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

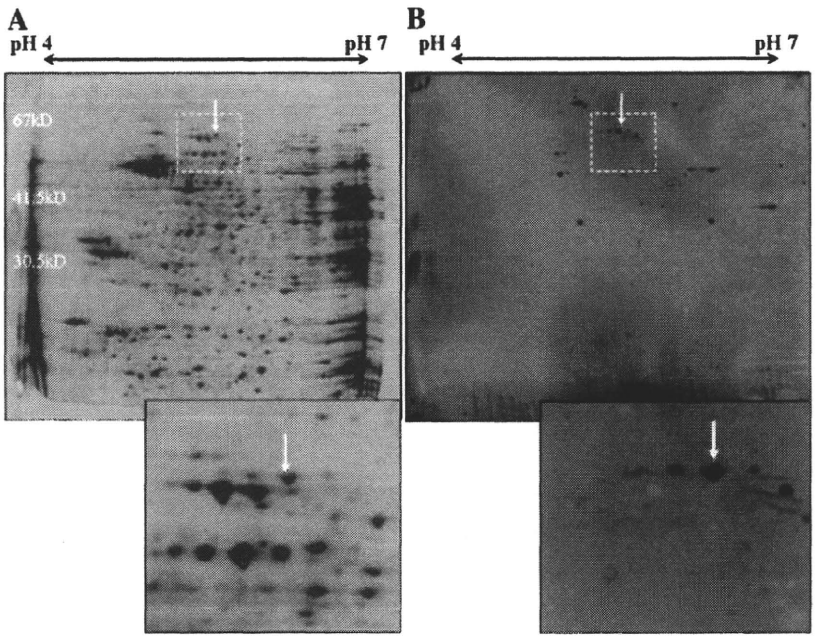

Fig. 1. Polyvinylidene difluoride membrane on to which separated proteins were transferred and stained with fluorescent total protein stain reagent, and two-dimensional immunoblotting result for multiple sclerosis patient with anti-mitochondrial heat shock protein 70 antibody. PVDF membrane on to which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

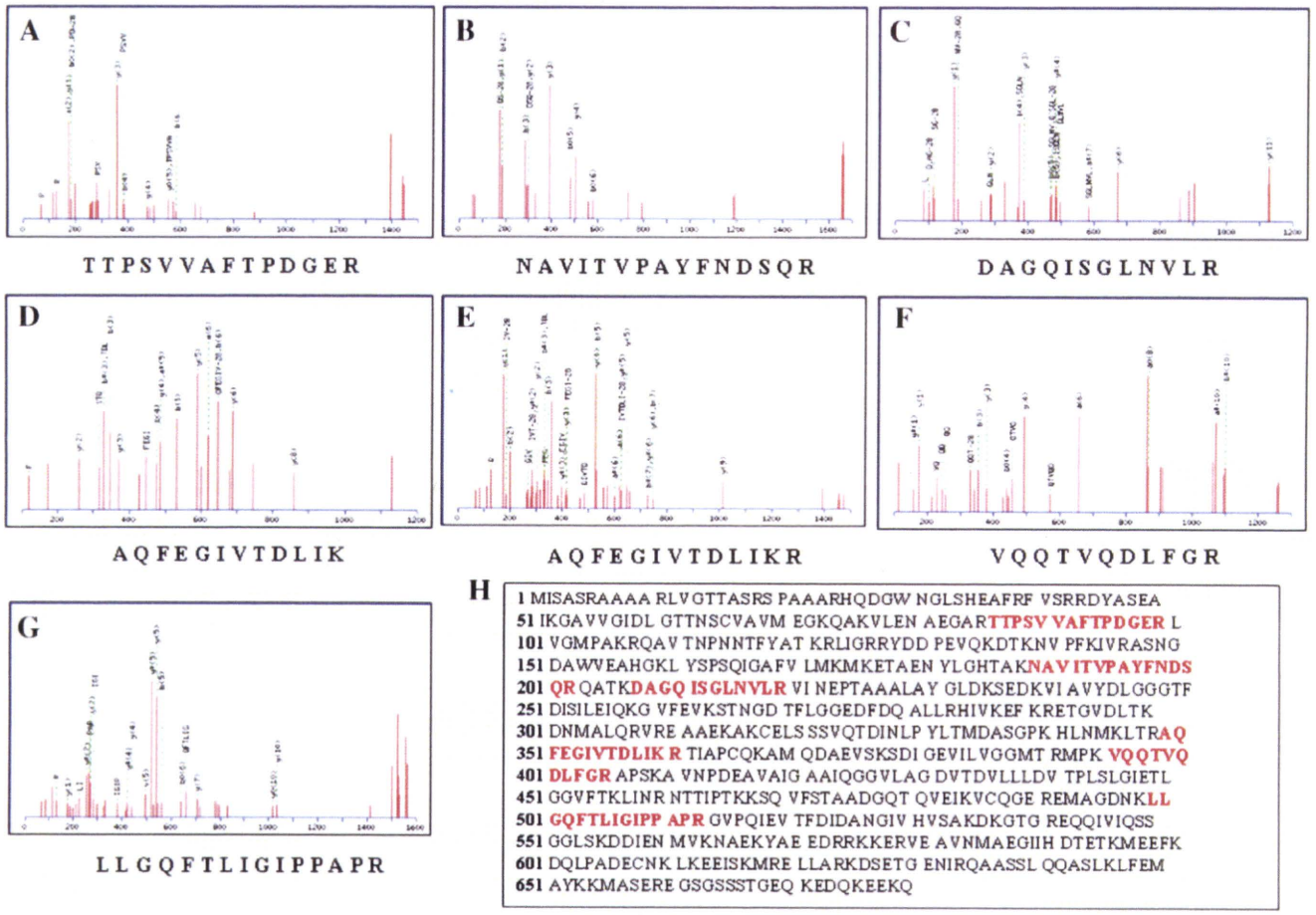

Fig. 2. Identification of mitochondrial heat shock protein 70 by mass spectrometry. MS/MS spectra of seven peptides of mtHSP70 (A–G) and total amino acid sequences of mtHSP70 (H). Sequences in bold red letters indicate the matched sequences of seven peptides. Abbreviations: MS/MS, tandem mass spectrometry;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

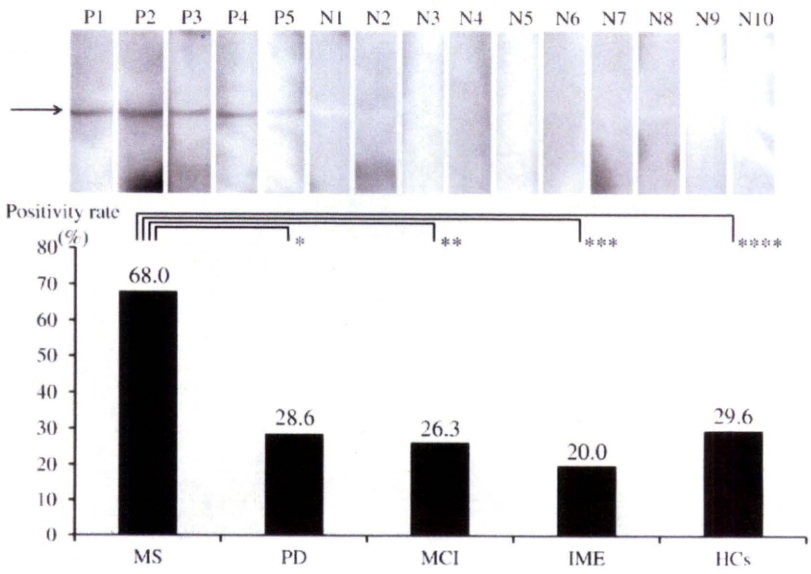

Fig. 3. Immunoblotting of human, mitochondrial heat shock protein 70, full-length, recombinant protein and prevalence of the anti-mitochondrial heat shock protein 70 antibody. Arrows indicate positive bands that immunoreacted with the anti-mtHSP70 antibody. P1–5, 1:2000-diluted serum from MS patients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