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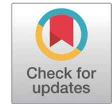

Identification of Medium-Length Antineurofilament Autoantibodies in Patients with Anti-N-Methyl-D-Aspartate Receptor Encephalitis

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Background and Purpose Anti-N-methyl-D-aspartate receptor (NMDAR) encephalitis is a severe central nervous system disorder mediated by NMDAR antibodies that damages neurons. We investigated the correlation between cytoskeletal autoantibodies and the clinical severity in patients with anti-NMDAR encephalitis.

Methods Non-NMDAR autoantibodies were identified by screening matched cerebrospinal fluid (CSF) and the serum samples of 45 consecutive patients with anti-NMDAR encephalitis and 60 healthy individuals against N-methyl-D-aspartate receptor 1-transfected and non-transfected human embryonic kidney 293T cells. Immunocytochemistry was performed to assess antibody binding in rat brain sections and primary cortical neurons. Cell-based assays and Western blotting were applied to identify autoantibodies targeting medium neurofilaments (NFMs). We compared clinical characteristics between patients with NMDAR encephalitis who were positive and negative for anti-NFM-autoantibodies.

Results Anti-NFM autoantibodies were detected in both the serum and CSF in one patient (2%) and in the serum only in six patients (13%). No antibodies were detected in the serum of healthy controls (7/45 vs. 0/60, p=0.0016). Four of the seven patients with anti-NFM autoantibodies in serum were children (57%), and three (43%) had abnormalities in brain magnetic resonance imaging. These patients responded well to immunotherapy, and either no significant or only mild disability was observed at the last follow-up. Anti-NMDAR encephalitis did not differ with the presence of anti-NFM autoantibodies.

Conclusions Anti-NFM autoantibodies may be present in patients with anti-NMDAR encephalitis, indicating underlying neuronal damage. A large cohort study is warranted to investigate the clinical differences between patients with NMDAR encephalitis according to their anti-NFM antibody status.

Key Words anti-N-methyl-D-aspartate receptor encephalitis, autoantibodies, HEK293 cells, magnetic resonance imaging.

INTRODUCTION

N-methyl-D-aspartate receptor (NMDAR) encephalitis is the most common type of autoimmune encephalitis. Mediated by autoantibodies against the N-methyl-D-aspartate receptor 1 (NR1) subunit of NMDAR,[1-3] it is clinically characterized by acute encephalopathy, psychosis, seizures, memory deficits, and motor dysfunction.[1,4,5] Our aim was to broadly determine whether cytoskeleton-specific autoantibodies were present in matched serum and cerebrospinal fluid (CSF) samples obtained from patients with anti-NMDAR encephalitis, since this information would be valuable in clinical practice.

The pathogenesis of NMDAR encephalitis remains unclear. Receptor internalization is gen-

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erally accepted as the cause of reduced NMDAR function,[6-9] and complement-mediated cell lysis is another potential mechanism. Although several studies involving patients with NMDAR encephalitis did not find complement in the brain, this putative mechanism cannot be dismissed because complement immunoreactivity has been observed in these patients.[10] Neurofilaments and neurofilament autoantibodies have recently attracted attention due to their potential value in predicting neuronal/axonal damage and disease outcomes. It is of great interest to determine whether there is an NMDAR encephalitis subgroup with antineurofilament autoantibodies and, if so, to identify the clinical significance of these autoantibodies. We hypothesized that such a subgroup of patients could develop more-severe central nervous system (CNS) inflammation and have worse outcomes due to antineurofilament autoantibodies entering the leaky membrane caused by NMDAR antibodies, resulting in secondary neuronal damage or, alternatively, that antineurofilament autoantibodies reflect the presence of severely injured neurons that release neurofilaments as self-antigens, and activate the neurofilament-reactive B-cells.

Neurofilaments are axonal cytoskeletal proteins localized within neurons that can be divided into three types based on the length of their protein subunits: light neurofilaments (NFL, 68 kDa), medium neurofilaments (NFM, 160 kDa), and heavy neurofilaments (NFH, 200 kDa). NFLs serve as the backbones to which NFM and NFH are more peripherally attached.[11-13] Neurofilaments are essential for the radial growth of axons and nerve conduction. Recent research has focused on the possibility that NFL in blood or CSF is a biomarker of disease progression in multiple sclerosis (MS),[14-16] radiologically isolated syndrome,[17] neuromyelitis optica spectrum disorders,[18] CNS injury,[19] and neurodegenerative diseases.[20] An experimental animal model of autoimmune dementia supports a role for NFH antibodies in neurodegeneration in Alzheimer's disease.[21] Bartos et al.[13] reported that anti-NFL antibodies in serum were a measurable biomarker for potentially predicting the disease activity and treatment outcome in MS. Neurofilament antigens have been detected in many CNS diseases and may be released in a secondary manner after an initial neuron injury. Moreover, they may cause the immune system to produce antibodies that are detectable in peripheral areas. However, it remains unclear whether antineurofilament antibodies for potentially predicting the disease severity are present in patients with anti-NMDAR encephalitis.

METHODS

Patients

Paired serum and CSF samples were obtained from 45 consecutive patients with anti-NMDAR encephalitis who fulfilled the diagnostic criteria proposed by Graus et al.[22] and visited our neurology laboratory between March 2014 and May 2018. Sixty individuals undergoing routine physical examinations were enrolled as healthy controls. The serum and CSF samples were centrifuged at 1,500 g for 10 min and then stored at -80°C until tested. Anti-NMDAR antibodies were detected using a commercially available cell-based assay (NMDA-R/EU 90, Euroimmun, Medizinische Labordiagnostika, Lübeck, Germany). Neurological outcomes were assessed using the 6-point modified Rankin Scale score as follows[23]: 0, no symptoms; 1, symptoms but no significant disability; 2, slight disability; 3, moderate disability; 4, moderate to severe disability; and 5, severe disability. Data on patient sex, age at onset, duration of disease, symptoms, treatment, magnetic resonance imaging (MRI) findings, laboratory tests, and outcomes were obtained from the clinical database. The study protocol was approved by the Ethics Committee of the Third Affiliated Hospital of Sun Yat-sen University [ethics number: (2014)2-15]. All of the included subjects provided informed consent before participating in the study, which complied with the principles of the Declaration of Helsinki (1964).

Immunohistochemistry

Frozen rat brain sections (with a thickness of 20 μm) were permeabilized with 0.1% Triton X-100 in phosphate-buffered saline (PBS) for 20 min before being blocked in 10% goat serum for 30 min. The sections were then exposed to human serum (1:100) with commercial antibodies to chicken anti-NFM (1:500; Aves Labs, Tigard, OR, USA) and mouse anti-microtubule-associated protein 2 (MAP2; 1:500; Merck Millipore, Billerica, MA, USA) as neuronal markers at 4°C overnight. After washing in PBS, the sections were incubated with secondary antibodies for 1 h at room temperature (RT). The sections were then washed before being mounted using Fluoromount-G (SouthernBiotech, Birmingham, AL, USA).

The following secondary antibodies were used: antihuman immunoglobulin G (IgG) (H+L) secondary antibody conjugated to Alexa Fluor® 488 (1:1,000; Invitrogen, Carlsbad, CA, USA), antichicken secondary antibody conjugated to Alexa Fluor® 546 (1:800; Invitrogen), and antimouse secondary antibody conjugated to Alexa Fluor® 546 (1:800). The slices were examined using a laser scanning confocal microscope (TCS SP8, Leica, Wetzlar, Germany).

Primary neuron cultures

Embryos at day 18 were removed (to prepare cortical neuron cultures) from a pregnant Sprague-Dawley rat and transferred to ice-cold Hanks' balanced salt solution. For neuron preparation, the cortex was isolated and incubated with 2.5% trypsin

at 37°C for 15 min, washed three times in blocking buffer [Dulbecco's modified Eagle's medium with 15% fetal bovine serum (Biological Industries, Beit HaEmek, Israel) and 1% penicillin/streptomycin], and placed in neuron medium. The cells were counted and plated in a poly-D-lysine-coated 24 multiwell plate at the appropriate density. The cortical neurons were fed once every 4 days by exchanging equal volumes (one third of the initial volume of culture medium) until day 18 in vitro. Arabinocytidine was added to the feeding medium at 10 μM on day 8 after seeding the neurons.

Cell-based assay

The presence of antibodies against NFM and the related NFH, NFL, and βIII-tubulin proteins was tested in serum from patients 3, 13, 24, 39, 43, 46, and 51 as well as CSF from patient 43 and healthy controls. Briefly, human embryonic kidney 293T (HEK293T) cells were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum (Biological Industries) and 1% penicillin/streptomycin in 48-well plates at 37°C and 5% CO_2. Cells were cotransfected with green fluorescent protein (GFP) and plasmids containing human NFM, NFH, NFL, or βIII-tubulin using Lipofectamine® 2,000 Reagent (Invitrogen). Transfected cells were cultured for 24–36 h until the transfection efficiency had increased to 90%. The cells were then washed with PBS and fixed with 3% paraformaldehyde in PBS for 30 min, permeabilized with 0.25% Triton X-100 in PBS, and blocked using 10% goat serum for 30 min. Next, the cells were incubated with serum (1:20) or CSF (without dilution) from the patients and healthy controls, along with commercial chicken anti-NFM (1:800), rabbit anti-NFH (1:800; Abcam, Cambridge, MA, USA), chicken anti-NFL (1:800; Abcam), or rabbit anti-βIII-tubulin (1:800; Abcam) antibodies for 1 h. The cells were then incubated with Alexa Fluor® 546 goat antihuman IgG (H+L) (1:1,000), Alexa Fluor® 546 goat antichicken IgG (H+L) (1:1,000), and Alexa Fluor® 546 goat antirabbit IgG (H+L) secondary antibodies for 1 h. Images were captured under a fluorescence microscope using Axiovision software (Zeiss, Oberkochen, Germany). Further gradient dilution of samples with visible positive signals was performed to determine the anti-NFM antibody titers (i.e., the maximum dilution at which positive results could be observed).

Western blotting

HEK293T cells expressing human NFM subunits were grown for 24–36 h, washed with PBS (pH 7.4), homogenized in ice-cold radio immunoprecipitation assay buffer [25 mM Tris (pH 7.4), with 150 mM NaCl, 1% Nonidet P 40, and 0.5% deoxycholate] containing a protease inhibitor, and centrifuged at 12,000 g for 10 min. The total protein concentrations in the collected supernatants were determined using the QuickStart™ Bradford Protein Assay kit (Bio-Rad Laboratories, Hercules, CA, USA). The extracted protein samples were heat-denatured and then electrophoresed in an 8% sodium dodecyl sulfate–polyacrylamide gel, and the separated proteins were transferred to nitrocellulose membranes (Bio-Rad Laboratories) using Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad Laboratories). The nitrocellulose membranes were blocked with 3% skim milk in Tris-buffered saline containing 0.05% Tween 20 (TBST) at RT for 1 h, and then incubated with patients' serum (1:100 in blocking buffer) overnight at 4°C. After three washes in TBST, the membranes were incubated with a secondary antibody (1:1,000 goat antihuman IgG conjugated to horseradish peroxidase, Bio-Rad Laboratories; or 1:1,000 goat antichicken IgG conjugated to horseradish peroxidase, Santa Cruz Biotechnology, Santa Cruz, CA, USA) at RT for 2 h. After three further washes in TBST, the protein bands were detected using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, Waltham, CA, USA) and analyzed with a high-resolution imaging gel for fluorescence and a chemiluminescence system (G-BOX Chemi XX6/XX9, Syngene, Bangalore, India).

Statistical analysis

The Mann-Whitney U test and Fisher's exact test were used for group comparisons. The statistical analyses were conducted using GraphPad Prism (version 7.0, GraphPad Software, La Jolla, CA, USA) and SPSS (version 22.0, IBM Corp., Armonk, NY, USA). A p value of <0.05 was considered statistically significant.

RESULTS

Non-NMDAR autoantibodies identified in some patients with anti-NMDAR encephalitis

We incubated serum and CSF IgG with NR1-transfected (Fig. 1A–D) and nontransfected (Fig. 1E–H) HEK293T cells from 45 patients with anti-NMDAR encephalitis or serum from 60 healthy controls. Serum from patients 3, 13, 24, 39, 43, 46, and 51 as well as the CSF from patient 43 showed filamentous-like patterns (Fig. 1D and H) that were distinctive from the normal "drop shape" pattern in the other patients who were positive for NMDAR antibodies (Fig. 1B, C, F, and G). Accordingly, we hypothesized that distinct cytoskeleton-like autoantibodies exist in these patients.

Reactivity of patients' serum with rat hippocampal and cerebellar tissues

It remained uncertain whether or not the aforementioned cytoskeleton-like non-NMDAR autoantibodies could bind

to brain sections. To address this question, serum from three patients who showed specific filamentous-like patterns and for whom anti-NMDAR antibodies were detected in their CSF but not in their serum was prepared and screened for binding to fixed rat brain sections (Fig. 2). Strong binding was detected in multiple regions of the hippocampus and cerebellum.

Non-NMDAR autoantibodies from patients colocalized with axons on rat primary cortical neurons and rat brain sections

Immunofluorescence staining was used to visualize the axon-like distribution of non-NMDAR autoantibodies from the aforementioned three patients on primary cultured neurons (Fig. 3A). Double-labeled images showed overlapping patterns with a commercially available chicken anti-NFM antibody, but no overlap with anti-MAP2 and anti-glial fibrillary acidic protein (GFAP) antibodies (Fig. 3B). The rat brain slices showed the serum IgG-bound tissue components of multiple rat hippocampal and cerebellar regions at ×200 magnification (Fig. 3C); the findings were consistent with the results of immunostaining of primary cortical neurons. Confocal images revealed that staining for non-NMDAR autoantibodies and NFM completely overlapped in the hippocampus and cerebellum, while that for anti-MAP2 antibody did not overlap. These results confirmed that non-NMDAR autoantibodies targeted an axon protein rather than other common epitopes such as astrocytic and dendritic neuronal elements. Since we did not find this filamentous-like pattern in a large number of patients with high titers of anti-NMDAR antibodies, we concluded that it was not caused by cross-reaction of certain NMDAR antibodies, indicating that there was a novel cytoskeletal autoantibody.

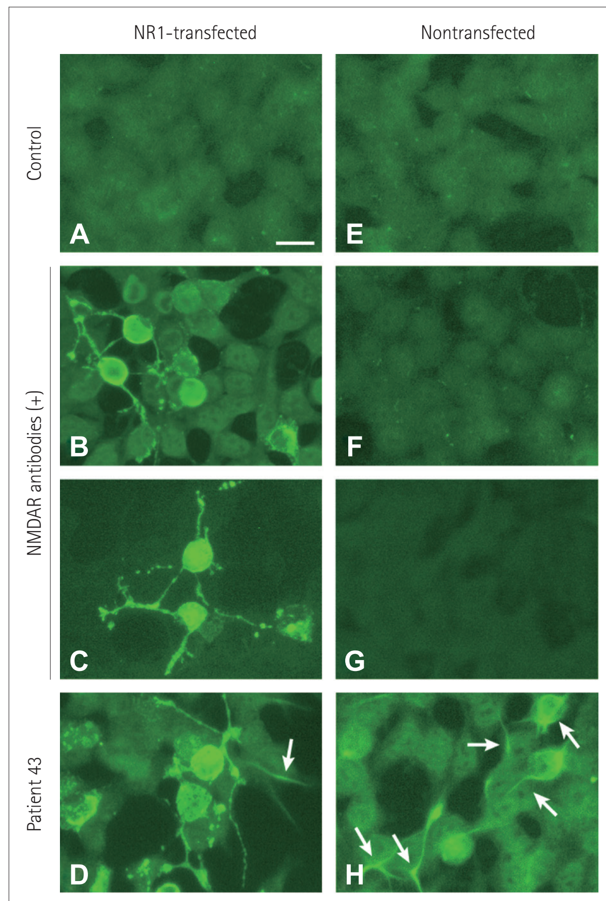

Fig. 1. Non-NMDAR autoantibodies identified in some patients with anti-NMDAR encephalitis. Non-NMDAR NR1-transfected and nontransfected cells after exposure to serum and CSF were observed in patients with positivity for anti-NMDAR antibodies using a commercially available cell-based assay. Healthy controls showed no binding (A and E). Representative images of anti-NMDAR-specific antibodies in NR1-transfected cells from the serum (B) and CSF (C) of a patient with anti-NMDAR encephalitis that did not react with nontransfected cells (F and G). Specific filamentous patterns of non-NMDAR autoantibodies in NR1-transfected (D, arrow) and nontransfected (H, arrows) cells in the serum of a patient with anti-NMDAR encephalitis (patient 43). Scale bar=20 μm. CSF: cerebrospinal fluid, NMDAR: N-methyl-D-aspartate receptor, NR1: N-methyl-D-aspartate receptor 1.

NFM identified as a target autoantigen in patients with anti-NMDAR encephalitis

We considered the possibility that axon-like staining patterns are not derived from NFM, but rather from other subtypes of neurofilaments (NFH and NFL) or βIII-tubulin, a microtubule protein that is a component of the cytoskeleton. We therefore applied further cell-based assays to identify the target antigen

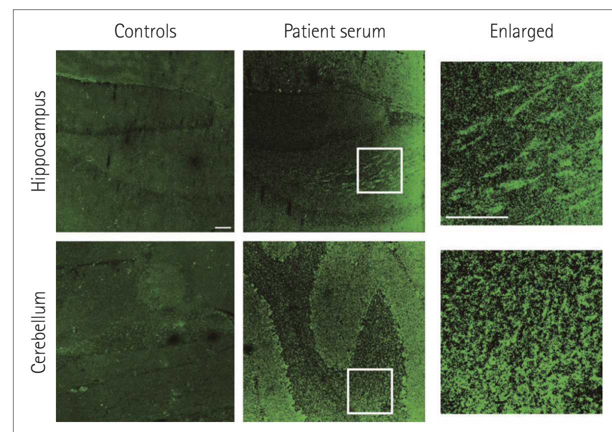

Fig. 2. Reaction between the serum from patients and the rat hippocampal and cerebellar tissues. Immunofluorescent staining of hippocampal and cerebellar sections of the rat brain using serum from three patients with non-NMDAR autoantibodies showed characteristic antibody binding in confocal microscopy, in contrast with the corresponding healthy controls (column 1). There was strong enrichment of non-NMDAR autoantibodies in the brain with large accumulation in the hippocampal and cerebellar regions (column 2). The boxed areas at higher magnification show specific binding of patient immunoglobulin G. Scale bar=100 μm. NMDAR: N-methyl-D-aspartate receptor.

in this axon-like staining. First, we confirmed that the plasmids were expressed by using commercially available antibodies to detect the expressions of NFM, NFH, NFL, and βIII-tubulin (Fig. 4A). Based on the colocalization of GFP and the red fluorescence signals of commercially available antibodies, which were distributed in the cytoplasm but absent from the nucleus, we confirmed that all plasmids were working. Second, the cell-based assay showed strong binding of antibodies from patients to cells transfected with human NFM, but not to cells transfected with NFH, NFL, or βIII-tubulin (Fig. 4B). In contrast, filamentous patterns unrelated to the transfection appeared, similar to the specific filamentous morphologies that we had previously observed in patients with NMDAR encephalitis (Fig. 1). No binding was observed using plasma from pooled healthy controls. Finally, we used Western-blot analysis to confirm the antigenic targets. The most-prominent bands detected by the serum samples from patients were found at approximately 160 kDa in the NFM-transfected cells, in accordance with the commercially available anti-NFM antibodies. However, no protein bands against the nontransfected cells (negative controls) were detected in any of the samples, while the healthy controls showed no binding (Fig. 4C).

Clinical characteristics in patients with anti-NMDAR encephalitis and anti-NFM autoantibodies

The clinical characteristics of the patients with anti-NFM autoantibodies are listed in Table 1. Four (57%) of the seven patients with NFM autoantibodies were children, accounting for 21% of the 19 pediatric patients in the study. Three patients had nonspecific symptoms with antecedent infection (fever and headache). Three patients presented with seizures; two patients exhibited involuntary movements and memory dysfunction; three patients developed obvious psychiatric and behavioral symptoms; one patient exhibited motor dysfunction, a speech disorder, and mild mental retardation; and two patients had decreased consciousness.

Brain MRI revealed that one of the two patients with CNS symptoms that included seizures showed increased bilateral T2-weighted signals involving the frontal cortex and the semioval center. The other patient presented increased T2-weighted signals in the right cerebellum and bilateral frontal temporal lobes, whereas the signals in the remaining four

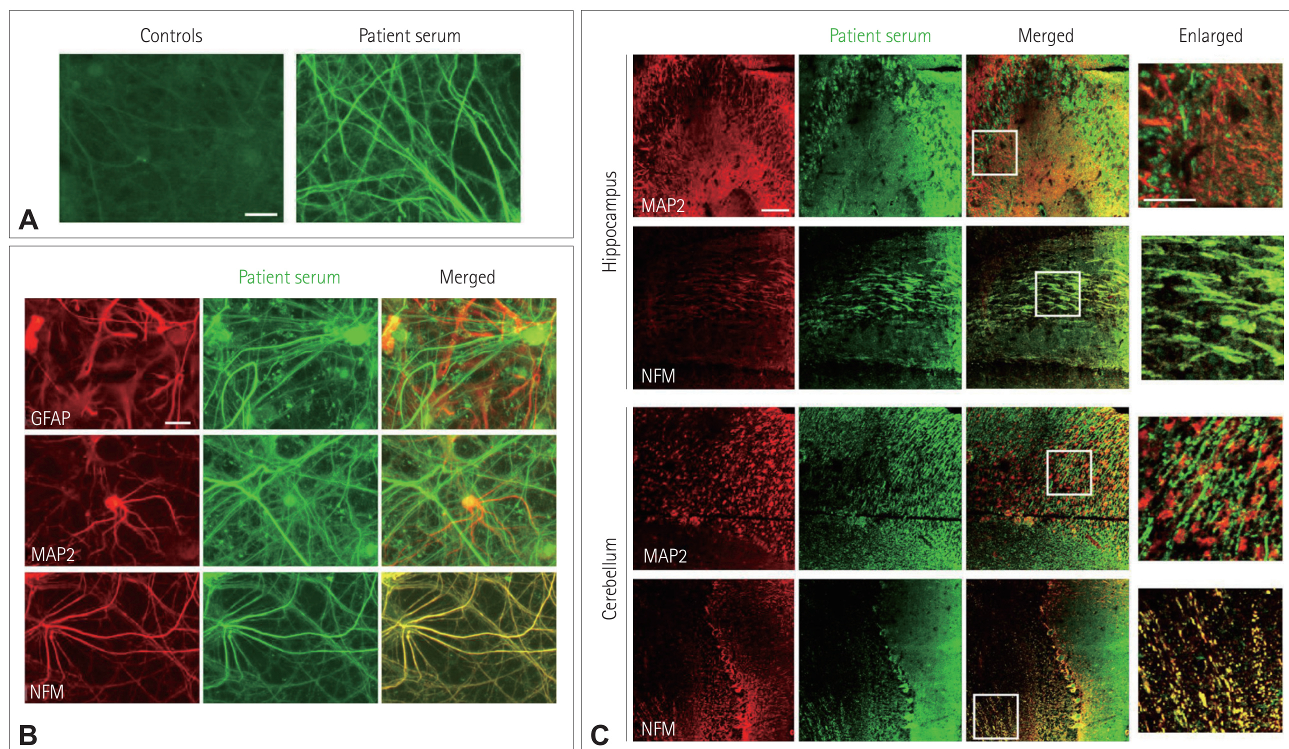

Fig. 3. Non-NMDAR autoantibodies from three patients with anti-NMDAR encephalitis colocalized with axons. Primary culture of rat cortical neurons after day 18 in vitro. Rat brain sections were analyzed using immunofluorescent staining. Serum from patients showed strong binding to the primary culture neurons, whereas that from healthy controls did not (A). Double labeling with anti-NFM antibody revealed the typical distribution of axons, overlapping with patients' serum (B, bottom). There was no colocalization with the dendritic marker MAP2 (B, middle) or the astrocyte marker GFAP that labels the astrocytic end feet in contact with the blood vessels (B, top). High magnification (×200) confocal images showed non-NMDAR antibody/NFM colocalization. Anti-MAP2 staining was clearly visible in the dendrites of pyramidal cells, whereas patients with non-NMDAR autoantibodies displayed the neurophil and axon staining that typically appears in the rat hippocampus and cerebellum (C, boxed areas are enlarged in the last column). Scale bar=20 µm (A and B). Scale bar=100 µm (C). GFAP: glial fibrillary acidic protein, NFM: medium neurofilament, NMDAR: N-methyl-D-aspartate receptor.

patients were normal. Seven patients improved with immunotherapy and methylprednisolone; these patients were either not significantly disabled [Expanded Disability Status Scale (EDSS) score of <2] or only mildly disabled (EDSS score of ≥2 and <3) at the last follow-up. Anti-NMDAR antibody titers in CSF also decreased (in one of the seven patients) or became negative (in four patients); there was no correlation between the NR1 antibody titers and the modified Rankin Scale scores (p=0.4010). Finally, there were no significant differences between patients with and without anti-NFM autoantibodies (Table 2).

DISCUSSION

The main finding of this exploratory study was that anti-NFM autoantibodies could be detected in some patients with anti-NMDAR encephalitis using a cell-based assay, whereas anti-NFL and anti-NFH autoantibodies were rare or absent. However, we also found that the study had insufficient statistical power for confirming correlations between the presence and titer of anti-NFM autoantibodies and the disease course of anti-NMDAR encephalitis.

Neurofilaments are expressed in neurons and maintain the axonal structural integrity, which establishes the stability of the neuronal cytoskeleton.[24] It has recently been found that neurofilament protein is released in many neurological diseases and is closely related to axonal degeneration and damage. In particular, the release of neurofilament antigens has been detected in patients with autoimmune encephalitis,[25] and this might be a predictor of the clinical outcome. Based

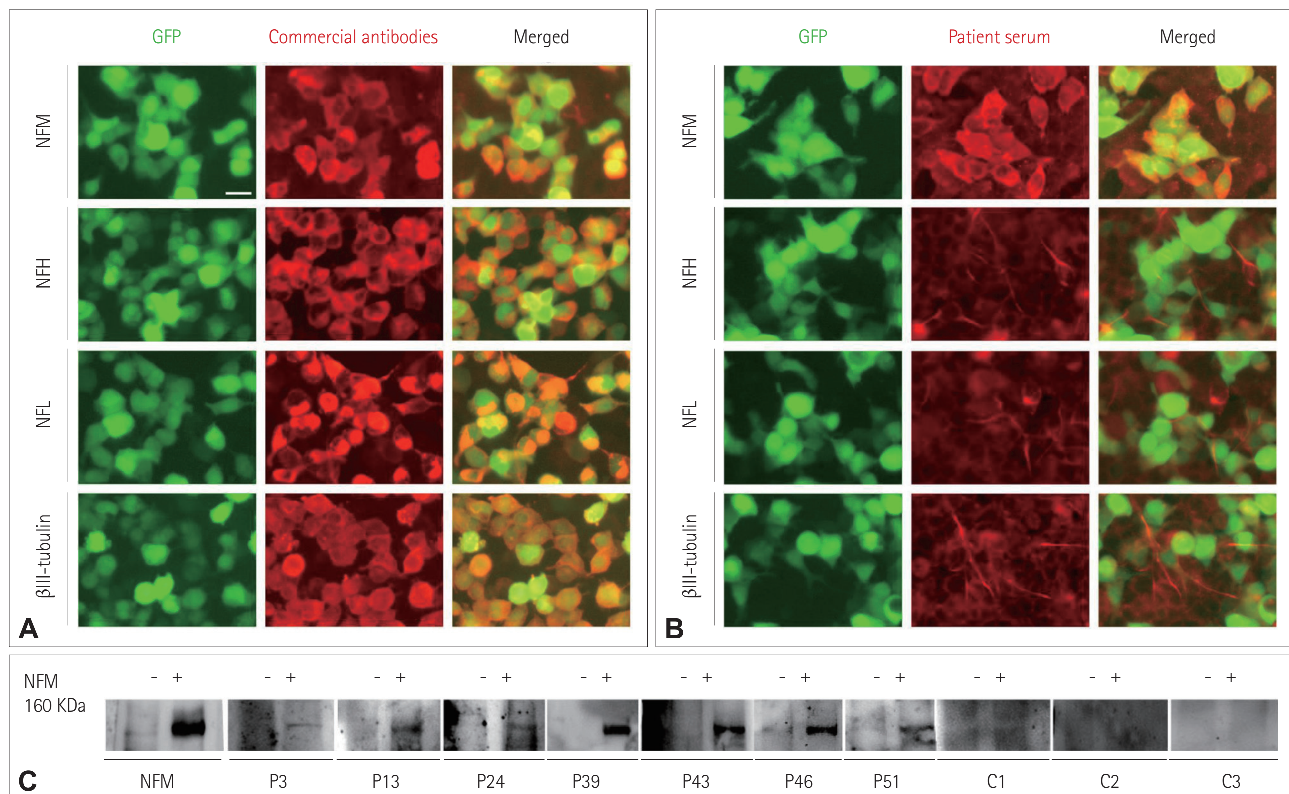

Fig. 4. Identification of NFM as the target antigen of non-NMDAR autoantibodies. We cotransfected HEK293T cells with GFP and NFM, NFH, NFL, or βIII-tubulin (A, column 1); column 2 shows the immunostaining for a commercially available antibody in red, and column 3 shows the merged reactivities. The non-NMDAR autoantibodies in the serum from patients reacted with HEK293T cells expressing human NFM in a fluorescence-based cell-binding assay (B). From top to bottom: binding of antibodies from patients' serum or CSF using an antihuman immunoglobulin G secondary antibody (red) to cells transfected with human NFM (green), merged images (yellow), and the absence of staining of nontransfected cells. Patients with anti-NMDAR encephalitis were negative for NFH, NFL, and βIII-tubulin antibodies, while cells displayed filamentous patterns. No binding was observed when using healthy serum. Representative results obtained by Western blotting ("P" and "C" indicate specific patients and controls, respectively) (C). Whole-cell lysates prepared from human HEK293T cells with (+) or without (–) transfection of the human NFM gene were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The blots were exposed to serum from seven patients with anti-NMDAR encephalitis and seven healthy controls (C1–C3) after a total of 20 μg of protein lysates from HEK293T cells. Antibodies that bound to the HEK293T cell lysates transfected with NFM that predominantly reacted with one antigen of approximately 160 kDa were seen in serum (diluted 1:100) from patients 3, 13, 24, 39, 43, 46, and 51. This binding was not observed in controls. No protein bands against the HEK293T cells without transfection (as negative controls) were detected in any of the samples. NFM was detected using commercially available anti-NFM antibodies as a positive control. Scale bar=20 μm. CSF: cerebrospinal fluid, GFP: green fluorescent protein, HEK: human embryonic kidney, NFH: heavy neurofilament, NFL: light neurofilament, NFM: medium neurofilament, NMDAR: N-methyl-D-aspartate receptor.

Table 1. Clinical features of anti-NMDAR encephalitis patients with anti-NFM antibodies

Patient no./sex/age at onset (years)	mRS score		CSF WBC ($\times 10^6$/L)	CSF anti-NMDAR antibody titer		Tumor	Symptoms	Brain MRI	Follow-up (months)	Treatment	Anti-NFM antibody titer
	At onset	After treatment		At onset	follow-up						
3/female/14	4	0	100	1:1	Negative	Negative	1 month of headache, 3 weeks of fever, and loss of consciousness	Normal	17	Steroids and tacrolimus	Serum 1:100
13/male/13	3	0	6	1:100	1:32	Negative	6 months of seizures, and 10 days of mental and behavioral abnormalities	Bilateral increased T2-weighted signals involving frontal cortex and semioval center	29	Steroids and RTX	Serum 1:100
24/male/6	4	0	22	1:100	Negative	Negative	8 months of seizures, movement disorders, and speech disturbances	Increased T2-weighted signals involving right cerebellum and bilateral frontal temporal lobes	13	Steroids, IVIG, and RTX	Serum 1:100
39/female/6	2	0	30	1:32	Negative	Negative	1 day of involuntary movements	Normal	19	Steroids and IVIG	Serum 1:640
43/female/39	2	1	6	1:10	1:1	Ovarian cysts	2 months of memory decline	Increased T2-weighted signals involving the bilateral thalamus, basal ganglia, right cerebral pedicle, left hippocampus, left radial crown, and cerebellum	3	IVIG	Serum 1:320 and CSF 1:100
46/female/26	4	2	86	1:100	Negative	Negative	1 month of fever, 20 days of psychiatric symptoms	Normal	8	Steroids and IVIG	Serum 1:320
51/female/26	3	0	26	1:100	-	Ovarian teratoma	1 week of fever, 4 days of seizures, and mental abnormality	Normal	32	Steroids and tumor removal	Serum 1:320

CSF: cerebrospinal fluid, IVIG: intravenous immunoglobulin, MRI: magnetic resonance imaging, mRS: modified Rankin Scale, NFM: medium neurofilament, NMDAR: N-methyl-D-aspartate receptor, RTX: rituximab, WBC: white blood cell count.

Table 2. Comparison between anti-NMDAR encephalitis patients with and without anti-NFM antibodies

	Anti-NFM (–) (*n*=38)	Anti-NFM (+) (*n*=7)
Age at onset, years	24 [2–64]	20 [6–39]
Pediatric onset	13 (34)	4 (57)
Sex ratio (female:male)	1:1	2.5:1
Onset symptoms		
Seizures	10 (26)	3 (43)
Fever	12 (32)	3 (43)
Psychiatric behavior	22 (58)	3 (43)
Movement disorder	3 (8)	2 (29)
Speech	2 (5)	1 (14)
Orofacial	0 (0)	0 (0)
Limb	0 (0)	1 (14)
Diplopia	1 (3)	0 (0)
Cognitive dysfunction	5 (13)	1 (14)
Autonomic dysfunction	0 (0)	0 (0)
MOG-IgG	4 (11)	2 (29)
Systemic antibodies	9 (24)	0 (0)
Concomitant autoimmunity	1 (3)	0 (0)
CSF WBC ($\times 10^6$/L)	11 [0–568]	28 [6–91]
CSF protein (g/L)	0.26 [0.07–1.18]	0.27 [0.11–1.57]
Abnormal brain MRI findings	24 (63)	3 (43)
Tumor	7 (18)	1 (14)
mRS score at onset	4 [1–5]	3.5 [2–4]
Follow-up duration, months	1 [0–24]	7 [1–32]
mRS score at last visit	2 [0–5]	0 [0–2]

Data are median [range] or *n* (%) values.
Concomitant autoimmunity, positive for anti-Sjögren's-syndrome-A antibody.
CSF: cerebrospinal fluid, IgG: immunoglobulin G, MOG: myelin oligodendrocyte glycoprotein, MRI: magnetic resonance imaging, mRS: modified Rankin Scale, NFM: medium neurofilament, NMDAR: N-methyl-D-aspartate receptor, WBC: white blood cell count.

on these observations, we hypothesized that patients with anti-NMDAR encephalitis can develop severe CNS inflammation or injury that leads to axonal degeneration and the release of cytoskeletal-like antigens, which in turn causes the immune system to produce autoantibodies that can be detected in the peripheral system. However, a correlation between these autoantibodies and the clinical course has not been confirmed. The age of the patients with anti-NFM autoantibodies in our study suggested that a pediatric onset is more common, which could be due to the incompleteness of the blood–brain barrier in children. We speculated that the axons were injured or that the blood–brain barrier was damaged or underdeveloped when anti-NFM autoantibodies were detected in the peripheral blood.

Human NFM is a 916-amino-acid protein with a molecular mass of 160 kDa that is encoded by the NEFM gene on chromosome 8 (8p21). The concentration of NFM protein has been found to be increased in the CSF and blood after brain injury, particularly after polytrauma.[26] NFM is associated with expression of the D1 dopamine receptor on the cell surface.[27] The level of intrathecal antibodies to NFM has been reported to be increased in MS patients, with axon damage occurring early in the disease process.[13] However, the anti-NFM antibody levels in patients with CSF were not related to clinical indicators such as disease severity.

Kreye et al.[28] reported a diverse range of antineural antibodies in NMDAR encephalitis, many of which did not bind to the NMDAR complex. To the best of our knowledge, the present study is the first to explore the possible coexistence of antineurofilament autoantibodies in patients with anti-NMDAR encephalitis, and it has expanded the catalogue of autoantibodies associated with this condition. We confirmed the presence of autoantibodies targeting NFM using Western blotting and immunofluorescence of brain slices, neurons, and other cells. We also screened 7 blood samples obtained from 45 patients with anti-NMDAR encephalitis, which revealed that the CSF was positive for anti-NFM autoantibodies in 1 patient, and remained so during follow-up even though the CSF became negative for anti-NMDAR antibodies. Unexpectedly, the antibody levels were higher in the serum than in the CSF. Since NFM proteins are strongly expressed in the CNS, we assumed that these antibodies are specific to CNS antigens in neurons. This finding is consistent with Bartos et al.[13] finding more anti-NFM antibodies in the periphery than in the CNS in MS patients. The origin of these serum antibodies is unclear, but anti-NFM autoantibodies may reflect the release of antigens.

The cytoskeleton is essential to cell morphology and the structure of neurons. The activation of NMDAR regulates the function of several proteins in the cytoskeleton, but the underlying mechanisms have been unclear. Fiumelli et al.[29] found that blockade of NMDAR can cause phosphorylation of NFM and increase its solubility. Moreover, regulation of NMDAR was hypothesized to change the stability of neurofilaments, thus affecting the growth of neuronal processes. Therefore, our research suggested that NMDAR encephalitis with subsequent neuronal and axonal loss can account for the presence of anti-NFM autoantibodies in these patients and also their presence in the peripheral nervous system when serum antibodies were produced in response to new antigen stimuli.

In conclusion, we have identified the presence of previously unreported anti-NFM autoantibodies in a subset of patients with anti-NMDAR encephalitis. However, this exploratory study did not have sufficient statistical power to confirm correlations between the presence and titer of anti-

NFM autoantibodies and the course of NMDAR encephalitis. Larger studies are required in the future to determine whether there are clinical differences between patients with NMDAR encephalitis who are positive and negative for anti-NFM autoantibodies. Future research would require multicenter collaboration to collect more clinical cases, especially for the CSF samples. As in the research on anti-GFAP[30,31] antibody, these antibodies may have more clinical significance in the CSF than in the peripheral blood.

Author Contributions

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Conflicts of Interest

The authors have no potential conflicts of interest to disclose.

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