Proteomic analysis of human brain identifies α-enolase as a novel autoantigen in Hashimoto's encephalopathy

Hirofumi Ochi[a,1], Izumi Horiuchi[a,1], Norie Araki[b,*], Tosifusa Toda[c], Tomohiro Araki[d], Kaori Sato[e], Hiroyuki Murai[a], Manabu Osoegawa[a], Takeshi Yamada[f], Ken Okamura[e], Tomoaki Ogino[g], Kiyohisa Mizumoto[g], Hirohumi Yamashita[h], Hideyuki Saya[b], Jun-ichi Kira[a]

[a] *Department of Neurology, Neurological Institute, Graduate School of Medical Sciences, Kyushu University, Fukuoka 812-8582, Japan*
[b] *Department of Tumor Genetics and Biology, Kumamoto University School of Medicine, Kumamoto 860-0811, Japan*
[c] *Proteomics Collaboration Center, Tokyo Metropolitan Institute of Gerontology, Tokyo 173-0015, Japan*
[d] *Department of Bioscience, School of Agriculture, Kyushu Tokai University, Kumamoto 869-1404, Japan*
[e] *Second Department of Internal Medicine, Graduate School of Medical Sciences, Kyushu University, Fukuoka 812-8582, Japan*
[f] *Department of Neurology, Aso Iizuka Hospital, Iizuka 820-8505, Japan*
[g] *Department of Biochemistry, School of Pharmaceutical Sciences, Kitasato University, Tokyo 108-8641, Japan*
[h] *Department of Neurology and Brain Pathology, Graduate School of Medicine, Kyoto University, Kyoto 660-8501, Japan*

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Abstract Hashimoto's encephalopathy (HE) is a rare autoimmune disease associated with Hashimoto's thyroiditis (HT). To identify the HE-related autoantigens, we developed a human brain proteome map using two-dimensional electrophoresis and applied it to the immuno-screening of brain proteins that react with autoantibodies in HE patients. After sequential MALDI-TOF-MASS analysis, immuno-positive spots of 48 kDa (p*I* 7.3–7.8) detected from HE patient sera were identified as a novel autoimmuno-antigen, α-enolase, harboring several modifications. Specific high reactivities against human α-enolase were significant in HE patients with excellent corticosteroid sensitivity, whereas the patients with fair or poor sensitivity to the corticosteroid treatment showed less reactivities than cut-off level. Although a few HT patients showed faint reactions to α-enolase, 95% of HT patients, patients with other neurological disorders, and healthy subjects tested were all negative. These results suggest that the detection of anti-α-enolase antibody is useful for defining HE-related pathology, and this proteomic strategy is a powerful method for identifying autoantigens of various central nervous system diseases with unknown autoimmune etiologies. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: α-enolase; Autoantigen; Proteomics; Two-dimensional electrophoresis; Brain

1. Introduction

Hashimoto's encephalopathy (HE) is a rare encephalopathy associated with autoimmune Hashimoto's thyroiditis (HT) [1]. To date only less than 40 cases of HE have been documented in the literature since the first report in 1966 [2,3]. It initially shows acute or sub-acute onset followed by relapsing-remitting courses, but eventually develops a severe dementia like Alzheimer's disease. As HE responds well to corticosteroids in early stage, its proper early diagnosis is critically important. The presence of anti-thyroid antibodies is suggestive for HE as well as HT; there are, however, no specific clinico-laboratory tests for defining HE-pathology, thus the HE patients have been found with very low frequency.

To analyze the clinical features of HE precisely, we have collected HE-like patients' data from several areas in Japan since 1992. To date, five patients are defined as HE, almost fulfilling the clinical Peschen–Rosin's criteria [3]. Although the mechanism of HE remains unclear, several lines of evidence suggest an autoimmune etiology of HE [4,5]. The presence of autoantibodies in patients is one of hallmarks of autoimmunity, yet identification of HE-relevant autoantigens in the central nervous system (CNS) has never been reported. Although the humoral immune response of HE patients has been analyzed by the serological analysis of a brain-derived cDNA expression library (SEREX) [6], this approach has not yet yielded any HE-relevant autoantigens.

Recently, we developed a mouse brain proteome map and applied it to the pathological study of brain diseases [7]. This strategy was thought to be applicable to the screening of autoantigens in human CNS diseases with autoimmune etiologies by using a human brain map. Thus we have newly developed a proteomic screening system for identifying any autoantigens by creating a human brain protein map of two-dimensional gel electrophoresis (2-DE), a sequential Western immunoblotting system with patient's sera, and subsequent sensitive methods for the identification of target spots by high-throughput matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometric analysis. By using this system, proteins immuno-reactive to the HE patients' sera were successfully identified as human α-enolase harboring various modified structures, and the high specificity was confirmed using a variety of control patients including

*Corresponding author. Fax: (81)-96-373 5120.
E-mail address: nori@gpo.kumamoto-u.ac.jp (N. Araki).

[1] These authors have equally contributed to this work.

Abbreviations: HE, Hashimoto's encephalopathy; HT, Hashimoto's thyroiditis; 2-DE, two-dimensional gel electrophoresis; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; CNS, central nervous system; BPB, bromophenol blue; IPG, immobilized pH gradient; PVDF, polyvinylidene difluoride; CBB, Coomassie Brilliant Blue; OND, other neurological disorder; HC, healthy subject

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HT and normal subjects. The possible relation between this autoantigen and an HE-etiology as well as the validity of screening using the brain proteomic strategy is discussed.

2. Materials and methods

2.1. Subjects

Sera from five patients with clinically diagnosed HE were studied. All had a corticosteroids-responsive reversible encephalopathy associated with euthyroid HT [8], and exhaustive exclusions of other causes of encephalopathy had been carried out. Four female patients (patients 1, 2, 3 and 4) had clinically definite HE with multiphasic courses according to Peschen–Rosin's criteria [3]. Patients 1, 2 and 3 showed excellent responses to corticosteroids, while patient 4 showed a fair response. The male patient (patient 5), because of a monophasic course, possibly had HE showing a poor corticosteroids response. All serum samples were from patients with an active, untreated disease. Apart from HT, none of the HE patients had other autoimmune diseases. Sera from 25 healthy subjects (HC), 54 patients with HT without encephalopathy and 20 patients with other neurological disorders (OND; 10 with encephalitis, 5 with neurodegenerative disorder, 4 with multiple sclerosis and one with acute disseminated encephalomyelitis) were used as controls.

2.2. Preparation of tissue proteins

Human parietal lobe tissues of cerebrum were obtained from a 68-year-old man who died of pneumonia and underwent an autopsy 6 h postmortem. He had no neurological diseases and there were no abnormalities found upon routine brain examination. Brain tissue (250 mg wet weight) was homogenized with 800 μl of a lysis solution, consisting of 9.8 M urea, 2% w/v NP-40, 2% v/v Pharmalyte, 100 mM DTT, 0.5 μg/ml E-64, 0.5 mM PMSF, 40 μg/ml TLCK, 1 μg/ml aprotinin, 10 μg/ml chymostatin, 0.5 mM EDTA, and 0.01% w/v bromophenol blue (BPB). The protein concentration was determined in the lysis solution with a dye reagent from Bio-Rad Laboratories (Richmond, CA, USA) using BSA as a standard, and adjusted to 140 μg/ml (50 μg protein per gel) for silver staining, and to 1.4 mg/ml (500 μg protein per gel) for protein identification. An aliquot of 350 μl was mixed with the internal marker proteins and subjected to 2-DE.

2.3. Two-dimensional electrophoresis (2-DE)

2-DE was carried out in a horizontal electrophoresis system, Maltiphor II (Amersham Pharmacia), for the first-dimensional isoelectric focusing using Immobiline dry strip (18-cm long for large gels and 7-cm for mini gels, linear gradient between pH 3–10, Amersham Pharmacia), and by a Giant slab gel electrophoresis system (Nihon-Eido, Tokyo, Japan) for the second-dimensional SDS–PAGE (12.0% gel). The first-dimensional immobilized pH gradient (IPG) on the rehydrated gel strip was carried out according to the manufacturer's instructions. After the first dimension, the IPG gel was equilibrated with equilibration buffer consisting of 50 mM Tris–HCl (pH 6.8), 6 M Urea, 3% SDS, 50 mM DTT and 0.01% BPB, and subjected to the second-dimensional SDS–PAGE (20×20×0.1 cm for large gels, and 8×10×0.1 cm for mini gels).

2.4. Western blotting and computer graphic analysis

The protein spots on the gels were electroblotted onto a polyvinylidene difluoride (PVDF) membrane (Fluortrans™, Pall Ultrafine Filtration, CA, USA) with a semi-dry blotting apparatus (Nihon-Eido, Tokyo, Japan) at 0.5 mA/cm^2 for 3 h at 4°C. The blotting buffer for amino acid sequencing of the PVDF membrane consisted of 10 mM CAPS-NaOH (pH 11.0) and 10% methanol. The PVDF membrane was stained with 0.1% Coomassie Brilliant Blue (CBB) in 50% methanol or with the fluorescent SYPRO ruby protein blot stain reagent (Bio-Rad). The stained gels and PVDF membranes were scanned and analyzed using Image Reader Ver. 1.3 and Mac Bas V2.5 (Fuji Photo Film), and analyzed using PDQUEST™ software.

2.5. Peptide mass fingerprinting and protein sequencing

Brain proteins separated on the 2-DE-gel were stained with CBB or fluorescent reagent for polyacrylamide gel (SYPRO ruby gel stain, Bio-Rad). The protein spots detected to be immuno-reactive with patient's sera were cut and sliced into small pieces. The gels were then subjected to reduced-*S*-pyrydilethylation, dehydrated in 100% acetonitrile, dried in a Speed-Vac, and soaked again with digestion buffer containing trypsin. After the overnight protein digestion, peptide fragments in the supernatant were desalted by Zip tips C18 (Millipore, Bedford, MA, USA) and subjected to MALDI-TOF mass spectrometry (MALDI-TOF, Micro Mass, London, UK) for peptide mass fingerprinting (PMF). The background noise was removed by subtraction of mass signals obtained from a control gel. Protein identification was carried out using the MS-fit search engine in ProteinProspector (UCSF) by sending a query of the subtracted PMF data [9]. For the N-terminal sequence, the stained protein spot on the PVDF membrane was cut out and transferred into the cartridge of the Procise™ 494cLC sequence system (Applied Biosystems, Foster City, CA, USA). The amino acid sequence obtained from the analysis was subjected to a search for sequence similarity against the PIR International Database and/or SWISS-PROT [7].

2.6. Detection of anti-human α-enolase antibodies in patients' sera

6×His-recombinant human α-enolase expression vector, pQE-hENOα [10] was expressed in *Escherichia coli* XL1-Blue strain and partially purified by a Ni-NTA super-flow affinity column (Qiagen, Chatsworth, CA, USA). Rabbit β-enolase (Sigma, St. Louis, MO, USA), human γ-enolase (Polysciences, Warrington, PA, USA), and the purified recombinant human α-enolase were run on 10% SDS–PAGE gel, transferred onto PVDF membrane, and probed with sera samples. Goat anti-human α-enolase antibody (Santa Cruz Biotechnology, CA, USA) was used as a positive control. The second antibody was horseradish peroxidase-conjugated sheep anti-human IgG (Amersham Pharmacia) or horse anti-goat IgG (VECTOR Laboratories, Burlingame, CA, USA), and bound conjugate was detected by enhanced chemiluminescence.

Table 1
Mass-fit analysis for tryptic fragments of spot 1-1

Signal no.	m/z^+ submitted	NH^+ matched	Delta Da	Start–end amino acid no.	Peptide sequences	Modifications
1	1143.9333	1143.6156	0.3171	183–192	(R)IGAEVYHNLK(N)	
2	1426.1017	1425.7260	0.3751	269–280	(R)YISPDQLADLYK(S)	
3	1526.1854	1525.7692	0.4156	358–371	(K)LAQANGWGVMVSHR(S)	
4	1541.1962	1540.7834	0.4128	239–252	(K)VVIGMDVAASEFFR(S)	
5	1557.1670	1540.7834	0.3887	239–252	(K)VVIGMDVAASEFFR(S)	Met-Oxidized
6	1682.2870	1681.8583	0.4287	345–359	(K)VNQIGSVTESLQACK(L)	
7	1908.4705	1907.9870	0.4829	162–178	(K)LAMQEFMILPVGAANFR(E)	
8	1924.4503	1907.9870	0.4678	162–178	(K)LAMQEFMILPVGAANFR(E)	Met-Oxidized
9	1940.4369	1907.9870	0.4594	162–178	(K)LAMQEFMILPVGAANFR(E)	2Met-Oxidized
10	2033.5356	2033.0549	0.4801	306–325	(K)FTASAGIQVVGDDLTVTNPK(R)	

Selected mass signals for tryptic peptides of spot 1-1 by MALDI-TOF-Mass analysis were completely matched (100%; 10/10) for the database sequence of tryptic fragments of human α-enolase with a high MOWSE Score (6.93e+007). The peptide sequences shown in the table were obtained from database sequences of α-enolase, of which peptide masses were matched to the search query of tryptic peptide-mass fingerprinting. The expected amino acid to be followed to the N-terminal or C-terminal sequence of each identified peptide is listed in parentheses. The MS-fit analysis for spots 1-2, and 1-3 (data not shown) also showed the same results as those for 1-1. The results of each spot were reproduced in the three independent analyses. Representative data of 1-1 are shown in this table.

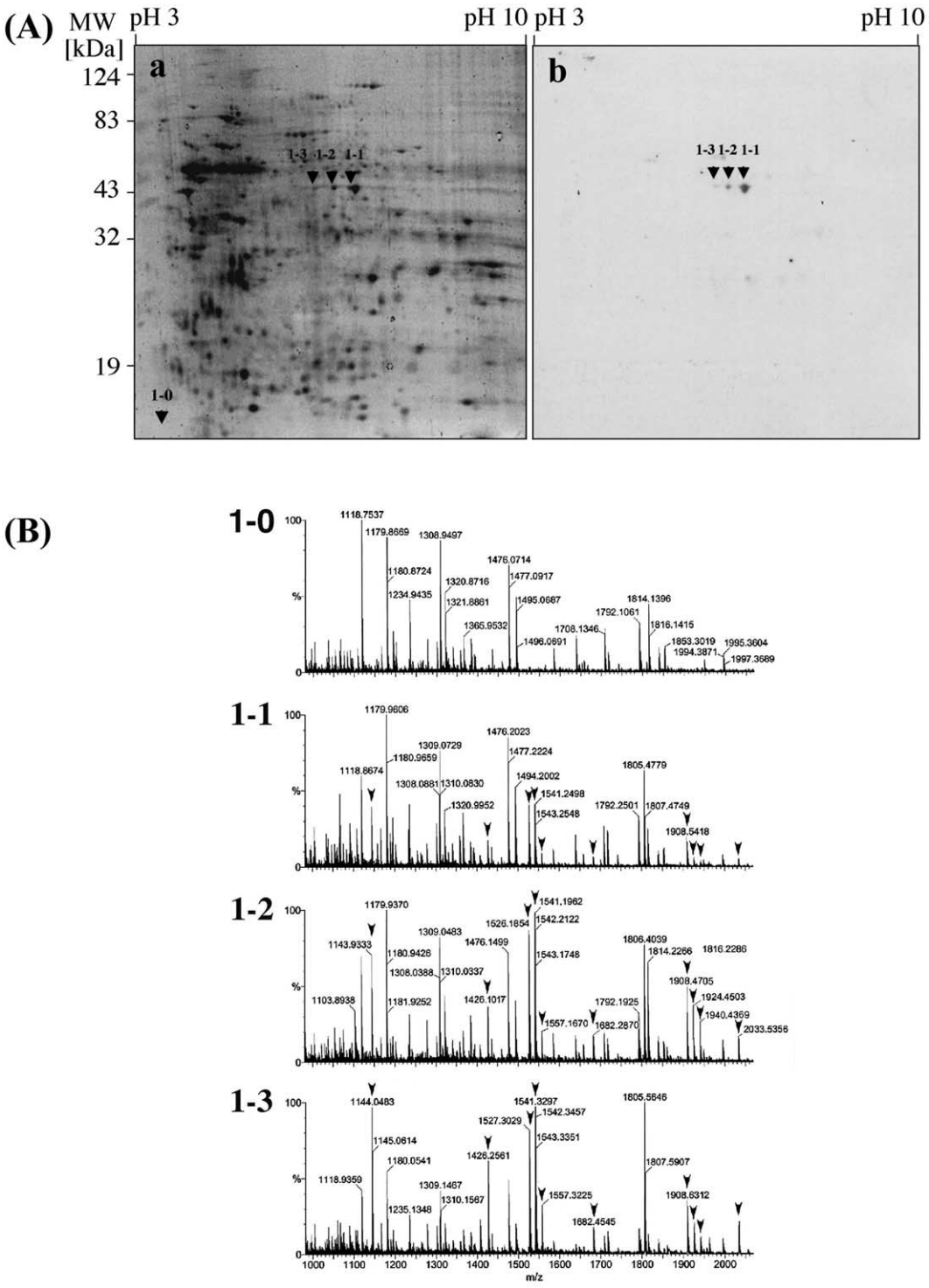

Fig. 1. A: 2-DE pattern of human brain proteins, and detection of immuno-reactive proteins against autoantibodies of HE patients by immunoblotting. The brain proteins on 2-DE-gel were stained with fluorescent protein staining reagent (a), and the immuno-positive proteins on the PVDF membrane were detected by immunoblotting with sera from HE patient 1 (b). The specific spots 1-1, 1-2 and 1-3 reactive to the autoantibody from the HE patient's sera and a negative control 1-0 on 2-DE-gel are indicated with arrowheads. B: MALDI-TOF mass spectra of immuno-reactive spots 1-1, 1-2, 1-3 and control spot 1-0. The immuno-reactive spots (1-1, 1-2, 1-3) and negative control gel (1-0) were excised, digested with trypsin, and subjected to MALDI-TOF mass analysis. Ten common signals of mono-isotopic peptides (*m/z* 1143.9, 1426.1, 1526.1, 1541.1, 1557.1, 1682.2, 1908.4, 1924.4, 1940.4, 2033.5) are detected in all three spectra for spots 1-1, 1-2 and 1-3 after subtraction of noise peaks that also exist in the tryptic digest of control spot 1-0. Mass-fit search for PMF was performed using the ten signals.

3. Results

3.1. Identification of brain proteins reacted with autoantibodies in HE patients

To detect the brain proteins that react with autoantibodies raised in HE patients, we first created a human brain 2-DE map. Brain extracts were separated on 2-DE (p*I* 3–10, 12% PAGE, 20×20 cm) and stained with fluorescent reagent (Fig. 1Aa) and then CBB. The protein spots on the 2-DE map were counted up to 2580 and the major proteins assigned according to the mouse brain map presented previously [7,11]. Aliquots of the brain proteins were subjected to 2-DE and transferred to PVDF membrane. Sera from HE patient 1 were reacted on the membrane, and the brain proteins that reacted with the patient's IgG were screened. Three spots of 48-kDa proteins with p*I* ranging from 7.3 to 7.8 were detected (Fig. 1Ab: 1-1, 1-2, 1-3), and all were subjected to MALDI-TOF mass spectrometric analysis. The signals derived from the contaminants such as autolytic fragments of trypsin in the negative control (1-0) were successfully subtracted as background noise from those of the protein spots 1-1, 1-2 and 1-3. As shown in Table 1, all of the ten specific signals (indicated by arrows in Fig. 1B) selected in the spectrometry of the tryptic peptide from each protein (Fig. 1B: 1-1, 1-2, 1-3) were well matched with the calculated masses of tryptic fragments of human α-enolase, EC.4.2.1.11 (2-phospho-D-glycerate hydrolase; non-neuronal enolase, NNE; phosphopyruvate hydratase; SWISS-PROT Accession No. P06733). The MOWSE Scores of the Mass-fit analysis for 1-1, 1-2, and 1-3 vs. human α-enolase were 6.93e+007, 1.098e+007, and 1.436e+006, respectively. The tryptic fragments of 162–178 and 239–252 of the brain α-enolase were included with the oxidation forms of 164/168Met and 243Met, respectively. N-terminal amino acid analysis of spots 1-1 and 1-2 revealed their N-termini Ser to be blocked. Thus, we concluded that the proteins of 48 kDa that reacted with HE patients' sera were varied modified forms of human α-enolase. The same results were obtained in the experiment using another 2-DE map of human temporal lobe of cerebrum from another woman aged 64 years who died of pneumonia (data not shown).

3.2. Immuno-reactivities against human α-enolase

Specific positive signals against recombinant human α-enolase were found by Western immunoblotting in serum samples from 3 of the 5 (60%) patients with HE (Fig. 2A,B); i.e. significant high immuno-reactivities were found in all of the three patients with clinically definite relapsing HE showing excellent corticosteroid responses (patients 1–3), while neither patient with clinically definite relapsing HE showing a fair corticosteroid response (patient 4) nor with monophasic possible HE showing a poor corticosteroid response (patient 5) had more reactivities than cut-off level against α-enolase (Fig. 2A). Fifty-one of the 54 HT (94.4%) patients without encephalopathy showed negative reactivities, although three (5.6%) of the HT patients showed faint immuno-reactivities (less than 1/20 intensity of HE patients 1–3) against α-enolase (Fig. 2A). None of the 20 patients with OND (10 with encephalitis, 5 with neurodegenerative disorder, 4 with multiple sclerosis, and one with acute disseminated encephalomyelitis) or the 25 HC were positive (0%). The positive rates in HE were significantly higher compared with those in HT without encephalopathy ($P = 0.021$), OND ($P = 0.026$), and HC ($P = 0.012$; Fig. 2B).

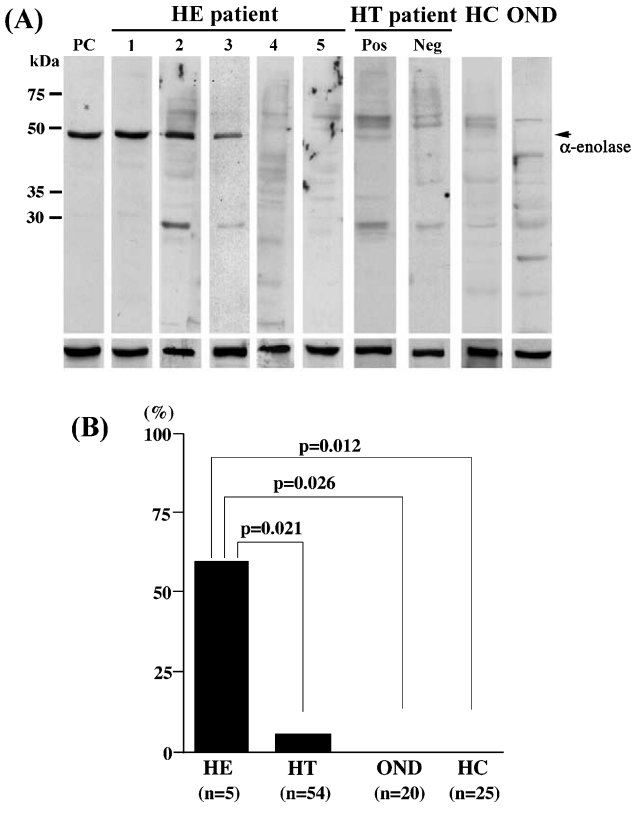

Fig. 2. A: Immuno-reactivities against recombinant human α-enolase. Partially purified bacterial 6×His-recombinant human α-enolase was separated on 10% SDS–PAGE, transferred on PVDF membranes, and probed with goat anti-human α-enolase antibody (upper panel; PC, positive control), or sera from HE patients 1, 2, 3, 4, and 5 (upper panel; lanes 1, 2, 3, 4 and 5) with a dilution of 1:200. Results of two representative HT (HT without encephalopathy) patients (upper panel; one is positive, Pos; another is negative, Neg), one representative HC (upper panel; HC) and one representative OND patient (upper panel; OND) are also shown. After stripping, the membranes were re-probed with goat anti-human α-enolase antibody (lower panel). B: The positive rates of anti-α-enolase antibody. Immuno-reactivities against human α-enolase were detected by Western blotting with sera samples from five patients with HE, 54 with HT (HT without encephalopathy), 20 with OND (10 with encephalitis, five with neurodegenerative disorder, four with multiple sclerosis and one with acute disseminated encephalomyelitis) and 25 with HC. The vertical line shows the % ratio of the sample numbers with positive immuno-reaction in all of the sample numbers tested in each group. Fisher's exact test was used for statistical analysis.

Additional immuno-positive reactivities against other proteins with M.W. about 30 kDa, besides human α-enolase (48 kDa), possibly derived from *Escherichia coli* XL1-Blue strain, were detected in some serum samples from HE patients, HT patients, OND patients and HC (Fig. 2A). However, there were no significant differences in the frequency against the additional signals for 30 kDa among HE, HT, OND or HC.

3.3. Specificity of anti-human α-enolase antibody

Goat anti-human α-enolase antibody showed five immuno-positive spots of 48-kDa brain proteins (Fig. 3A). Subsequent sequence analysis of these spots revealed that the very acidic one (p*I* 5), absent in the immunoblotting with the patient's sera, was γ-enolase, suggesting the cross reactivity of this antibody against γ-enolase of human brain. On the other hand,

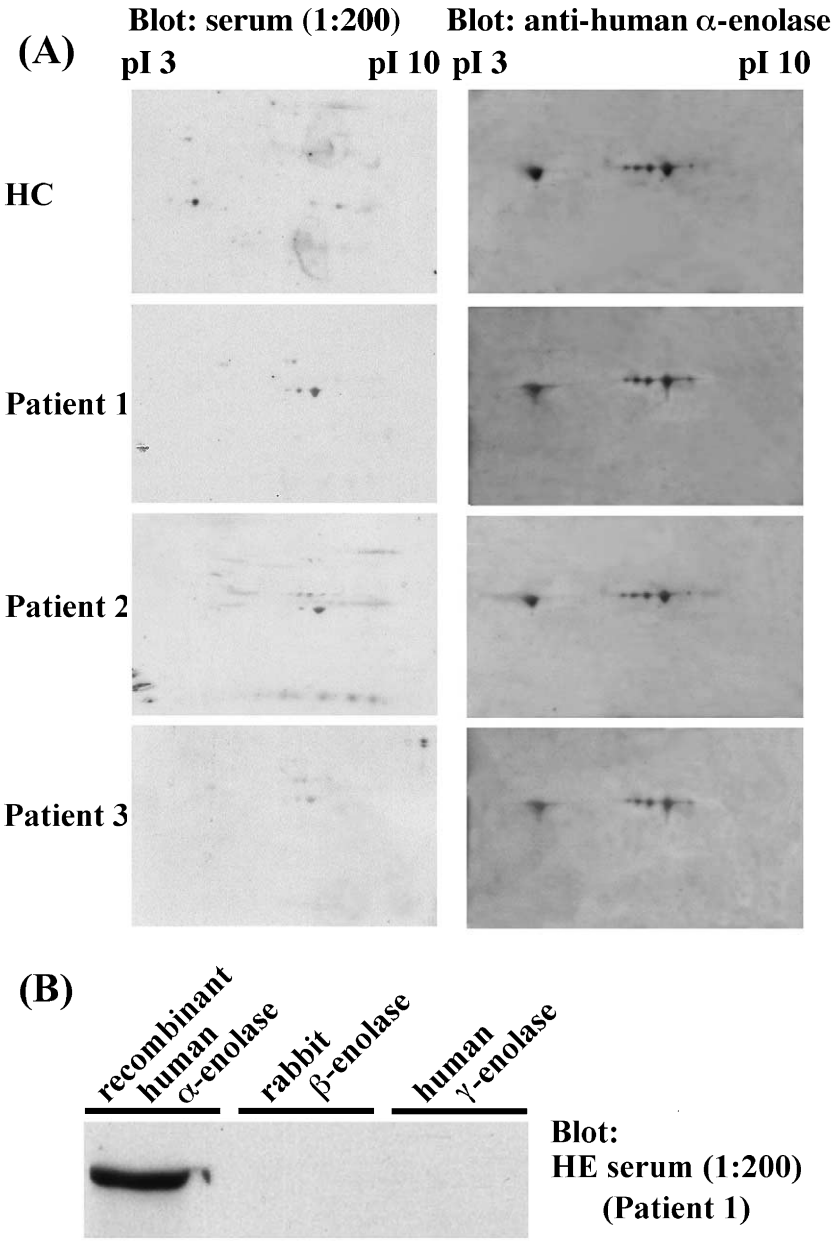

Fig. 3. A: Characterization of immuno-reactivities of HE patients' sera by 2-DE-immunoblotting. Human normal brain extracts were separated on mini-scaled 2-DE, transferred on the PVDF membrane, and probed with sera samples from patients 1, 2 and 3 and a control subject (HC) (left panels). Immuno-positive spots were visualized with enhanced chemiluminescence. After stripping, the membranes were re-probed with goat anti-human α-enolase antibody (right panels). B: Immuno-reactivities of HE patients' sera against α-, β- and γ-enolase isoforms. Recombinant human α-enolase, rabbit β-enolase and human γ-enolase were separated on 10% SDS–PAGE gel, transferred onto the PVDF membrane, and probed with HE patient sera containing anti-α-enolase antibodies with a dilution of 1:200. Representative data of HE patient 1 are shown. Totally identical results were obtained from two other HE patients (patients 2 and 3).

sera from three HE patients (patients 1, 2 and 3) reacted with only the basic two or three spots of α-enolase but not with the acidic one of γ-enolase (Fig. 3A). Furthermore, sera from these patients reacted neither with rabbit β-enolase (human α-enolase shares 85% amino acid identity to rabbit β-enolase) nor human γ-enolase (Fig. 3B). These results suggest that autoantibodies from HE patients react with the specific epitope structure of human α-enolase.

4. Discussion

This study, by developing a new screening system for the autoantigen in human brain, is the first to identify α-enolase as an autoantigen in HE that exists both in the thyroid gland and in the brain. It is important that the autoantigen detected in this screening system is highly relevant for HE. Only three of the 54 patients with HT contained anti-α-enolase antibodies with much weaker reactivities than those of immuno-positive HE patients. Anti-α-enolase antibody is thus considered to be specific for HE, especially for multiphasic HE, rather than HT.

Enolase is a ubiquitous glycolytic enzyme highly conserved through evolution [12]. It exists as three highly homologous isozymes: α, β and γ; isozyme α is found in most tissues,

isozyme β in muscle, and isozyme γ is present mainly in nervous tissue [13]. Despite about 83% homology between amino acids among three isozymes [14], the antibodies in HE only reacted with α-enolase. Furthermore, sera from HE patients reacted with various modified forms of α-enolase, the results suggesting that these antibodies may recognize unique structural epitopes on this protein. However, further biochemical study will be needed regarding the specific epitope structure of α-enolase.

One possible mechanism of HE is autoimmune cerebral vasculitis [15]. Discovery of an anti-α-enolase antibody in HE further supports this notion, since this antibody was found in various autoimmune vasculitic diseases such as systemic lupus erythematosus with renal disease, anti-neurotrophil cytoplasmic antibody-associated vasculitis and rheumatoid arthritis [16]. Another theory holds that the anti-α-enolase antibody may induce global cerebral hypoperfusion, resulting in encephalopathy through a disruption of cerebral microvasculatures, because α-enolase is highly expressed in the endothelium [17]. These hypothetical roles might need examination in animal models in which anti-α-enolase antibodies have been actively induced or passively administrated.

Identification of relevant autoantigens by immuno-screening of a brain-derived cDNA library usually is exhaustive work. The present study confirmed that two-dimensional immunoblotting of human brain proteins followed by mass spectrometric identification of target proteins is a powerful tool for searching for unknown autoantigens in ill-defined autoimmune CNS diseases. As in whole brain extract more than 2500 proteins are identifiable on 2-DE, this proteomic strategy may be applicable to a wide variety of other autoimmune CNS diseases.

In summary, we have identified α-enolase as a novel autoantigen for HE using a brain proteomic screening system. Detection of the anti-α-enolase antibody in conjunction with the anti-thyroid antibodies may help proper diagnose HE as well as predict corticosteroids response.

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