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Development of a cell-based assay for the detection of anti-aquaporin 1 antibodies in neuromyelitis optica spectrum disorders

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ABSTRACT

Objective: To develop a cell-based assay (CBA) to detect aquaporin 1 (AQP1) antibodies and determine sensitivity/specificity in patients with neuromyelitis optica (NMO) spectrum disorders.
Methods: A HEK-293T transfected cell model expressing AQP1 was established and detected to be serum AQP1 antibodies.
Results: AQP1 antibodies were present in 73/98 (74.5%) AQP4 antibody-positive patients. Some AQP4 antibody-negative patients were also AQP1 antibody-positive. Test sensitivity was 74.5% in 98 AQP4 antibody-positive patients. Test specificity was 79.6% in 67 multiple sclerosis (MS) patients and 31 controls.
Conclusion: A sensitive and simple CBA was developed to detect serum AQP1 antibodies. AQP1 antibodies were mainly present in NMO and its high-risk syndrome, but also in some MS patients.

1. Introduction

Neuromyelitis optica immunoglobulin G (NMO-IgG) is used as a biomarker to differentiate neuromyelitis optica (NMO) from multiple sclerosis (MS). Several pathological studies revealed that this autoantibody was an important contributor to NMO pathology (Misu et al., 2007, 2013; Saji et al., 2013). The target antigen of NMO-IgG was identified as aquaporin-4 (AQP4), the main water channel protein in the central nervous system (CNS), which is mainly expressed on astrocyte end-feet at the blood–brain barrier (BBB) (Lennon et al., 2004, 2005).

However, although a number of highly sensitive immunoassays for the detection of AQP4 antibody in patients with NMO have been developed, NMO patients negative for anti-AQP4 antibodies have been identified, indicating that other autoantibodies might be involved in NMO pathogenesis (Kitley et al., 2012). However, it is unclear how anti-AQP4 antibodies cross the intact BBB from the serum into the CNS to cause inflammatory responses. In a previous study, Shimizu et al. (2012) demonstrated that anti-brain microvascular endothelial cell antibodies in the sera from NMO patients disrupted the BBB, providing a new pathological explanation of the triggers for BBB breakdown and trafficking of AQP4 antibodies into the CNS during the acute stage of NMO. This indicates that sera antibodies against microvessels other than anti-AQP4 antibodies are necessary to disrupt the BBB.

AQP1, another AQP family member, is highly expressed in human CNS astrocytes (Misu et al., 2013) and AQP1 may also selectively be lost around active NMO lesions, reflecting a role for AQP1 in NMO pathology (Misu et al., 2013). Furthermore, AQP1 is also highly expressed in microvascular endothelia (Verkman, 2002), although the functional significance of AQP1 is unclear. These studies suggested that AQP1 antibodies might induce BBB destruction and cause astrocyte injury. Recently, Tzartos et al. (2013) found that anti-AQP1 auto-antibodies were highly sensitive and specific to NMO spectrum disorders (NMOSD) using a radioimmunoprecipitation assay (RIPA). However, they failed to develop a useful cell-based assay (CBA) using AQP1-transfected HEK293 cells.

Here, according to a previous study (Gao et al., 2005), we aimed to establish a CBA for anti-AQP1 antibody detection in NMOSD patients. We also analyzed the diagnostic value and clinical significance of AQP1 antibodies in a large number of Chinese patients with NMOSD and controls.

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2. Patients and methods

The study protocol was approved by the Ethics Committee of the Second Affiliated Hospital of Guangzhou Medical University. Written informed consent was provided by all participants.

NMO/NMOSD was diagnosed as previously described (Wingerchuk et al., 2006; Fujihara and Sato, 2013). MS was diagnosed according to the 2010 criteria (Polman et al., 2011). Patients with transverse myelitis (TM) fulfilled the inclusion and exclusion criteria proposed by the Transverse Myelitis Consortium Working Group (2002) (Anon., 2002). Longitudinally extensive transverse myelitis (LETM) patients were certified by magnetic resonance imaging (Scott et al., 2006). Optic neuritis (ON) was defined by acute or subacute visual loss in unilateral or bilateral eyes. Other neurological disorders were diagnosed according to their criteria.

Until December 2013, 249 samples from consecutive patients (showed in Table 1) who had undergone anti-AQP4 antibody determination were used to detect AQP1 antibodies. Patient data were retrospectively evaluated by medical record reviews and recent interviews.

According to the serum profiles, 98 patients positive for AQP4 antibodies were diagnosed with NMOSD (mean age: 34.5 ± 12.5 years [12–70]). This group consisted of 89 women and 9 men (female/male = 9.9). Furthermore, 63 patients had NMO, 21 patients had recurrent LETM (rLETM), five patients had monophasic LETM, five patients had recurrent optic neuritis (RON), and four patients without ON and TM had brainstem or brain NMO lesions.

AQP4-negative patients consisted of 85 women and 66 men (female/male = 1.3). This group consisted of 11 patients with NMO, 67 patients with MS, 8 patients with rLETM, 8 patients with monophasic LETM, 12 patients with non-LETM, 14 patients with RON, and 31 controls. The control group consisted of 31 patients with other neurological diseases including cerebral infarction (n = 20), CNS infectious disorders (n = 5), and other disorders (n = 6).

Data acquired from each patient's previous record and recent interviews included age, sex (shown in Table 1), medication, number of demyelinating events, clinical characteristics, and their Expanded Disability Status Scale score (Kurtzke, 1983).

2.1. Cell culture

Human embryonic kidney (HEK) 293T cells were obtained from ATTC. According to a previous study (Gao et al., 2005), HEK-293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) nutrient mixture supplemented with 10% heat inactivated fetal bovine serum (HyClone, Logan, UT, USA), 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 °C in a humidified atmosphere of 5% CO_2.

2.2. Transfection of HEK-293T cells with a construct containing the human aquaporin-1 gene

The HEK-293T cell line was transduced with pcDNA3.1 Vectors (Invitrogen, IA, USA). AQP1 was amplified from a human gene cDNA library (Yingshen Company, Guangzhou, China) by polymerase chain reaction using the following primers: forward 5′-CGGGATCCGCCACCATGGCCAGCGAGTTCAAGAAG-3′, and reverse 5′-GGAATTCCTATTTGGGCTTCATCTCCACC-3′ and cloned into a vector. HEK-293T cells were transfected with a vector carrying the human AQP1 gene or a control vector without human AQP1 by lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). In brief, HEK-293T cells were plated and grown until 70–80% confluent. For each 35-mm well, 1 μg AQP1 or empty vector was diluted with 100 μL serum-free DMEM in one tube, and 1 μL lipofectamine 2000 was diluted with 100 μL serum-free DMEM in another tube. The DNA solutions and lipofectamine 2000 were then mixed from the two tubes and incubated at room temperature for 20 min. The DNA-lipofectamine 2000 complex mixture was added to each well. After 24 h, the medium containing the DNA-lipofectamine 2000 complex mixture was removed and replaced with complete growth medium.

2.3. Reverse-transcription polymerase chain reaction

Total RNA was isolated from HEK293/AQP1 or HEK293/vector control cells using Trizol reagent (Invitrogen). First-strand cDNA was synthesized from total RNA using a reagent kit (Invitrogen) according to the manufacturer's instructions. The primer sequences were: AQP1-F: 5′-ACCTCCTGGCTATTGACTACA-3′ and AQP1-R: 5′-CAGAAAATCCAGTGGTTGCT-3′. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), as an internal control, was amplified using primers F: 5′-GGGAAACTGTGGCGTGAT-3′ and R: 5′-GAGTGGGTGTCGCTGTTGA-3′.

2.4. Western blot

HEK-293T cells were lysed and centrifuged at 10,000 ×*g* for 10 min. For western blot analysis, a 50-μg sample was solubilized at 60 °C for 15 min, resolved by 12% polyacrylamide gel electrophoresis, and electrotransferred to a polyvinylidene difluoride membrane. The membrane with blotted protein was blocked for 1 h with blocking buffer containing 5% nonfat dry milk, followed by incubation with a rabbit anti-human AQP1 antibody (Bioss, Beijing, China) diluted 1:200 in blocking buffer at 4 °C overnight. After three 5-min washes with blocking buffer, the membrane was incubated with a goat anti-rabbit antibody (Southern Biotech) diluted 1:500 in blocking buffer for 2 h at room temperature. The stained bands were scanned and pixel density was quantified using a Gel Image System (Bio-Rad).

2.5. Expression of AQP1 on the surface of transfected cells and tissues

AQP1-transfected cells and empty cells were fixed and sectioned. Monkey brain and stomach tissues were purchased from Euroimmun Company (Lübeck, Germany). After incubation with primary antibodies for 1 h, the substrates were washed three times in phosphate-buffered saline (PBS) and incubated with anti-human or anti-rabbit IgG (Bioss Company, Beijing, China) for 30 min, washed in PBS, and stained with 4′,6-diamidino-2-phenylindole. Images were captured using a Leica microscope.

Table 1
Comparison among NMOSD, MS and others patients.

Characteristic	NMOSD (n = 98)	AQP4 antibody negative patients						p[a]
		N-NMO (n = 11)	MS (n = 67)	RON (n = 14)	LETM (n = 16)	Non-LETM (n = 12)	Control (n = 31)	
Female/male	89/9	6/5	37/30	8/6	8/8	8/4	14/17	<0.0001
Median age, y (range)	36 (12–70)	39 (28–65)	32 (10–68)	19 (14–46)	31 (12–55)	32 (4–72)	40 (17–72)	0.521
AQP1 antibody, n (%)	73/98 (74.5%)	7/11 (63.6%)	19/67 (28.4%)	11/14 (78.6%)	7/16 (43.8%)	4/12 (33.3%)	1/31 (3.2%)	<0.0001

NMO, neuromyelitis optica; NMOSD, NMO spectrum disorders; N-NMO, AQP4 antibodies negative NMO; MS, multiple sclerosis; RON, recurrent optic neuritis; LETM, longitudinal extensive transverse myelitis.

[a] Compared between NMOSD and MS.