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Aquaporin-4 IgG Antibody Detection in Neuromyelitis Optic Spectrum Disorder

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Abstract: *Objective*: To develop a sensitive and reliable diagnostic approach for optic neuromyelitis (ONM), an overexpressing cell line capable of detecting aquaporin 4 (AQP4) antibodies was established. Subsequently, immunofluorescence was employed to detect AQP4 antibodies in serum and cerebrospinal fluid (CSF) samples from patients. Additionally, the clinical utility of AQP4 antibodies in the detection of ONM patients was analyzed. *Methods*: An aquaporin 4 expression plasmid was constructed and transfected into cell lines. Subsequently, indirect immunofluorescence was utilized to detect anti-AQP4 antibodies in serum and cerebrospinal fluid samples. *Results*: The indirect immunofluorescence detection system exhibited high sensitivity in the detection of 2241 clinical samples, with a positive rate of 16.8%. The positive proportion was in line with epidemiological data, and the positive situation was consistent with clinical symptoms. *Conclusion*: The engineered cell line exhibits superior detection performance for identifying antibodies present in serum and cerebrospinal fluid samples. The capability to detect anti-aquaporin 4 antibodies is pivotal for improving the efficiency of screening and diagnosing neuromyelitis optica, thereby possessing considerable potential for clinical application.

Keywords: Aquaporins; Autoantibodies; Ophthalmoneuromyelitis; Indirect immunofluorescence assay; Cell transfection

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1. Introduction

The aquaporin-4 (AQP4) protein is widely distributed in the central nervous system and primarily mediates the transport of water molecules in the brain tissue. In 2004, Lennon et al. confirmed the presence of self-antibody NMO-IgG in the serum of patients with neuromyelitis optica (NMO) [1]. This antibody can specifically bind to the microvasculature and other parts of the brain tissue, leading to neuromyelitis optica spectrum disorders (NMOSD). This specific antibody has been identified as an anti-AQP4 antibody. With further advancements in research, the detection of the anti-AQP4 antibody has begun to be applied in the auxiliary diagnosis of NMO. As noted in the 2021 Diagnostic and Treatment Guidelines for Neuromyelitis Optica Spectrum Disorders, AQP4-IgG is a highly specific diagnostic marker with a specificity of up to 90% and sensitivity of approximately 70%. Furthermore,

guidelines recommend the use of cell-based transfection immunofluorescence assays (CBAs) or flow cytometry techniques for serological examinations [2].

This study aimed to construct an AQP4 overexpressing cell line using the cell transfection technique mentioned in the diagnostic guidelines. Subsequently, an indirect immunofluorescence assay was performed to establish a detection protocol for AQP4-IgG antibodies in the samples. A total of 2,241 samples collected between January 2021 and December 2022 in the laboratory were tested for AQP4-IgG antibodies. The test results were then analyzed and summarized to assess the positivity rate of anti-AQP4 antibodies in central nervous system diseases, as well as the sex distribution among affected individuals. Additionally, this study discussed the clinical value of this antibody in the diagnosis and treatment of neuromyelitis optica spectrum disorder. To evaluate the effectiveness of the CBA method established in this study, a comparative experiment was conducted using commercial Elisa kits (RSR Limited: AQP4/96/2).

2. Materials and methods

2.1. Sample information

A total of 2,241 serum and cerebrospinal fluid samples were collected from January 2021 to December 2022. The samples were all patients with suspected NMO who were referred to the laboratory from neurology departments in various regions of China. The sex distribution among the samples was approximately 1:1.28 (male-to-female ratio). The mean age of the individuals included in the study was 50.44 ± 14.73 years. Additionally, the samples were carefully collected by nurses adhering to standard procedures and stored in designated tubes. The specimens were promptly transported to the research lab via cold chain logistics, ensuring their integrity for further analysis.

2.2. Gene synthesis

Retrieve the mRNA sequence for the AQP4-M23 (NM_001364286) gene from the NCBI database, followed by gene synthesis conducted by Nanjing Kingsley Company utilizing pcDNA3.1-eGFP plasmid vector.

2.3. Extraction and validation of the plasmid

The synthesized plasmid was introduced into *Escherichia coli* DH5α cells through transformation, followed by cultivation in an aminobenzyl medium. Subsequently, positive monoclonal colonies were carefully selected for amplification and further culturing. After harvesting bacterial cultures, the recombinant expression vector was isolated using an endotoxin-free plasmid extraction kit (Tiangen).

The extracted plasmids were subjected to double digestion with NheI and EcoRV at 37°C for 15 min and 80°C for 20 min to inactivate the enzyme using the following digestion system. At the same time, the extracted plasmids were sent to Qingke Biological Company for sequencing verification.

Table 1. Digestion system

Digestion system	Dosage
pcDNA3.1(+)-C-eGFP-human AQP4	5 μL
NheI	1 μL
EcoRV	1 μL
10×Buffer	2 μL
ddH_2O	11 μL

2.4. Cell culture and transfection

Hek293 (human embryonic kidney cells) cells were cultured and transfected with AQP4 gene expression plasmid according to the instructions using Lipofectamine3000 transfection reagent when 80%–90% confluence was reached. After 48 hours, the transfection effect was observed.

2.5. Fixation and permeabilization

Isolated cells with a transfection efficiency of up to 60%, and subjected them to structural fixation using 4% paraformaldehyde. Perform cell permeabilization using 0.2% Triton X-100, followed by blocking the cell slides with 3% BSA.

2.6. Validation using antibodies

First, the treated cell slides were incubated with a rabbit monoclonal AQP4-IgG antibody (Abcame catalog number: ab259318) at 35°C for 1 hour, followed by three washes with PBST. Next, goat anti-rabbit IgG labeled with TRITC (Abcame catalog number: ab6718) was added to the slides and incubated at 35°C for another hour, after which three washes with PBST were performed. Finally, the DAPI sealing reagent was applied and the cells were observed under a fluorescence microscope to verify transfection and protein expression.

2.7. Testing of samples

Before the test, the experimenters were unaware of the expected results of the samples. Serum samples (1:10) and cerebrospinal (1:1) were diluted, and the treated cell slides were incubated for 1 hour. The slides were washed three times with PBS, followed by incubation with Cy3-labeled anti-human IgG antibody (Jackson Immuno catalog number: 109-165-003) for 1 hour. The slides were washed three times with PBST again, DAPI sealing reagent was added, and the results were examined and recorded using a fluorescence microscope. The results were judged by two trained experimenters. In addition, for samples with very high fluorescence, further dilution can be performed.

2.8. Statistical analysis

Employing SPSS 19.0 statistical software for data processing and analysis, count data are presented as case numbers, age data as mean ± standard deviation ($X \pm s$), and the Chi-square test was conducted for comparison between male and female groups, represented by (n (%)). A *P*-value less than 0.05 signifies a statistically significant difference.

2.9. Comparative experiment

Commercial ELISA kits (RSR Limited: AQP4/96/2) were procured for comparative studies, and the protocols were followed according to the manufacturer's instructions. Samples were randomly chosen and analyzed concurrently using both CBA and ELISA methods. It is worth noting that this commercially available kit can quantitatively detect AQP4 antibodies in samples, and the commercially available kits have already obtained medical device licenses within China, indicating their widespread utilization and acceptance within the domestic market. This ensures their reliability and effectiveness for various applications in the medical field.

3. Results

3.1. PCR validation results

Double-enzyme digestion and sequencing validation of the plasmids are presented in **Figure 1**. The digested

fragment larger than 6000 bp corresponds to the plasmid vector pDNA3.1(+)-C-eGFP (6176 bp), whereas the fragment smaller than 1000 bp represents the mRNA sequence of AQP4 (906 bp). Furthermore, the plasmid was subjected to sequencing analysis, and a comparison of the sequencing results with the theoretical sequence demonstrated a 100% alignment.

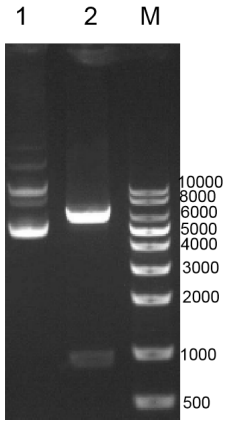

Figure 1. Enzyme digestion and electrophoresis. Lane 1: AQP4-pcDNA3.1(+)-C-eGFP: Recombinant plasmid; Lane 2: AQP4-pcDNA3.1(+)-C-eGFP: NheI and EcoRV Double Digestion; Lane M: 10KB Ladder

3.2. Results of the primary antibody reaction

Due to the presence of EGFP protein in the plasmid vector, transfected cells exhibited green fluorescence under the blue excitation wavelength range (420–485 nm) of a fluorescence microscope. Transfection efficiency can be analyzed based on the proportion of green fluorescent cells observed. Furthermore, incubation with the primary antibody and the addition of a red fluorescent-labeled secondary antibody resulted in red fluorescence in the green excitation wavelength range (460–550 nm), indicating successful expression of human aquaporin 4, as shown in **Figure 2**.

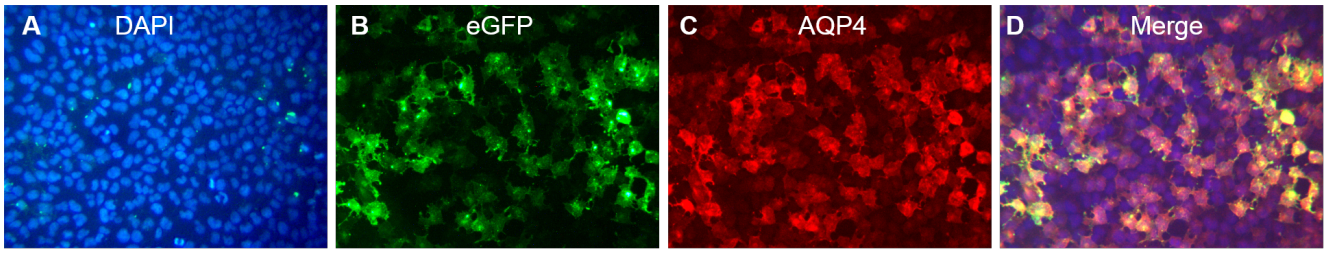

Figure 2. The expression of AQP4 protein in transfected cells was confirmed using an AQP4-igg antibody. A. DAPI staining of nuclei; B. eGFP was expressed in the cells; C. The antibodies in the sample bound to cellular antigens, followed by a secondary antibody labeled with fluorescence; D. A, B, and C were merged

3.3. Positive sample reaction results and their implications

Similarly, transfected cells exhibited green fluorescence in the blue excitation group (420–485nm). In the presence of an AQP4-IgG antibody-containing sample (positive sample), the antibody was bound to the AQP4 protein (antigen) expressed by the transfected cells, which was then bound by a Cy3-labeled anti-human IgG secondary antibody. Under the green excitation group (460–550nm) of the fluorescence microscope, red fluorescence was observed, which corresponded to the position of the green fluorescence. Conversely, in samples without AQP4-IgG antibodies (negative samples), only EGFP green fluorescence expressed by the cells was visible, devoid of any red fluorescence, as illustrated in **Figure 3**.

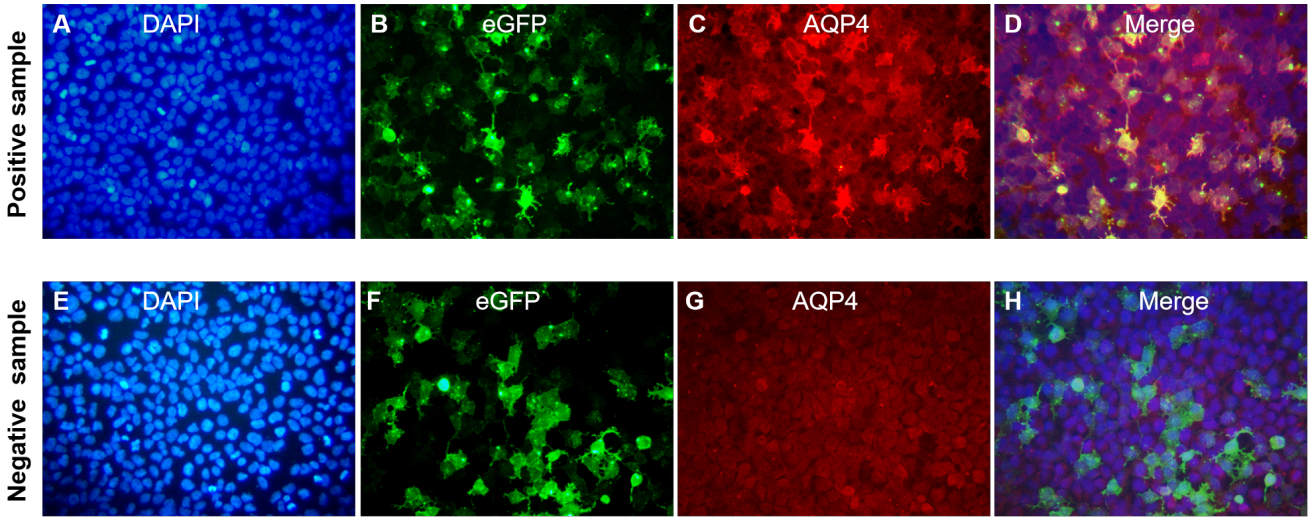

Figure 3. Fluorescence imaging of the positive and negative samples. A–D. The results of the positive samples under fluorescence microscope; A. DAPI staining of nuclei; B. eGFP tagged at the AQP4 C-terminus showing green fluorescence for the positive sample; C. The anti-AQP4 antibody in the positive samples binds to AQP4 on the cell surface, followed by binding to the Cy3-labeled human IgG, which shows red fluorescence; D. Images A, B, and C were merged. E–H. The results of the negative samples under fluorescence microscope; E. DAPI staining of nuclei; F. eGFP tagged at the AQP4 C-terminus showing green fluorescence for the negative sample; G. The negative samples with absent AQP4 antibodies fail to bind fluorescent secondary antibodies to the cell surface; H. Image E, F, and G were merged.

3.4. The proportion of positive samples among different age groups and genders

The total positive rate was 16.15% (362/2241), with 5.8% (57/2241) for males and 24.23% (305/2241) for females. The Chi-square test result was $x^2 = 138.22$, with a P-value < 0.05, indicating a significant difference between the groups. In other words, the incidence rate among females was notably higher than that among males. The mean age of onset was 50.44 ± 14.73, with 51.35 ± 16.82 for males and 50.28 ± 14.33 for females. The results are presented in **Table 2**.

Table 2. Positive rate statistics of the samples

Total sample size	Total positive rate (%)	Male positive rate (%)	Female positive rate (%)	x^2	*P*	Mean age of onset
2241	362 (16.15%)	57 (5.8%)	305 (24.23%)	138.221	$P < 0.05$ ($6.5195E^{-32}$)	50.44±14.73

3.5. Comparison with commercially available ELISA kits

Discrepancies were observed between the results obtained using the commercial kits (RSR Limited) and the CBA method for clinical samples (**Table 3**). Among the 15 groups of samples tested, four yielded differing results, and one serum sample was not detected by the commercial kits. Moreover, the results for the 3 cases of cerebrospinal fluid samples were incongruous with those obtained using the CBA method in the laboratory. To assess the concordance between the novel methodology employed in this study and the commercially available kits, the kappa test was utilized to meticulously analyze the agreement rate between the two approaches. **Table 4** provides the data about the concordance rate. The statistical analysis revealed a kappa value of 0.722, indicating a substantial level of agreement, with a P-value of 0.000, which is statistically significant.

Table 3. Test results of CBA and ELISA

Serum	Commercially ELISA kits cut off > 3.0	CBA method in the laboratory	CSF	Commercially ELISA kits cut off > 3.0	CBA method in the laboratory	Conclusion
NMO23476-1	0.30	1:10	NMO23476-2	3.13	1:1	Serum results showed inconsistencies
NMO23575-1	33.89	1:32	NMO23575-2	26.85	1:3.2	paired samples positive
NMO23582-1	20.50	1:32	NMO23582-2	2.64	1:1	CSF results showed inconsistencies
NMO23592-1	36.88	1:100	NMO23592-2	3.73	1:10	Paired samples positive
NMO23684-1	0.33	negative	NMO23684-2	2.67	negative	Paired samples negative
NMO23685-1	0.38	negative	NMO23685-2	1.22	negative	Paired samples negative
NMO23686-1	0.43	negative	NMO23686-2	2.37	negative	Paired samples negative
NMO23687-1	0.41	negative	NMO23687-2	1.43	negative	Paired samples negative
NMO23688-1	0.35	negative	NMO23688-2	2.27	negative	Negative
NMO22619-1	9.63	1:32	NMO22619-2	11.04	1:10	Paired samples positive
NMO22363-1	0.12	negative	NMO22363-2	3.25	negative	CSF results showed inconsistencies
NMO22366-1	0.15	negative	NMO22366-2	3.38	negative	CSF results showed inconsistencies
NMO22377-1	7.65	1:10	NMO22377-2	3.65	1:1	Paired samples positive
AQP22074-1	0.45	negative	AQP22074-2	2.94	negative	Paired samples negative
NMO23642-1	0.33	negative	NMO23642-2	2.59	negative	Paired samples negative

Table 4. Concordance statistics

Method 1 (CBA)	Method 2 (ELISA)		Total
	Positive	Negative	
Positive	10	2	12
Negative	2	16	18
Total	12	18	30

4. Discussion

Neuromyelitis optica (NMO) is an autoimmune central nervous system disease characterized by acute or subacute demyelination, that can affect the optic nerve and spinal cord, resulting in single or recurrent optic neuritis and transverse myelitis. Neuromyelitis optica and multiple sclerosis have several clinical manifestations. However, the principles of treatment differ significantly between the two conditions. As such, it is crucial to make an early and accurate diagnosis of neuromyelitis optica to ensure effective treatment for patients. In recent years, immunopathological research has demonstrated a significant association between antibodies against aquaporin-4 (AQP4) and the pathogenesis and development of neuromyelitis optica spectrum disorder (NMOSD). Detection of anti-AQP4 antibodies in patient serum and cerebrospinal fluid (CSF) may contribute to early diagnosis of NMOSD [3]. The 2021 version of the “Diagnosis and Treatment Guidelines for Neuromyelitis

Optica Spectrum Disorder" proposes that AQP4-IgG antibodies are highly specific diagnostic biomarkers, with approximately 70% to 80% of NMOSD patients expressing AQP4-IgG. In 1994, Jing et al. obtained aquaporin-4 (AQP4) by homologous cloning of the aquaporin family [4]. The protein is located on the cell membrane, and its monomer is composed of eight transmembrane structures (M1–M8) with five ring structures. Three ring structures (A, C, and E) are located outside the cell, and two ring structures (B, D), carboxyl, and amino termini are located inside the cell. Among them, loops B and E contain highly conserved asparagine-proline-alanine (NPA) repeat tandem sequences, and the two NPA loops return to and from the bilayer of the cell membrane to form a hydrophilic channel [5]. AQP4 is highly expressed in astrocytes throughout the central nervous system (CNS), with a particular abundance at the interface between the brain parenchyma, cerebral ventricle, and cerebrospinal fluid in the subarachnoid space [3]. The primary function of AQP4 facilitate water transport between the blood and brain as well as between the brain and cerebrospinal fluid (CSF) compartments. As a bidirectional channel protein, AQP4 can promote edema formation in cases of cytotoxic edema caused by ischemia or poisoning when the blood-brain barrier remains intact. Conversely, it can help alleviate excessive hydrocephalus in cases of vasogenic and interstitial edema when the blood-brain barrier is compromised [6]. Further studies have confirmed that the AQP4-IgG antibody is mainly of the IgG1 isotype [6]. The mechanism of action of AQP4-IgG antibody in patients with neuromyelitis optica involves antibody binds to the extracellular segment of the AQP4 protein on the surface of astrocyte foot processes in a nonlinear three-dimensional conformation, activating complement, and subsequently forming a membrane attack complex to damage astrocytes. Investigations have revealed that the expression of AQP4 in the optic nerve, spinal cord, and other brain tissues is significantly elevated compared to that in peripheral tissues and can aggregate to form larger-volume composite structures that are more easily recognizable by antibodies [7].

There are several methods for AQP4-IgG antibody detection: tissue-based indirect immunofluorescence assay (TBA), cell-based indirect immunofluorescence assay (CBA), and enzyme-linked immunosorbent assay (ELISA). Among them, TBA and CBA methods ensure the native conformation of the protein, but the operation is relatively complicated. In terms of positive detection rate, compared with the three methods, the CBA method had the highest positive detection rate and sensitivity. Although the ELISA method is more sensitive, the specificity is reduced. The guidelines do not recommend it as a detection method to establish the diagnosis, but it has a certain value in the longitudinal monitoring of antibody titers for the evaluation of disease progression and treatment [3]. Chinese scholar Li Min also found that when detecting AQP4-IgG antibodies in serum and cerebrospinal fluid samples of patients with neuromyelitis optica, the positive detection rate of CBA was 73.1% and 85.1%, respectively, followed by IIF, and the positive detection rate of ELISA was lower [8]. The CBA assay is the preferred method for AQP4-IgG antibody detection.

This study performed an indirect immunofluorescence assay based on cytology. The method involves expressing the AQP4 protein in eukaryotic cells through cell transfection, which not only preserves the native conformation of the protein but also enhances its expression level, thereby ensuring detection sensitivity. This approach demonstrated excellent performance in detecting clinical samples in our laboratory.

Detection of AQP4-IgG antibodies has emerged as a crucial reference indicator for the diagnosis, treatment, and prognosis of neuromyelitis optica spectrum disorder (NMOSD). Enhancing the sensitivity of AQP4-IgG antibody detection has attracted significant attention from researchers. This is particularly important during the early stages of the disease, as it holds great clinical application value in assisting physicians in making precise diagnoses and providing the best possible treatment opportunities for patients.

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Disclosure statement

The authors declare no conflict of interest.

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