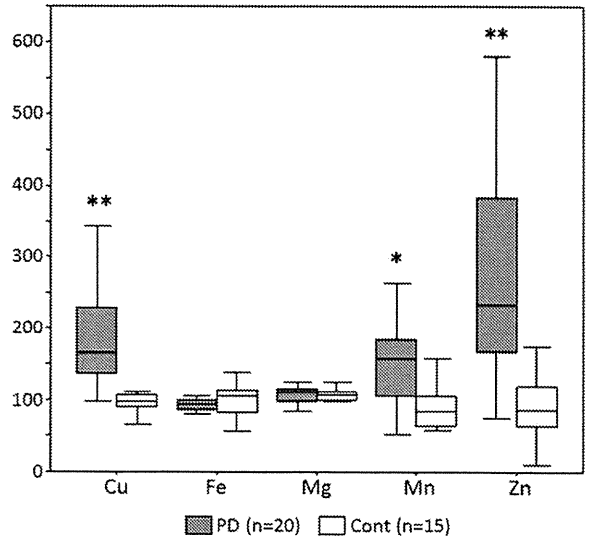

Fig. 3. The levels of biological metals in the CSF of patients with PD. The average levels of Cu, Fe, Mg, Mn, and Zn in the CSF in patients and controls are shown in the box-and whisker type figure using SPSS. The average levels of Mg, Fe, Cu, Zn, and Mn in the CSF in controls are shown to be set at 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

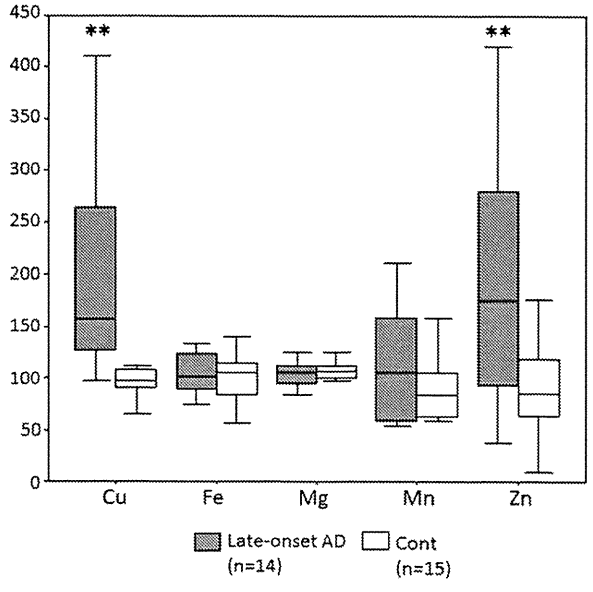

Fig. 2. The levels of biological metals in the CSF of patients with late-onset AD. The average levels of Cu, Fe, Mg, Mn, and Zn in the CSF in patients and controls are shown in the box-and whisker type figure using SPSS. The average levels of Mg, Fe, Cu, Zn, and Mn in the CSF in controls are shown to 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.

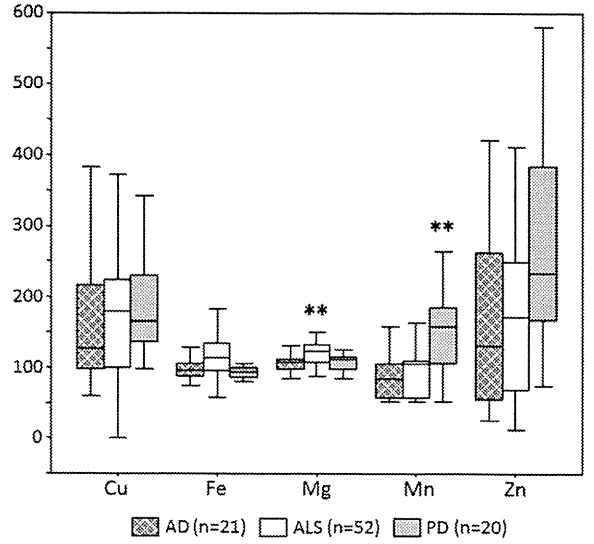

Fig. 4. The levels of the biological metals in the CSF among patients with ALS, AD and PD. The level of Mg in ALS patients was significantly higher than those in AD and PD patients (** $p<0.01$), and the levels of Mn were significantly higher than those in ALS and AD patients (** $p<0.01$) according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

untreated PD. It is needed to evaluate the function of the GI tract in patients with early-stage, untreated PD.

Recently, the ^{13}C-acetate breath test (^{13}C-ABT) has been widely recognized as useful for evaluating gastric emptying because it is less invasive than isotope or acetaminophen methods [10]. It was reported that the ^{13}C-ABT was a reliable and non-invasive tool for the analysis of gastric emptying rates of liquid phases without radiation exposure [11]. Though the ^{13}C-ABT is an isotopic method, it uses a stable isotope not emitting ionizing radiation and is feasible methods for PD patients [12].

The aim of this study is to compare the gastric emptying between patients with early-stage, untreated PD and patients with advanced-stage, treated PD, and healthy volunteers using the ^{13}C-ABT. We tested whether there is the delayed gastric emptying of patients with early-stage, untreated PD.

Methods

Patients

Our study population consisted of 60 patients with an initial diagnosis of PD on the basis of the UK Parkinson's Disease Society Brain Bank Clinical diagnostic criteria [13, 14] and 20 healthy volunteers (control group). The control group was ten men and ten women, median age 69.0 years (range 63–73 years). The patients were divided into two groups: 20 patients with early-stage, untreated PD [eight men and 12 women; median age 70.5 years (range 54–82 years); disease duration 0.9 years (range 0.3–2.5 years)] and 40 patients with advanced-stage, treated PD [14 men and 26 women; median age 67.0 years (range 42–86 years); disease duration 6.0 years (range 3.0–31.0 years)]. Each group of the PD patients was consecutively consulted at our hospital. Modified Hoehn and Yahr classification of the patients with early-stage, untreated PD was stage 1–2, according to the Unified Parkinson's Disease Rating Scale [15, 16]. Modified Hoehn and Yahr classification of the patients with advanced-stage, treated PD was stage 3–4. All PD patients with early-stage were not treated with any medications at first visit, and were followed up for at least 1 year after this study in order to rule out atypical parkinsonism. All PD patients with advanced-stage were being treated with antiparkinsonian medications (long-term L-dopa therapy). No patient was treated with drugs that might alter gastric emptying. None of the PD patients had basic diseases such as liver dysfunction, renal failure, cardiopulmonary disease, diabetes mellitus, GI disease or history of gastric surgery. Clinical characteristics (including age, gender, body mass index) were not significantly different among the PD groups with early-stage and with advanced-stage, or the control group. The results of blood examinations were within normal range. In addition, there were no differences between the PD groups in terms of pepsinogen I, II, and serum gastrin levels, hemoglobin A1c (HbA1c), which might affect gastric motility [17]. The positive ratio of immunoglobulin G anti-Helicobacter pylori antibody did not differ significantly between the PD groups and no patients had past history of peptic ulcer. The positive ratio of orthostatic hypotension (OH) [18] and coefficient of variation of R-R intervals ($CV_{R\text{-}R}$) [19], heart/mediastinum (H/M) ratio of I-[123]-metaiodo-benzylguanidine (MIBG) scintigraphy [20] were not significantly different between the PD groups.

Informed consent was obtained from each subject prior to participation in this study. The study protocol was approved by the Ethical Committee of Gifu University, and was carried out in accordance with the 1975 Declaration of Helsinki.

Gastric emptying examination

The GE examination was carried out using the ^{13}C-breath test according to Ghoos [10] with slight modifications. PD patients and healthy volunteers were tested after an overnight fast of 12 h. All PD patients did not take any antiparkinsonism drug over 24 h. Early in the morning, PD patients and healthy volunteers took the liquid test meal (Racol: TM, 200 kcal/200 ml; Otsuka Pharmaceuticals Co., Ltd., Tokyo, Japan) containing 100 mg ^{13}C-sodium acetate. Thereafter, an expiration breath sample was collected every 10 min for 4 h and analyzed for $^{13}CO_2$ using an IR spectrophotometer (UBiT-IR300; Otsuka Electronics Co., Ltd., Tokyo, Japan). During the examination, all subjects were in a sitting position.

The principle of ^{13}C-ABT is ingestion of a liquid test meal containing ^{13}C-acetate, gastric emptying, absorption from the digestive tract, metabolism in the liver (production of $^{13}CO_2$), expiration from the lung, and increase of $^{13}CO_2$ in expired breath.

Mathematical analysis

The data were used for mathematical curve fitting. A best fit curve of expired $^{13}CO_2$ was constructed for each subject. The %-$^{13}CO_2$ cumulative excretion in the breath was assessed using a non-linear regression formula [21, 22]: $y = m\ (1 - e^{-kt})^{\beta}$ to fit the curve of the cumulative ^{13}C recovery. The %-$^{13}CO_2$ excretion per hour was fitted to the formula $mkbe^{-kt}\ (1-e^{-kt})^{\beta-1}$. T is time and m, k, and β are constants. The value of m represents the total cumulative $^{13}CO_2$ recovery when the time is infinite. The half emptying time (HET) was calculated using the formula: $HET = -1/k\ \ln(1 - e^{-1/\beta})$. T_{max} is the peak time of the ^{13}C%–dose-excess curve (%–dose/h) based on a time