Original Article

Identification and evaluation of high-titer anti-Sox Group B antibody in limbic encephalitis

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We evaluated a high titer autoantibody detected in a limbic encephalitis patient with possible lung carcinoma. The patient's autoantibody reacted with BrdU-incorporated dividing cells in the subventricular zone of lateral ventricle and dentate gyrus of the hippocampus, the neurogenic regions of the adult brain, and most of the immunoreactive cells are thought be proneural cells. Characterization of the autoantigen by Western blotting and immunoabsorption has shown that the antibody in the patient's serum binds to Sox Group B proteins but not to authentic paraneoplastic antigens. Our results indicate that the neural stem/progenitor cell population would be a target of the autoantibody in the adult brain.

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Introduction

A feature of autoimmune diseases is an immune response manifested by the presence of autoantibodies targeted against cellular components, such as proteins and nucleic acids. Human autoantibodies have been utilized to screen cDNA libraries to identify many target autoantigens that have aided the clinician to differentially diagnose many autoimmune diseases. One of the best studied examples is paraneoplastic neurologic disorders (PND)[1]. PND are believed to be autoimmune neuronal degeneration caused by the remote effects of cancer, and are most commonly associated with small cell lung cancer (SCLC). Laboratory studies have demonstrated that an autoimmune response links the neurologic disorder and the cancer, and established a model whereby the cancer is believed to initiate the syndrome by expressing a protein antigen normally expressed in the nervous system, leading to an anti-tumor immune response followed by autoimmune neurologic symptoms[2]. Most symptomatic PND are rare, affecting perhaps 0.01% of patients with cancer[3] and are

accompanied by autoantibodies against neural antigens. These antibodies, detected in the serum and cerebrospinal fluid (CSF), sometimes define specific clinical syndromes and/or specific underlying cancers[4], and have been used as reagents to clone and characterize a number of target antigens, including Nova, HuB, HuC, HuD and Cdr2[1].

Hu proteins, a neuronal specific RNA binding protein, are autoimmune targets in paraneoplastic encephalomyelitis and sensory neuropathy (PEM/SN) patients with SCLC who harbor a high titer anti-Hu antibody[5]. All SCLCs appear to express Hu antigens, but the syndrome is rare, probably complicating considerably less than 0.1 % of SCLC cases[6]. In SCLC patients, *Sox* Group B (*Sox B*) family gene (*Sox1*, *Sox2*, *Sox3* and *Sox21*) had been identified by serological analysis of expression cDNA libraries (SEREX) using pooled sera of SCLC patients. Although PND have been associated with several SCLC antigens, neurologic symptoms have not been observed in patients with anti-Sox B antibodies[7,8].

In this study, we evaluated a high titer autoantibody detected in a limbic encephalitis patient with possible lung carcinoma. Characterization of the autoantigen by Western blotting, immunohistochemistry and immunoabsorption has shown that the antibody in the patient's serum (PS) and CSF binds to Sox B proteins.

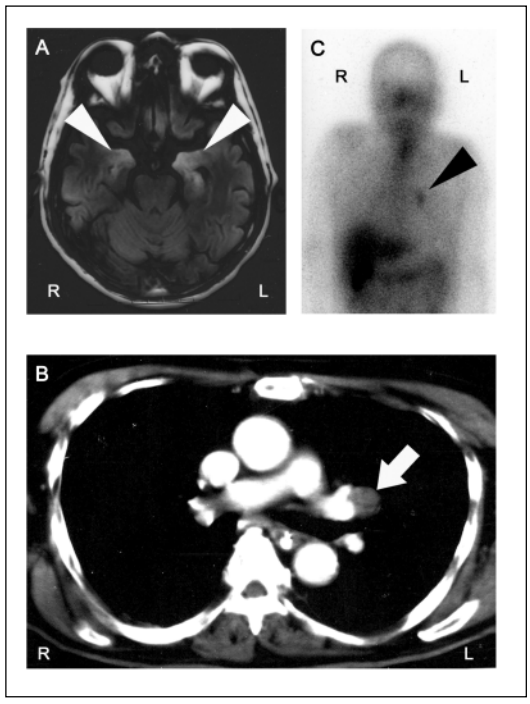

Fig.1
(A) Axial FLAIR MRI image. Arrowheads indicated high signal intensity in the bilateral medial temporal lobes.
(B) Chest computed tomography scan. Arrow indicated a left hilar tumor of the patient's lung.
(C) Gallium scintigraphy. Arrowhead indicated high signals on the lung lesion.

Patient and Methods

1)History of illness

A 66-year-old retired man who had a loss of consciousness several days before was reporting headache and somnolence, so he admitted to a local hospital. Since he had an attack of generalized seizure in the hospital, head magnetic resonance imaging (MRI) and lumbar puncture were performed. The T2-weighted and fluid-attenuated inversion recovery (FLAIR) MRI demonstrated high signal intensity in the bilateral medial temporal lobes (Fig.1A), and CSF examination showed pleocytosis (mononuclear cell, 222/ μl; polynuclear cell, 5/ μl). As he was diagnosed as limbic encephalitis with unknown origin, his treatments with acyclovir, methylprednisolone, and anticonvulsants were started. However, quantitative PCR method could not detect herpes simplex virus DNA.

After he was referred to Keio University Hospital, no focal symptom was detected without his confusion. Chest computed tomography showed a left hilar tumor of his lung (Fig.1B), and gallium scintigraphy indicated high signals on the same lesion (Fig.1C). Additionally, blood examination revealed high tumor markers (neuron-specific enolase 14.8 ng/ml, pro-gastrin releasing peptide 46.1 pg/ml, soluble interleukin 2 receptor 1740 U/ml) and normal tumor markers (carcinoembryonic antigen 5.2 ng/ml, carbohydrate antigen 19-9 19 U/ml, squamous cell carcinoma related antigen 0.6 ng/ml, sialyl Lewis X-i antigen 32.1 U/ml, cytokeratin fragment 1.3 ng/ml). Paraneoplastic limbic encephalitis with lung tumor was strongly suspected, but lung biopsy was not performed because of technical difficulty. Pleocytosis of CSF was improved to normal range, but his disorientation remained.

His whole blood was spun down at 15,000 g for 15 minutes, and the supernatant was used as serum in the following studies. All experimental procedures were performed with the approval of the ethical committee of the Keio University School of Medicine.

2)Western blot analysis

P0 and adult C57B/6 mouse (CLEA Japan) tissue extracts were prepared with lysis buffer (1% TritonX-100, 10 mM Tris-HCl [pH 7.6], 50 mM NaCl, 30 mM sodium pyrophosphate, 50 mM

sodium fluoride, 20 mM β-glycero phosphate, 1 mM EDTA, 1 mM EGTA and Complete protease inhibitor (Roche)). 293T cells were transfected with HuB, HuC, HuD, Nova, Sox2, Sox5, Sox6, Sox21, deletion mutants of Sox21 (Sox21 high-mobility group (HMG) (1-92) and Sox21 C-terminal (CT) (93-277))/ Myc pcDNA3 vector, Sox1/ p3 x FLAG CMV vector (SIGMA), Sox3/ pFLAG CMV vector (SIGMA) or Cdr2/ T7tag pcDNA3 vector and harvested with lysis buffer at 48 hours after transfection. These extracts were centrifuged at 15,000 g for 15 minutes and used for Western blotting with PS (1:2,000), anti-β-actin (mouse, 1:5,000; SIGMA), anti-FLAG (mouse, 1:1000; SIGMA), anti-Myc (mouse, 1:1,000; SIGMA) or anti-T7 (mouse, 1:3,000; Novagen) antibodies. For titer check the PS was diluted at 1: 5,000, 1:10,000 or 1:50,000 before using for Western blotting.

3)Absorption assay

293T cells were transfected with Sox21 or HuC/ pFLAG CMV vector (SIGMA). 48 hours after transfection, all the cells were harvested with lysis buffer. Immunoprecipitates prepared with ANTI-FLAG M2 affinity gel (SIGMA) were used for the absorption of the PS, diluted at 1:500, for overnight at 4℃. After centrifugation, the absorbed PSs were used for Western blotting (1:4) and immunohistochemistry (1:4).

4)BrdU labeling and immunohistochemistry

For short-term labeling, mice were injected intraperitoneally with BrdU (200 mg/kg dissolved in PBS) and sacrificed 2 hours after injection, and then the brains were processed for immunohistochemistry.

Embryonic and adult mice were fixed with 4% paraformaldehyde at 4℃ for overnight, and then placed in a 30% sucrose/ PBS solution at 4℃ for overnight. Frozen sections (14 μm) were cut along the coronal plane. Subsequently, the sections were then boiled in buffered citric acid (pH 6.0) in a microwave oven for 10 minutes. For BrdU staining, the sections were additionally treated with 1.0 N HCl for 30 minutes. The following primary antibodies were used: PS (1:500), anti-NeuN (mouse, 1:100; CHEMICON), anti-proliferating cell nuclear antigen (PCNA) (mouse, 1:200; Oncogene), anti-BrdU (rat, 1:1000; Abcam) and anti-Sox Group B1 [Sox1/(2)/3][9] (rabbit, 1:1000; provided by Dr. H. Kondoh (Osaka University)) antibodies. Immunolabeled samples were examined using confocal laser scanning microscopy (LSM 510, Carl Zeiss) or light microscopy (Axiophoto 2, Carl Zeiss).

Results

1)High-titer autoantibody in limbic encephalitis patient

To evaluate autoantibodies in the PS, we performed Western blotting using extracts from various mouse organs. With the PS diluted at 1:2000, two specific bands of approximately 37 kDa were detected in the mouse brain extract and its expression was restricted in the nervous system, including the olfactory bulb, hippocampus, cortex, cerebellum and brain stem. The immunoreactivities were stronger in early developmental stages and were very weak in adult brain (Fig.2A). No immunoreactivity was detected with normal human serum (data not shown).

2)Autoantibody targeting neural stem/progenitor cells in adult brain

Although we failed to detect target proteins for the PS in adult mouse brain extract by Western blotting, some immunoreactive cells were found in the limited regions of the adult mouse brain by immmunohistochemical analysis. A double-immunofluorescent analysis with NeuN, a marker for mature neurons, showed that a small number of neurons in the striatum and cortex were stained with the PS, however, most immunoreactive cells were NeuN-negative and were found particularly in the subventricular zone (SVZ) of lateral ventricle and dentate gyrus (DG) of the hippocampus (Fig.2B). The PS immunoreactive cells in the SVZ appeared to locate along the surface with one or two cell layers apart from the wall of the lateral ventricle, corresponding to the area in which the neural stem/progenitor cells reside. In the hippocampus, a few cells with strong PS immunoreactivity were observed in the subgranular cell layer of the DG, which were negative for NeuN (Fig.2C). We next determined whether the autoantibody binds to proliferating cells in these regions. Adult mice were injected with BrdU to label dividing cells, and the brain sections were double-stained with the PS and anti- BrdU or anti-PCNA antibody (Fig.3). All of the BrdU or PCNA immunoreactive cells, both in the SVZ and DG, were labeled with the PS, strongly supporting the conclusion that the neural stem/ progenitor cell population is the target of the autoantibody in the adult brain.

3)Autoantibody to Sox B proteins

To elucidate whether the PS contained autoantibodies to authentic PND antigens (HuB, HuC, HuD, Cdr2 and Nova) or Sox proteins (Sox1, Sox2, Sox3, Sox5, Sox6 and Sox 21), fusion proteins of these antigens were generated and probed with the PS by Western blotting. The reason we chose these proteins was that PND antigens and Sox B proteins have been characterized as autoantigens in small cell lung cancer. As a result, the PS did not bind to any PND antigens, Sox5 or Sox6 but strongly reacted with Sox1, Sox2, Sox3 and Sox21, demonstrating the specificity of the autoantibody to Sox B proteins. The same result was obtained using the patient's CSF, diluted at 1:10 (data not

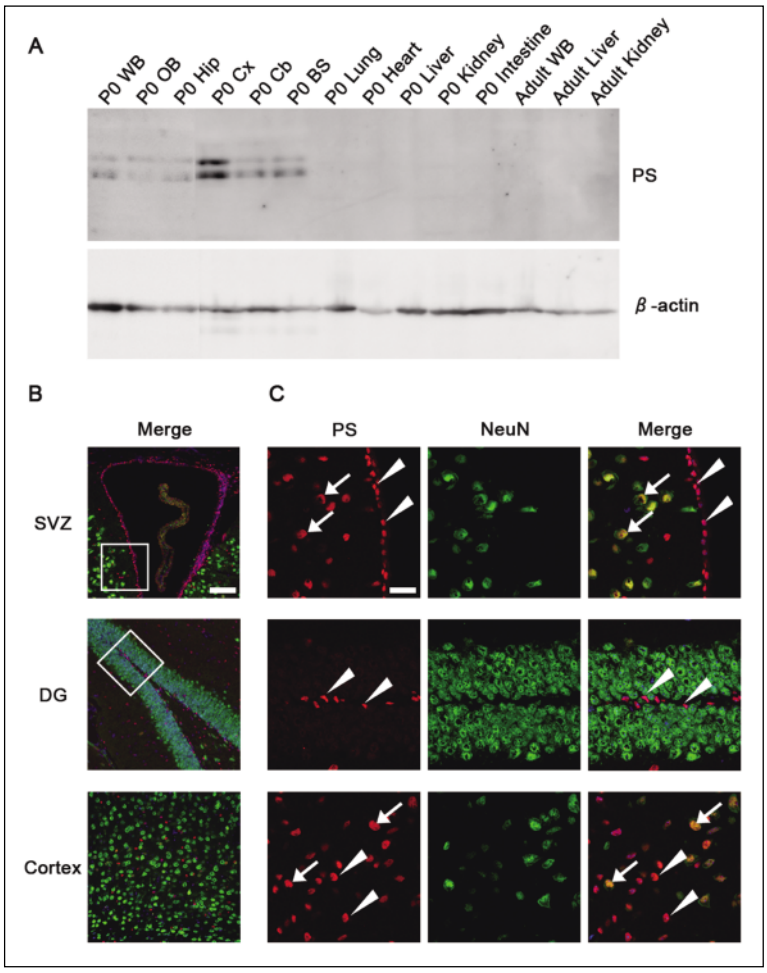

Fig.2 Detection of autoantibody in the patient's serum (PS)

(A) Western blotting using PS in P0 and adult mouse tissue extracts. WB: whole brain, OB: olfactory bulb, Hip: hippocampus, Cx: cortex, Cb: cerebellum, BS: brain stem.

(B) Triple immunohistochemistry with PS (red), anti-NeuN antibody (green) and Hoechst 33258 (blue) in adult mouse brain. SVZ: subventricular zone, DG: dentate gyrus.

(C) High-magnification views of inset in B. Most of the PS immunoreactive cells were NeuN negative (arrowheads), but a very few cells were double-positive with NeuN (arrows).

(Scale bars: 100 μm in B and 25 μm in C)

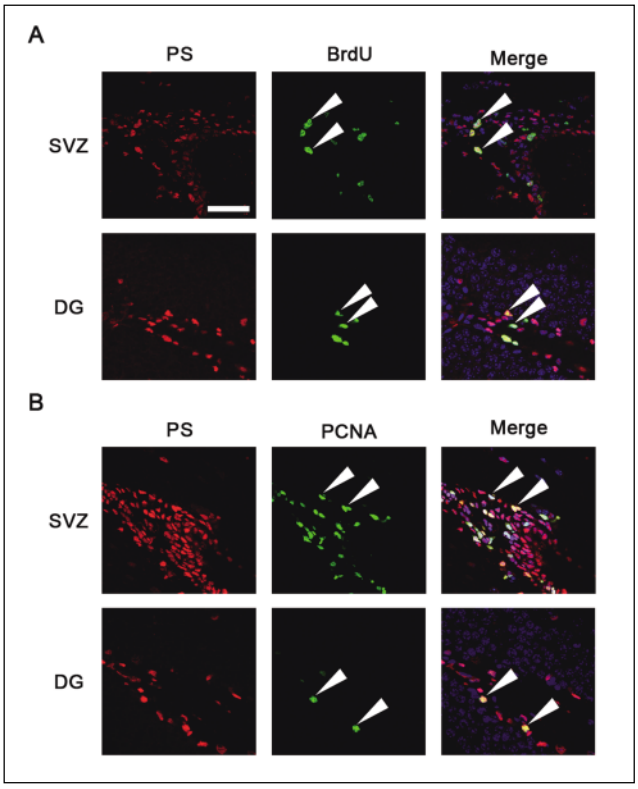

Fig.3 Characterization of PS immunoreactive cells in adult mouse brain

(A) Double immunohistochemistry with PS (red) and anti-BrdU antibody (green) in the SVZ (upper slides) and DG (lower slides).

(B) Double immunohistochemistry with PS (red) and anti-PCNA antibody (green) in the SVZ (upper slides) and DG (lower slides). The arrowheads indicate double-positive cells.

(Scale bar: 50 μm in A and B)

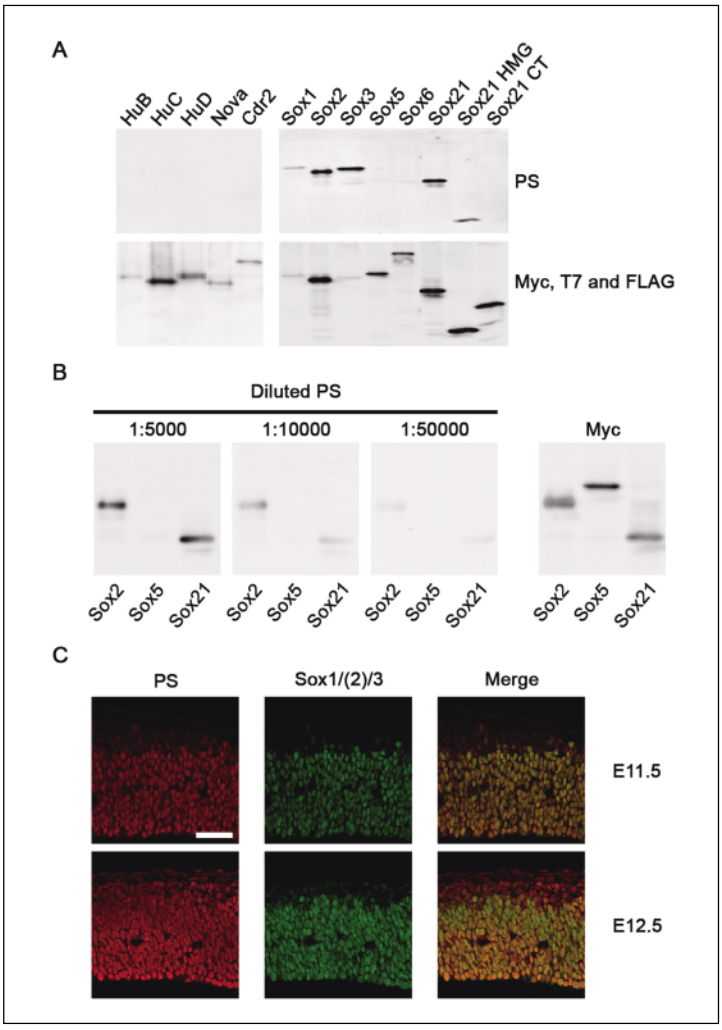

Fig.4 Evaluation of target proteins for autoantibodies in the PS

(A) Western blotting using fusion proteins of PND antigens and Sox proteins. The PS was used at 1:2000 dilution. The filters were reprobed with anti-Myc, T7 or FLAG tag antibodies.

(B) Titer check of the PS with serial dilutions.

(C) Double immunohistochemistry for PS (red) and anti-Sox1/(2)/3 antibody (green) in the E11.5 (upper slides) and E12.5 (lower slides) mouse ventricular zone.

(Scale bar: 50 μm in C)

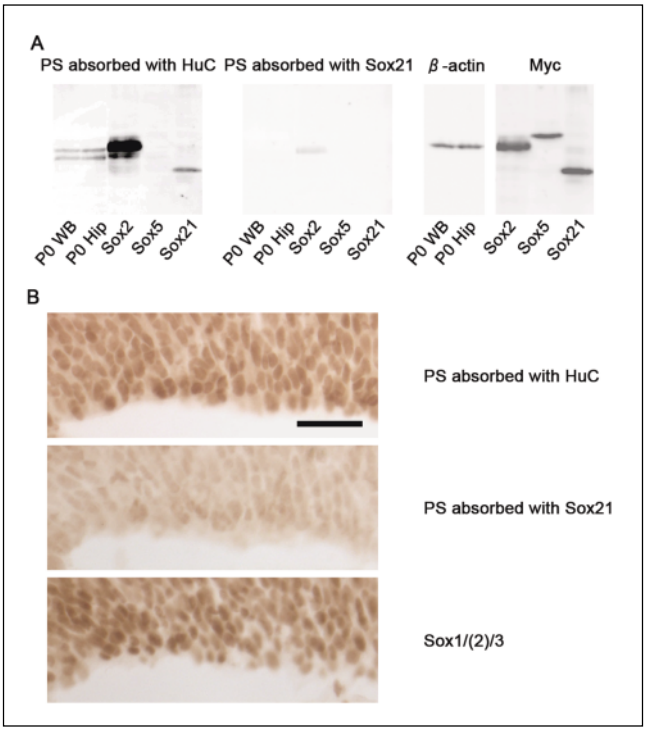

Fig.5 Absorption assay. The PS was preabsorbed with either HuC or Sox21 protein before using for Western blotting or immunostaining

(A) Extracts of P0 mouse whole brain, hippocampus, Sox2, Sox5 and Sox21 protein were probed with preabsorbed PS at 1:2000 dilution. The filters were reprobed for β-actin or Myc tag.

(B) Immunostaining for the pre-absorbed PSs and anti-Sox1/(2)/3 antibody in the ventricular zone of E14 mouse brain.

(Scale bar: 30 μm in B)

shown). Further analysis using truncated Sox21 fusion proteins demonstrated that the PS recognized HMG domain but not C-terminal domain, suggesting that the autoantibody in the PS binds to shared epitopes within the highly conserved HMG box of Sox B proteins (Fig.4A).

We next prepared serial dilutions of the serum to measure a titer of anti-Sox B antibody in the PS. Western blotting using the fusion proteins of Sox2 and Sox21 showed that the titer of the antibody remained as high as $\geqq$ 1:0.5 x 10^5 (Fig.4B). Immunohistochemical analysis of mouse embryos of embryonic day 11.5 and 12.5 using the PS demonstrated an intense reactivity in the ventricular zone (VZ) of the central nervous system (Fig.4C). The immunoreactive cells showed predominantly nuclear staining, which is consistent with previous reports demonstrating the expression pattern of Sox B proteins[9,10]. To examine the specificity of the patient's autoantibody to Sox B proteins, we performed double-immunofluorescent staining using anti-Sox1/(2)/3 antibody[9]. The staining pattern of Sox1/(2)/3 and the PS in the VZ of the embryonic brain were almost identical, supporting the conclusion that a target of the autoantibody is Sox B proteins.

Furthermore, to confirm the specificity of the autoantibody to Sox B proteins, we performed an absorption assay. The PS was pre-absorbed with Sox21 or HuC protein produced in 293T cells before used for Western blotting. The reactivity of the serum pre-absorbed with HuC protein remained as strong as the PS, however the one pre-absorbed with Sox21 protein almost completely lost its immunoreactivity to mouse brain extracts and the fusion proteins of Sox2 and Sox21 (Fig.5A). Staining with HuC-absorbed PS showed a similar immunoreactivity with anti-Sox1/(2)/3 antibody in a limited number of cells in the VZ of the lateral ventricle, whereas reactivity was almost absent with Sox21-absorbed PS in the same region (Fig.5B). These results indicated that the PS contains a high-titer autoantibody against Sox B proteins and that Sox B proteins are the only targets of the autoantibody produced in this patient.

Discussion

In this study, we identified a high-titer autoantibody that specifically recognizes Sox B proteins in the serum of a limbic encephalitis patient with possible lung carcinoma. PND has not yet been diagnosed in this case, but a brain MRI and a test for oncological markers suggested that the patient might be paranaeoplastic limbic encephalitis associated with a lung hilus cancer. Western blot analysis using fusion proteins for various PND antigens and Sox proteins resulted in the detection of an autoantibody to Sox B proteins that targeted HMG domain as its antigenic epitopes. Remarkably, the anti-Sox B antibody was the only autoantibody found in the PS and no other autoantibodies especially against well known PND antigens, Hu, Nova or Cdr2, were detected. Other paraneoplastic limbic encephalitis antigens, Ma2 and voltage-gated potassium channel[2,4], would not be targets of the autoantibody in this patient because the PS immune response to the brain extract disappeared completely by the absorption assay with Sox21 protein. Furthermore, the autoantibody was shown to be extremely specific to Sox B proteins and did not react with other Sox proteins of different group, including Sox5 and Sox6. In a previous report, Sox B proteins, except for Sox14, were identified as targets of autoantibodies produced in approximately 40% of SCLC patients without neurologic symptoms[7,8]. It is noteworthy to emphasize that this patient may be the first reported case of limbic encephalitis with a high titer autoantibody to Sox B proteins.

Sox genes encode evolutionarily conserved transcription factor and have been found to play key roles in cell fate decisions during developmental processes. The Sox family comprises more than 30 members that share the 79-amino acid HMG domain. The *Sox* gene family is classified into ten groups (A-J) based upon their HMG box sequences[11]. The five *Sox* Group B genes (*Sox1*, *Sox2*, *Sox3*, *Sox14* and *Sox21*) share maximum homology with the HMG domain and have been identified in mammals and other species including birds, fish and *Xenopus*. Sox B proteins can be further divided into group B1 and B2 and have been shown to be involved in developmental processes in several organs, including the central nervous system[12]. All Sox B1 proteins, Sox1, Sox2 or Sox3, continue to be expressed in self-renewing neuroepithelial progenitors throughout CNS development and share functions to maintain an immature state and inhibit neuronal differentiation[13,14]. Sox B2 proteins are closely related to Sox B1 proteins that possess a C-terminal repression domain[12]. Recent reports have shown that Sox2 plays a critical role in neurogenesis and the maintenance of mature neurons in the adult mammalian CNS[15,16]. *Sox2* deficiency causes reduced proliferation and impaired neurogenesis in the SVZ and DG, the neurogenic regions of adult mouse brain. Interestingly, in *Sox2* deficiency mice, 40% of the mutants showed epileptic spikes in the cortex and hippocampus and the mutant animals exhibited abnormal behavior typically seen in striatal neurodegeneration[15]. Since the persistent expression of Sox2 and Sox3 is observed in some mature neurons, undefined roles for Sox B proteins in neuronal function is suggested[15,17].

In the present study, we showed that PS immunoreactive cells were observed in specific regional patterns in the adult mouse

brain, particularly in the SVZ and DG. The patient's autoantibody reacted with numerous undifferentiated cells and few postmitotic differentiated cells in the striatum and cortex. Most of the PS immunoreactive cells were thought be proneural cells, which are also immunoreactive for anti-Sox 1/(2)/3 specific antibody.

Neurogenesis generally occurs during the developmental period, but new neurons can also be generated in the DG and SVZ into the postnatal period and adulthood. Neurons in the DG are generated throughout life from dividing progenitor cells residing in the subgranular region in the rodent hippocampus. The newly generated neuronal cells migrate into the granule cell layer where they extend axons and differentiate into mature neurons. Interestingly, a reduction in neurogenesis in the DG of adult hippocampus impaired hippocampal-dependent forms of associative memory formation[18]. Hippocampal-dependent learning processes may involve conscious awareness or recollection. A previous report demonstrated that new neurons are generated in the DG of adult humans as well, and that the human hippocampus retains its ability to generate neurons throughout life[19]. Although it is not clear whether the Sox B expressing progenitor cells in the DG were attacked by the autoantibody in the PS and CSF, limbic encephalitis, including hippocampus dysfunction, was evident in this patient. Indeed an autoimmune mechanism is one of the possible underlying pathophysiology of this case.

PND patients have autoantibodies in their serum and CSF that react with both the nervous system and the underlying cancer. Although several PND antibodies, including Ri, Hu and Yo, have been reported, many unidentified antibodies still exist in the serum of neurodegenerative patients with various types of carcinoma. The identification of these antibodies and their target neural antigens has substantially advanced our ability to make an early diagnosis and has led to the concept that PND are immune-mediated[4].

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