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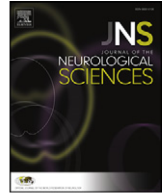

Paraneoplastic cerebellar degeneration associated with an onconeural antibody against creatine kinase, brain-type

Syuichi Tetsuka [a,b], Kaoru Tominaga [b], Eriko Ohta [b], Kenji Kuroiwa [b], Eiji Sakashita [b], Katsumi Kasashima [b], Toshiro Hamamoto [b], Michito Namekawa [a], Mitsuya Morita [a], Shinsuke Natsui [c], Tatsuo Morita [c], Keiko Tanaka [d], Yoshihisa Takiyama [e], Imaharu Nakano [a], Hitoshi Endo [b,*]

[a] Division of Neurology, Department of Internal Medicine, Jichi Medical University, 3311–1 Yakushiji, Shimotsuke, Tochigi 329–0498 Japan
[b] Department of Biochemistry, Jichi Medical University, 3311–1 Yakushiji, Shimotsuke, Tochigi 329–0498 Japan
[c] Department of Urology, Jichi Medical University, 3311–1 Yakushiji, Shimotsuke, Tochigi 329–0498 Japan
[d] Department of Neurology, Kanazawa Medical University, 1–1 Daigaku, Kahoku, Ishikawa 920–0293, Japan
[e] Department of Neurology, University of Yamanashi, 1110 Shimokato, Chuo-shi, Yamanashi 409–3898, Japan

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ABSTRACT

Onconeural immunity, a cancer-stimulated immune reaction that cross-reacts with neural tissues, is considered to be the principal pathological mechanism for paraneoplastic neurological syndromes (PNS). A common PNS is paraneoplastic cerebellar degeneration (PCD). We had encountered a PCD patient with urothelial carcinomas (UC) of the urinary bladder who was negative for the well-characterized PNS-related onconeural antibodies. In the present study, we aimed to identify a new PCD-related onconeural antibody, capable of recognizing both cerebellar neurons and cancer tissues from the patient, and applied a proteomic approach using mass spectrometry. We identified anti-creatine kinase, brain-type (CKB) antibody as a new autoantibody in the serum and cerebrospinal fluid from the patient. Immunohistochemistry indicated that anti-CKB antibody reacted with both cerebellar neurons and UC of the urinary bladder tissues. However, anti-CKB antibody was not detected in sera from over 30 donors, including bladder cancer patients without PCD, indicating that anti-CKB antibody is required for onset of PCD. We also detected anti-CKB antibody in sera from three other PCD patients. Our study demonstrated that anti-CKB antibody may be added to the list of PCD-related autoantibodies and may be useful for diagnosis of PCD.
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1. Introduction

Paraneoplastic neurological syndromes (PNS) are unique examples of naturally occurring antitumoral immune responses and are a diverse group of human neurodegenerative diseases associated with cancer and antitumor immunity. Ectopic expression of neural antigens in cancer tissues from PNS patients triggers a potent immune response that eventually attacks the neurons expressing the antigen shared with the cancers (onconeural antigen) [1]. Although it is estimated that PNS affects 1%–25% of cancer patients [2], this prevalence will likely increase as cancer patients live longer and diagnostic methods for PNS are improved. One of the most common types of PNS is paraneoplastic cerebellar degeneration (PCD) [3], which is characterized initially by subacute cerebellar ataxia and ultimately by severe pancerebellar syndrome due to the death of Purkinje cells in the cerebellum. Conditions for the development of PCD are divergent, and PCD can be associated with many different types of cancers, including small cell lung cancer (SCLC), breast or ovarian carcinoma, and lymphomas [2,3]. Antibodies such as anti-Yo [4], Hu [5], Tr [6], and Ri [7] antibodies have been identified as autoantibodies causing PCD. Shams'ili et al. reported that only approximately 36% of PCD patients are positive for the known onconeural antibodies associated with PCD [8]. After this report was published, an anti-amphiphysin antibody that targets neuropil in some cases of SCLC was identified as an onconeural antibody associated with PCD [9]. However, in many cases, the PCD-related onconeural antibodies still remain unresolved. Thus, we believe that new onconeural antibodies associated with PCD should be identified to elucidate the molecular mechanism of pathogenesis for PCD and to establish a new strategy for treatment to PCD patients.

We recently encountered a PCD patient with urothelial carcinomas (UC) of the urinary bladder. We examined serum from the patient for well-characterized onconeural antibodies (anti-Yo, Hu, Ri, Tr antibodies, etc.), but found none. Therefore, we considered that an unidentified onconeural antibody exists in the serum from this patient. Hence, in the present study, we aimed to identify a new onconeural antigen recognized by a novel onconeural antibody in this PCD. To this end, we used a proteomic approach, employing mass spectrometry (MS) after two-dimensional electrophoresis (2-DE) and western blotting (WB). These procedures have been shown by others to be useful in identifying

* Corresponding author at: Department of Biochemistry, Jichi Medical University, 3311–1 Yakushiji, Shimotsuke, Tochigi, 329–0498, Japan. Tel.: +81 285 58 7322; fax: +81 28 44 1827.
E-mail address: hendo@jichi.ac.jp (H. Endo).

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anti-neuropil antibodies [10,11], although identifying a new onconeural antibody related to PCD using this approach has not been reported yet. We identified an autoantigen that has not previously been reported as a PCD-related antigen and also confirmed that this autoantigen was expressed in both the cancer tissue of the patient (immune sites) and in mouse cerebellum (potential target sites). We further evaluated the specificity of the newly identified onconeural antibody using the sera from other PCD and non-PCD patients.

2. Materials and methods

2.1. Patients and controls

A 66-year-old male patient diagnosed with UC of the urinary bladder a month before, was admitted to our Neurology Department with progressive gait imbalance. Neurological examination disclosed dysarthria, bilateral horizontal nystagmus, dysmetria in both legs, an ataxic gait and the inability to walk with a tandem gait. Although well-characterized onconeural antibodies (anti-Yo, Hu, Ri, Tr, CV2, Ma1, Ma2, and amphiphysin) were negative, we diagnosed the patient as having PCD according to the criteria defined by the Paraneoplastic Neurological Syndrome Euronetwork [12]. We obtained the serum, cerebrospinal fluid (CSF), and a frozen biopsy specimen of UC of the urinary bladder, which had been excised in bladder cancer surgery, from the patient. Sera from ten healthy blood donors served as controls, and sera from 20 other patients with cerebellar ataxia (two cerebellitis, 14 spinocerebellar degeneration [SCD], two cerebral infarction, one opsoclonus myoclonus, and one progressive supranuclear palsy [PSP]) were used as negative controls. Anti-Yo positive sera from three PCD patients were also used as negative controls. We also obtained sera from three other patients diagnosed with PCD (two SCLC and one non-Hodgkin's lymphoma). Well-characterized onconeural antibodies were negative in these sera. During sample collection, all PCD patients were in the active phase of the disease, presenting with symptomatic cerebellar ataxia. The study was conducted with the approval of the ethics committees at the participating institutions.

2.2. Animal tissues and reagents

Normal C57BL/6 J male mice, aged 6–9 weeks, were used. Animal experiments were conducted in a humane manner after receiving approval from the Institutional Animal Experiment Committee of the Jichi Medical University, and in accordance with the Institutional Regulation for Animal Experiments and Fundamental Guideline for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions under the justification of the Ministry of Education, Culture, Sports, Science, and Technology. Mice were sacrificed by dislocation of cervical vertebrae, and the cerebellum and liver were removed immediately. After the tissues were quickly frozen under liquid nitrogen, these samples were stored at −80 °C until used.

Purified human CKB, purified human creatine kinase, muscle-type (CKM), rabbit monoclonal antibody against CKB (anti-CKB antibody), and mouse anti-β-actin monoclonal antibody were obtained from Abcam (Cambridge, UK). To probe the expression of CKB in UC of the urinary bladder, we purchased a bladder tissue array of UC (US Biomax, Inc., Rockville, MD, USA). In addition, paraffin-embedded mouse brain sagittal sections for immunohistochemistry were obtained from Genostaff Co., Ltd. (Tokyo, Japan).

2.3. WB and 2-DE

Total cell lysates were prepared from mouse cerebellum and liver using NP-40 lysis buffer [1% NP-40, 20 mM Tris–HCl (pH 7.5), 1 mM EDTA, 150 mM NaCl, and 10% glycerol] supplemented with Protease Inhibitor Cocktail Set I (539131, Calbiochem, San Diego, CA). The lysates (100 μg proteins) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), after which separated proteins were transferred onto nitrocellulose membranes (GE Healthcare). The sera from the patients and healthy blood donors were diluted 1:1000 in blocking buffer [2% fetal bovine serum (FBS), 0.5% skim milk in phosphate-buffered saline with 0.05% Tween20 (PBST)] and used for overnight incubation with the membranes at 4 °C. The membranes were then incubated with horseradish peroxidase (HRP)-conjugated anti-human Ig secondary antibody (GE Healthcare) at a 1:500 dilution for 1 h at room temperature, and then treated with ECL reagent (GE Healthcare). The chemiluminescent bands of interest were detected using a LAS mini 4000 (GE Healthcare). Beta-actin, detected by mouse anti-β-actin antibody (1:2000, Abcam), was used as an internal control. For final evaluation of the presence of anti-CKB antibody in the sera, we performed WB using commercially available human CKB and CKM proteins instead of lysates.

Two-DE was performed in duplicate; one gel was used for MS analysis, and the other for immunodetection. Two hundred micrograms of mouse cerebellum protein extract was purified and concentrated using Ettan 2-D Clean-up kit (GE Healthcare) according to the instruction manual. Samples were applied onto Immobiline Drystrips (pH 3–11 NL, 18 cm, GE Healthcare). Isoelectric focusing was performed using an Ettan IPGphor System (GE Healthcare) according to the step gradient protocol (rehydration for 12 h, 500 V 1 h, 1000 V 1 h, 8000 V 8 h). The proteins then underwent the second dimension separation by 10% SDS-PAGE, and then stained with Flamingo™ Fluorescent Gel Stain (Bio-Rad Laboratories, Hercules, CA, USA) or transferred onto PVDF membrane filter (Millipore, Bedford, MA, USA).

2.4. MS analysis

Protein spots were excised from a fluorescently stained gel using 2-DE Gel-Picker FluoroPhoreStar 3000 (Anatech, Tokyo, Japan) according to the results of ECL images of WB in which patient antiserum was used for detection. The proteins in the gel pieces were digested by trypsin (12.5 ng/μl, Promega, Madison, WI, USA).

Liquid chromatography-mass spectrometry (LC-MS/MS) using FINNIGAN LTQ (Thermo Fisher Scientific, Rockford, IL, USA) was performed as described previously [13]. Protein identification was performed by MS/MS ion search using MASCOT software program version 2.2 (Matrix Science, London UK) and National Center for Biotechnology Information (NCBI) database. Proteins with a MASCOT score of >50 were considered as statistically significant ($p < 0.05$).

2.5. Antibody absorption test by recombinant CKB protein

To generate expression vector for glutathione S-transferase (GST)-CKB fusion protein, DNA fragment for human CKB was amplified by PCR using Pfu ULTRA DNA polymerase (Agilent, USA) and pCAGGS-hCKB as a template [14] and subcloned into Eco RI and Xho I sites of pGEX-4 T1. GST and GST-CKB fusion protein were expressed in *E. coli* BL-21. GST and GST-CKB fusion protein in the soluble fraction were purified using glutathione sepharose 4B ™ (GE Healthcare). Purified GST or GST-CKB fusion protein (50 μg) was incubated with 60 μl of 50% slurry of glutathione sepharose beads at 4 °C for 1 h and then beads were washed with washing buffer (20 mM Tris–HCl [pH 7.5], 150 mM NaCl, 1 mM EDTA, and 0.5% NP-40) three times. Serum (20 μl) from the PDC patient was added to the beads, incubated at 4 °C for 1 h, and then the serum was recovered. WB for proteins from mouse cerebellum (100 μg each) and purified human CKB (0.5 μg) was performed using the absorbed sera at 1:1000 dilution.

2.6. Immunohistochemistry

A bladder tissue array, in which bladder cancer tissues containing UC are spotted, was deparaffinized and rehydrated using a graded ethanol series (100%–70%). Frozen sections of UC of the urinary bladder from

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