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Detection of anti-aquaporin-4 autoantibodies in the sera of Chinese neuromyelitis optica patients★

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Abstract

In this study, we recruited 10 neuromyelitis optica patients, two multiple sclerosis patients and two myelitis patients. Chinese hamster lung fibroblast (V79) cells transfected with a human aquaporin-4-mCherry fusion protein gene were used to detect anti-aquaporin-4 antibody in neuromyelitis optica patient sera by immunofluorescence. Anti-aquaporin-4 autoantibody was stably detected by immunofluorescence in neuromyelitis optica patient sera exclusively. The sensitivity of the assay for neuromyelitis optica was 90% and the specificity for neuromyelitis optica was 100%. The anti-aquaporin-4 antibody titers in sera were tested with serial dilutions until the signal disappeared. A positive correlation was detected between Expanded Disability Status Scale scores and serum anti-aquaporin-4 antibody titers. The anti-aquaporin-4 antibody assay is highly sensitive and specific in the sera of Chinese neuromyelitis optica patients. Detection of aquaporin-4 autoantibody is important for the diagnosis and treatment of neuromyelitis optica.

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Key Words

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Research Highlights

(1) Chinese hamster lung fibroblast (V79) cells transfected with a human aquaporin-4-mCherry fusion protein gene were used to detect anti-aquaporin-4 antibody in neuromyelitis optica patient sera by immunofluorescence. Anti-aquaporin-4 autoantibody was stably detected by immunofluorescence in neuromyelitis optica patient sera exclusively. The sensitivity of the assay for neuromyelitis optica was 90% and the specificity for neuromyelitis optica was 100%.
(2) A positive correlation was detected between Expanded Disability Status Scale scores and serum anti-aquaporin-4 antibody titers in neuromyelitis optica patient sera.
(3) Detection of aquaporin-4-autoantibody is important for the diagnosis and treatment of neuromyelitis optica.

INTRODUCTION

Neuromyelitis optica is a neurological autoimmune demyelinating disease that usually involves severe optic neuritis and transverse, longitudinally extensive myelitis[1-4]. Neuromyelitis optica was hard to diagnose for a long time until aquaporin-4 was found to be important in this disease. Aquaporin-4, which is expressed abundantly in the central nervous system, is considered to be the autoimmune antigen of neuromyelitis optica. Therefore, detection of anti-aquaporin-4 antibody in the sera of patients is potentially an effective approach to diagnosing neuromyelitis optica. Neuromyelitis optica-immunoglobulin G (IgG) detected in patients' serum reacts with the microvessels and subpiamater of the central nervous system[5]. Subsequently, the target antigen of neuromyelitis optica-IgG was identified as aquaporin-4[6-9], the most abundant water channel in the central nervous system[10]. Aquaporin-4, which is expressed in astrocyte foot processes, the glia limitans and ependyma, induces water movement in the brain, accelerates astrocyte migration and alters neuronal excitation phenomena[11-14]. Deficiency of aquaporin-4 causes brain edema and obstructive hydrocephalus[15-17].

For proper diagnosis and treatment of neuromyelitis optica, it is important to detect aquaporin-4 autoantibody. The routine methods are neuromyelitis optica-IgG assay and anti-aquaporin-4 antibody assay, the latter of which is more sensitive[18]. There have been several studies of the relationship between aquaporin-4 autoantibody titer and neuromyelitis optica in Europe and Japan[1, 10, 18-21], but there have been few in China. In the present study, we used a sensitive and specific method to detect aquaporin-4 autoantibodies in the sera of Chinese patients with neuromyelitis optica, and investigated the relationship between the titer of anti-aquaporin-4 antibody and diagnosis of neuromyelitis optica.

RESULTS

Quantitative analysis of subjects and clinical information

This study included 10 neuromyelitis optica patients, two multiple sclerosis patients and two myelitis patients. Clinical data of patients are shown in Table 1.

Anti-aquaporin-4 autoantibody was stably detected in Chinese neuromyelitis optica patients' sera

Anti-aquaporin-4 antibody was detected and titrated in 9 of 10 neuromyelitis optica patients' serum by indirect immunofluorescence. Anti-aquaporin-4 antibody in the positive sera bound to aquaporin-4, and stained the surfaces of aquaporin-4-transfected Chinese hamster lung fibroblast (V79) cells (Figure 1).

High sensitivity and strong specificity of the anti-aquaporin-4 antibody assay

Nine of the ten patients diagnosed with neuromyelitis optica were seropositive for anti-aquaporin-4 antibody (Table 2). The two multiple sclerosis patients and two myelitis patients were seronegative. Thus, the sensitivity of the assay for neuromyelitis optica was 90% and the specificity for neuromyelitis optica was 100%.

Positive correlation between Expanded Disability Status Scale scores and serum anti-aquaporin-4 antibody titers

Six different dilutions of neuromyelitis optica patients' sera from 1:100 to 1:16 000 underwent immunofluorescence assay. Among the sera of nine neuromyelitis optica patients, aquaporin-4 was detected in two at a dilution of 1:16 000, two at a 1:8 000 dilution, two at a 1:4 000 dilution, one at a 1:1 000 dilution, and two at a 1:400 dilution (Table 2). Pearson's correlation analysis revealed a significant positive correlation (r = 0.904, P = 0.001) between Expanded Disability Status Scale scores and the titers (Figure 2).

Table 1 Clinical profiles of included patients

Item	Neuromyelitis optica	Multiple sclerosis	Myelitis
n	10	2	2
Gender (male/female, *n*)	2/8	0/2	0/2
Age at onset (year) [median (IQR)]	41 (14–68)	28 (26–30)	35 (25–45)
Disease duration (year) [median (IQR)]	5 (1–12)	6.5 (6–7)	6 (5–7)
Annual relapse rate [median (IQR)]	0.88 (0.64–1)	0.69 (0.67–0.71)	1 (The two patients experienced onset for the first time)
Expanded disability status scale [median (IQR)]	4.8 (3.5–6.5)	2.8 (2.5–3.0)	7.8 (7.5–8)

IQR: Interquartile range.

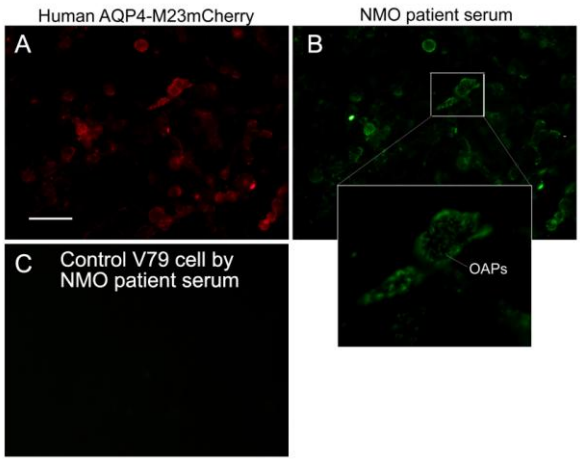

Figure 1 V79 cells transfected with human aquaporin-4 (AQP4)-M23-mCherry in neuromyelitis optica (NMO) patient sera (fluorescent microscope).

The continuous NMO patient serum and fluorescein isothiocyanate (FITC)-conjugated secondary antibody incubation caused green fluorescence to emerge on the membranes of AQP4-mCherry transfected V79 cells, which strongly matched the mCherry red fluorescence pattern. The stain was FITC. Scale bar: 100 μm.

(A) The cells show mCherry red fluorescence on the cell membrane.

(B) Immunofluorescence of the AQP4-M23-mCherry-transfected V79 cells. The secondary antibody is conjugated to FITC. OAPs: Orthogonal arrays of particles (scale 1: 3.5).

(C) No fluorescence in control V79 cells.

MRI revealed that five or more vertebral segments in each of the six neuromyelitis optica patients with serum titers of 1:4 000–16 000 were affected by spinal cord lesions. The concentration of anti-aquaporin-4 antibody in neuromyelitis optica serum positively correlated with the degree of myelitis. The two patients with a titer of 1:16 000 dilution experienced permanent complete blindness (Table 2).

DISCUSSION

We detected anti-aquaporin-4 autoantibody titers in Chinese neuromyelitis optica patients, and revealed the clinical and immunological implications of the autoantibody titer for neuromyelitis optica. The anti-aquaporin-4 antibody assay we used is highly sensitive for neuromyelitis optica, consistent with European and Japanese reports[19, 22-24]. First, we demonstrated that anti-aquaporin-4 antibody is exclusively detected in Chinese neuromyelitis optica patients, and found that the majority of them were positive for the autoantibody. This finding strongly suggested that anti-aquaporin-4 antibody is strongly associated with neuromyelitis optica. In Europe and Japan, there have been many studies of neuromyelitis optica; however, in China, there have been few. Our results showed that the anti-aquaporin-4 antibody assay is also sensitive for Chinese neuromyelitis optica patients, and that it will be a good means of diagnosis and an important appraisal of diagnosis in the clinic[25]. The anti-aquaporin-4 antibody assay was highly sensitive. As a result, the sensitivity of our anti-aquaporin-4 antibody assay was 90% (9 of 10) and the specificity in neuromyelitis optica was 100%. Takahashi and colleagues reported 91% sensitivity and the 100% specificity for neuromyelitis optica of their anti-aquaporin-4 antibody assay[18]. Lennon and colleagues reported 73% sensitivity and 91% specificity of their neuromyelitis optica-IgG assay[26].

Table 2 Patient information and aquaporin-4 (AQP4) autoantibody titers

Serum No.	Diagnosis	Degree of blindness	Spinal cord injury degree	Expanded Disability Status Scale	Positive and negative test	AQP4 autoantibody titer test (1:)
1	Multiple sclerosis	0	2	2.5	Negative	
2	NMO	0	6	3.5	Positive	400
3	NMO	2	8	6	Positive	16 000 (still visible)
4	NMO	0	3	4	Positive	400
5	Multiple sclerosis	0	3	3	Negative	
6	NMO	0	3	5	Positive	1 000
7	NMO	0	6	4.5	Positive	4 000
8	NMO	2	6	6.5	Positive	16 000 (still visible)
9	NMO	0	3	3.5	Negative	
10	NMO	1	7	5.5	Positive	8 000
11	Myelitis	0	3	7.5	Negative	
12	NMO	0	10	4.5	Positive	4 000
13	Myelitis	0	2	8	Negative	
14	NMO	0	8	5	Positive	8 000

The higher the Expanded Disability Status Scale scores is, the more severe the patient's disease is. The same applied to the severity of blindness and spinal cord injury[18]. NMO: Neuromyelitis optica.

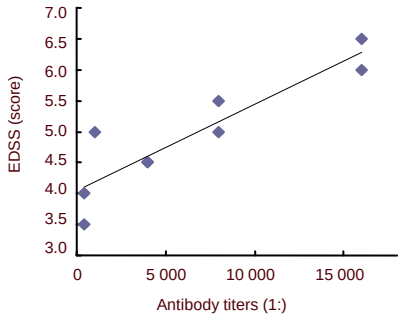

Figure 2 A significantly positive correlation between Expanded Disability Status Scale (EDSS) scores and anti-aquaporin-4 autoantibody titers.

The higher the EDSS score is, the more severe the patient's disease is. $r = 0.904$, $P = 0.001$ (Pearson's correlation analysis).

The neuromyelitis optica-IgG assay uses mouse brain slices[27-28]. Although human aquaporin-4 is highly homologous to mouse aquaporin-4, the amino acid sequences in the extracellular domains are different between the two species. This difference influences the binding of anti-aquaporin-4 autoantibodies in patients to the surfaces of mAQP4-transfected cells. As a result, it is hard to detect neuromyelitis optica-IgG in the sera of neuromyelitis optica patients using the neuromyelitis optica-IgG assay[28]. The present study used the V79 cell line stably transfected with hAQP4-M23-mCherry. Aquaporin-4 is expressed as two major isoforms, M1 and M23, depending upon the transcriptional start site[29]. Aquaporin-4-M23 particles form orthogonal arrays in the plasma membrane, whereas aquaporin-4-M1 is unable to form orthogonal arrays[30-31]. Orthogonal arrays of particles assemblies are required for neuromyelitis optica-IgG to recognize aquaporin-4[27, 32-33]. Thus, the stably transfected V79 cell line with hAQP4-M23-mCherry we used can bind to the anti-aquaporin-4 autoantibodies well, enabling us to achieve higher sensitivity and stronger specificity compared with previous approaches.

Our results revealed a positive correlation between Expanded Disability Status Scale scores and serum anti-aquaporin-4 antibody titers. The higher the anti-aquaporin-4 antibody titer, the more severe the condition of neuromyelitis optica is[34]. Five patients whose serum anti-aquaporin-4 autoantibody titers were in the range 1:4 000–1:16 000 had spinal cord lesions spanning ≥ 5 vertebral segments, as measured by MRI, and the two cases with the highest antibody titers (1: 16 000) had permanent complete blindness. Our results are similar to those of Takahashi *et al* [18, 35]. In Japanese neuromyelitis optica patients, serum anti-aquaporin-4 autoantibody titers were higher in cases with permanent complete blindness or ≥ 3 vertebral segments of spinal cord lesions, as measured by MRI[18, 36-37]. In Chinese patients, the anti-aquaporin-4 autoantibody titer is more strongly correlated with spinal cord lesions, but in Japanese patients, the anti-aquaporin-4 autoantibody titer is more strongly correlated with blindness[18, 35].

The anti-aquaporin-4 autoantibody assay is highly sensitive for the autoantibody in the sera of Chinese neuromyelitis optica patients. The presence of anti-aquaporin-4 autoantibody has significant implications for neuromyelitis optica. Detection of aquaporin-4-autoantibody is important for the diagnosis and treatment of neuromyelitis optica. A high anti-aquaporin-4 autoantibody titer is strongly correlated with spinal cord lesions.

SUBJECTS AND METHODS

Design

An immunological, molecular biological and diagnostic study.

Time and setting

The study was performed at the Northeast Normal University and Department of Neurosurgery, China-Japan Union Hospital, Jilin University, China from October 2010 to December 2011.

Subjects

Ten patients were diagnosed as having neuromyelitis optica, two as having multiple sclerosis and two as having myelitis. The patients were hospitalized in Beijing XuanWu Hospital, People's Hospital of Liaoning Province and the China-Japan Union Hospital of Jilin University. The criteria for diagnosis of neuromyelitis optica were as follows: optic neuritis, acute myelitis, and no symptoms implicating other central nervous system regions[1, 19]. We obtained informed consent from the patients and their family members.

Methods

V79 cell culture and transfection

The V79 cell line (a gift from Bari University, Italy) was grown in Dulbecco's modified Eagle's medium (Sigma-Aldrich, St. Louis, MO, USA) containing 100 U/mL penicillin, 100 g/mL streptomycin, and 10% fetal bovine serum. The cells were maintained at 37°C and 5% CO_2. The pcDNA3.1-hygromycin plasmid with hAQP4-M23-mCherry insertion was constructed by our lab previously[38]. This plasmid was transfected into V79

cells using Lipofectamine 2000 Transfection Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol[39]. Medium containing 500 g/mL hygromycin B (Invitrogen) was used to select transfected cells. Limited dilution was performed. hAQP4-M23 expression can be seen easily by mCherry red fluorescence[40]. Finally, a stable transfected V79 cell line with hAQP4-M23-mCherry expression was established.

Collection of patient sera

We collected sera from 14 Chinese patients. Blood was extracted from the ulnar vein or forearm vein, centrifuged, and then the supernatant was obtained.

Indirect immunofluorescence for the detection of anti-aquaporin-4 autoantibodies in the sera of neuromyelitis optica patients

Indirect immunofluorescence experiments were performed as previously described[38]. Briefly, V79 cells were seeded on glass coverslips and grown to confluence. The cells were fixed with 4% paraformaldehyde in PBS for 15 minutes, then washed with PBS and permeabilized with 0.1% Triton X-100 in PBS for 10 minutes. The cells were blocked by incubation in PBS containing 2% bovine serum albumin (Sigma-Aldrich) for 1 hour at room temperature. The cells were then exposed to neuromyelitis optica patient sera at serial dilutions for 2 hours at room temperature. After washing, an FITC-conjugated goat anti-human secondary antibody (Sigma-Aldrich; dilution 1:1 000) was applied for 1 hour at room temperature. The cells were washed with PBS and photographed using an Olympus IX71 fluorescent microscope (Tokyo, Japan).

Correlation of Expanded Disability Status Scale scores and serum anti-aquaporin-4 autoantibody titers

Expanded Disability Status Scale scores of patients were provided by source hospitals; the measurement of Expanded Disability Status Scale scores was performed as previously described[22]. We analyzed the linear relationship between Expanded Disability Status Scale scores and serum anti-aquaporin-4 autoantibody titers in nine neuromyelitis optica patients by Pearson's correlation analysis (SPSS software version 17.0, SPSS, Chicago, IL, USA). Sera tested for anti-aquaporin-4 autoantibody titers were obtained without any treatment.

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Author contributions: Miao Li and Tonghui Ma designed the research. Miao Li and Weiheng Su wrote the manuscript. Miao Li and Jie Wang collected neuromyelitis optica patient sera. Miao Li, Weiheng Su and Francesco Pisani performed the research. Antonio Frigeri and Tonghui Ma were the principal investigators on the grant, No. PGR00011. All authors approved the final version of the paper.

Conflicts of interest: None declared.

Ethical approval: This study was approved by the Medical Ethics Committee of Northeast Normal University with the reference No. NAC11870012.

Author statements: The manuscript is original, has not been submitted to or is not under consideration by another publication, has not been previously published in any language or any form, including electronic, and contains no disclosure of confidential information or authorship/patent application/funding source disputations.

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