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Clinical trial

Astrocytic damage in glial fibrillary acidic protein astrocytopathy during initial attack

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ABSTRACT

Objective: Determination of glial fibrillary acidic protein (GFAP), aquaporin 4 (AQP4), and myelin oligodendrocyte glycoprotein (MOG) levels in cerebrospinal fluid (CSF), and astrocytic damage analysis in patients with GFAP astrocytopathy (GFAP-A) and other conditions.

Methods: GFAP, AQP4, and MOG levels in CSF were detected via enzyme-linked immunosorbent assays. Anti-GFAP, anti-AQP4, and anti-MOG IgGs were detected via indirect immunofluorescence assays.

Results: In 32 GFAP-Astrocytopathy patients, CSF GFAP was significantly higher during acute exacerbation than it was in patients with MOG encephalomyelitis, multiple sclerosis, autoimmune encephalitis, and an "other inflammatory neurological disorders" group (all $p < 0.0001$). CSF GFAP levels were slightly higher in the GFAP-A group than in an anti-AQP4 IgG-positive neuromyelitis optica spectrum disorder group ($p = 0.012$). There were no significant differences between the CSF MOG and AQP4 levels in the GFAP-A group and those of other groups. CSF GFAP levels were significantly reduced after steroid treatment ($p = 0.011$). CSF GFAP levels differed significantly in GFAP-Astrocytopathy patients with and without encephalitis ($p = 0.016$). In GFAP-Astrocytopathy patients, CSF GFAP was correlated with Expanded Disability Status Scale (EDSS) score during attack ($r = 0.545$, $p = 0.001$). In follow-up examinations however, in GFAP-Astrocytopathy patients CSF GFAP level was not correlated with EDSS score 6 months later.

Conclusions: CSF GFAP level and pathological examination of GFAP-Astrocytopathy patients revealed astrocyte damage. CSF GFAP level was associated with steroid treatment at the acute stage, therefore CSF GFAP may be a sensitive biomarker with respect to the effects of therapy during the acute stage.

1. Introduction

Common autoimmune astrocytopathy of the central nervous system (CNS) includes neuromyelitis optica spectrum disorder (NMOSD) and autoimmune glial fibrillary acidic protein (GFAP) astrocytopathy (GFAP-A), which was first reported by Fang *et al.* (2016) in 2016 and is characterized by a biomarker of GFAP—an important intermediate filament protein expressed by astrocytes. Although both NMOSD and GFAP-A are identified as astrocytopathies, they may have different pathological characteristics. Prominent astrocytic damage induced by aquaporin 4 (AQP4) antibody is a typical pathological feature in NMOSD, and is associated with loss of AQP4 and GFAP, deposition of activated complement, inflammatory infiltration, and subsequent demyelination (Lucchinetti et al., 2014). Unlike the membranous expression of AQP4, GFAP is an intracellular skeleton protein expressed in astrocytes, and anti-GFAP IgG theoretically does not give rise to disease. In previously reported pathological studies however, there was striking astrocytic impairment in GFAP-A during the initial attack (Long et al., 2018), in conjunction with a loss of AQP4 and GFAP, which was similar to NMOSD. In contrast, in a study reported by Shu et al. (2018)

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Table 1
Cerebral spinal fluid GFAP, AQP4 and MOG level in a variety of neurological diseases.

Diagnoses	Median Age (y)	F/M	Median GFAP (ng/ml)	p1	Median AQP4 (ng/ml)	p2	Median MOG (pg/ml)	p3
GFAP-A (n = 32)	44(14–86)	19/13	6.87 (0–134.96)		0.34 (0.27–1.62)		104.88 (38.54–904.34)	
P-NMOSD (n = 71)	43(18–82)	67/4	2.40 (0–932.2)	0.012	0.35 (0.24–6.02)	0.075	129.08 (38.34–1517.52)	0.239
N-NMOSD (n = 11)	32(24–64)	9/2	2.54 (0–9.54)	0.124	0.36 (0.32–0.81)	0.215	114.24 (101.43–215.74)	0.344
MOG-EM (n = 35)	34(12–70)	23/12	1.92 (0–36)	<0.0001	0.34 (0.26–1.34)	0.979	181.31 (41.44–762.35)	0.148
MS (n = 40)	35(8–76)	20/20	1.69 (0–9.3)	<0.0001	0.35 (0.26–1.96)	0.316	182.16 (43.56–379.11)	0.066
AE (n = 26)	32(13–77)	16/10	2.29 (2–68.95)	<0.00001	0.32 (0.25–6.2)	0.082	177.44 (40.52–2571.57)	0.328
OIND (N = 195)	45(11–84)	91/104	1.55 (0–48.8)	<0.0001	0.34 (0.26–1.63)	0.418	180.87 (37.84–1720.75)	0.337

AQP4: aquaporin-4; AE: Autoimmune encephalitis F: female; M: male; GFAP: glial fibrillary acidic protein; MOG: myelin oligodendrocyte glycoprotein; MOG-EM: MOG encephalomyelitis; MS: Multiple sclerosis N−NMOSD: negative AQP4-IgG neuromyelitis optica spectrum disorder;; OIND: other inflammatory neurological disorders; P-NMOSD: positive AQP4-IgG neuromyelitis optica spectrum disorder.
p1: Comparison of CSF GFAP level in GFAP-A group and other disorders groups.
p2: Comparison of CSF AQP4 level in GFAP-A group and other disorders groups.
p3: Comparison of CSF GFAP level in GFAP-A group and other disorders groups.

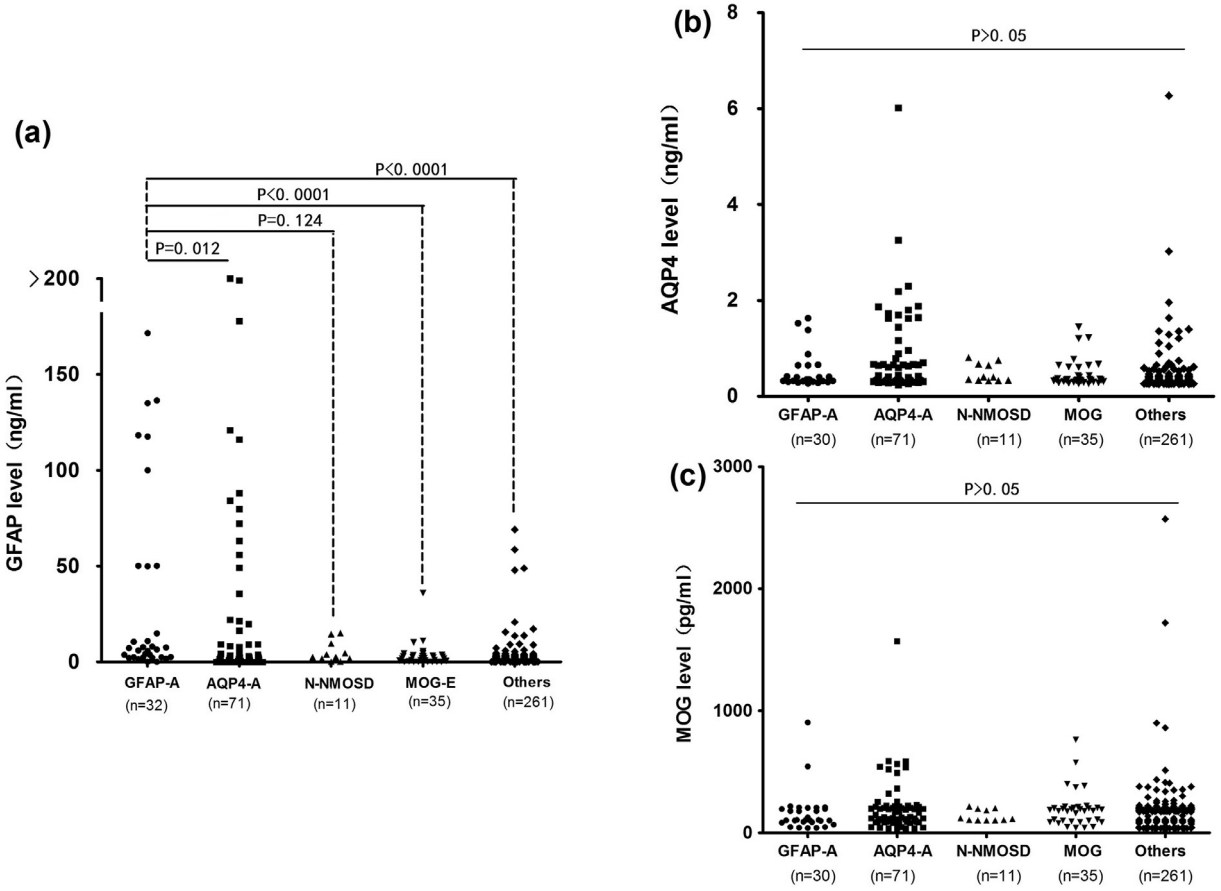

Fig. 1. CSF levels of glial fibrillary acidic protein (GFAP), aquaporin 4, and aquaporin 4 (AQP4), and myelin oligodendrocyte glycoprotein (MOG) in each patient group.
(a) Patients with GFAP astrocytopathy had significantly higher levels of CSF-GFAP than those with positive AQP4-IgG neuromyelitis optica spectrum disorders (NMOSD), MOG-IgG related encephalomyelitis and other neurologic disease ($p < 0.05$). (b) The CSF-AQP4 levels in GFAP astrocytopathy, NMOSD, MOG-IgG related encephalomyelitis, and other neurologic diseases were no significant difference ($p > 0.05$). (c)The CSF-MOG levels in GFAP astrocytopathy, NMOSD, MOG-IgG related encephalomyelitis, and other neurologic diseases were no significant difference ($p > 0.05$). GFAP-A: GFAP astrocytopathy; AQPA-A: AQP4 astrocytopathy; N−NMOSD: negative AQP4-IgG NMOSD; MOG-E: MOG-IgG related encephalomyelitis.

investigating GFAP-A, they did not refer to this aspect of neuropathology. Therefore, whether astrocytic damage or loss occurs in GFAP-A remains controversial.

The aims of the current study were to detect GFAP, AQP4, and myelin oligodendrocyte glycoprotein (MOG) levels in the cerebrospinal fluid (CSF) of patients with GFAP-A, NMOSD, and other conditions and analyze the pathologic value of CSF GFAP assessment with regard to astrocytic damage in GFAP-A.

2. Methods

2.1 Patients and samples

From August 2013 to August 2018, a total of 410 CSF samples were obtained from 32 patients with GFAP-A, 71 NMOSD patients who were positive for anti-AQP4 IgG (P-NMOSD), 11 NMOSD patients who were negative for anti-AQP4 IgG (N−NMOSD), 35 encephalomyelitis patients

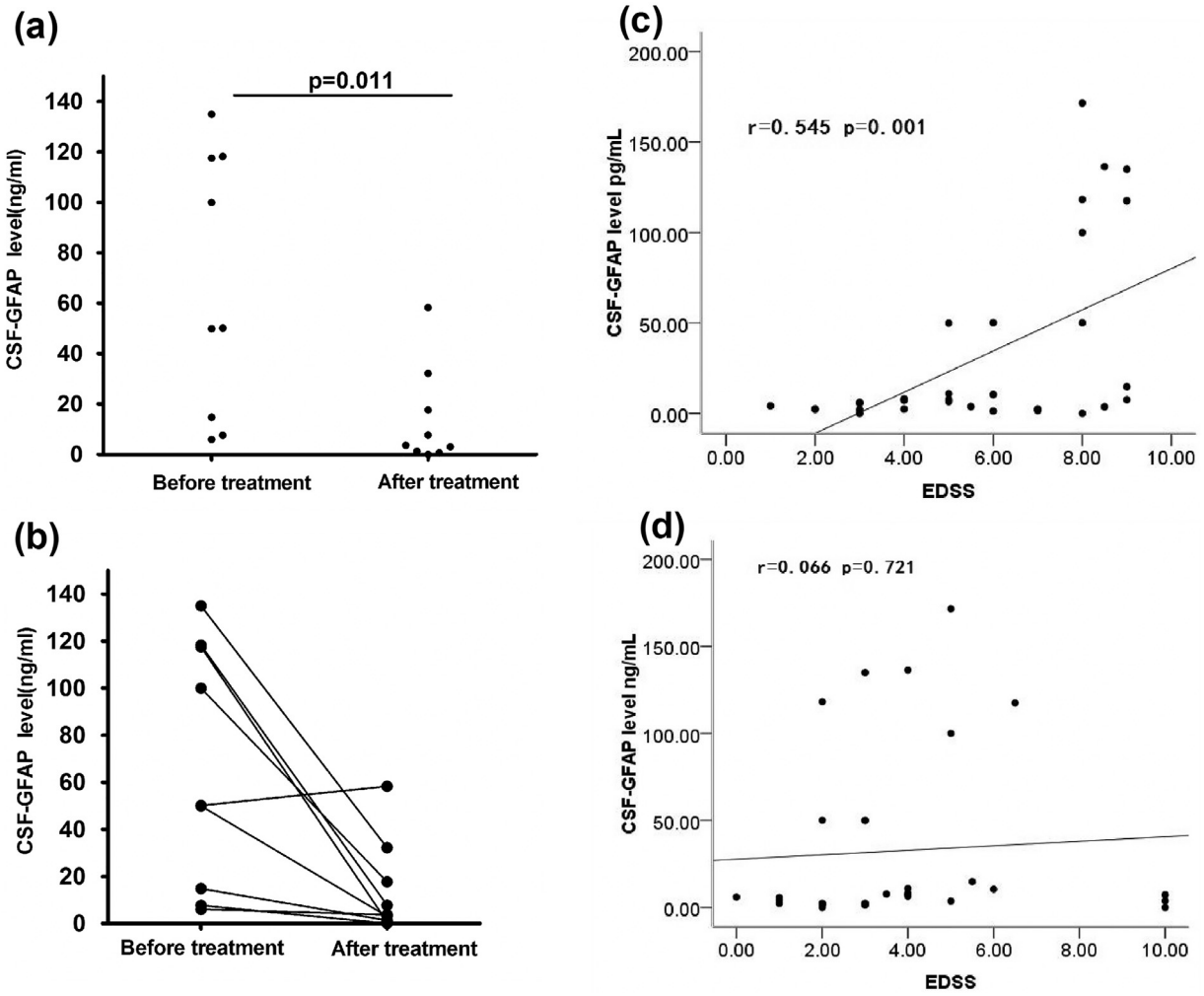

Fig. 2. CSF levels of glial fibrillary acidic protein (GFAP) in exacerbation and after intravenous methylprednisolone in 9 patients with GFAP astrocytopathy and the correlation between CSF-GFAP and expanded disability status scale during exacerbation and remission. (a)Patients with GFAP astrocytopathy had significantly decreased levels of CSF-GFAP after treatment ($p = 0.011$). (b) Almost all the samples had decreased CSF-GFAP after treatment. (c) The EDSS in exacerbation of 32 cases with GFAP astrocytopathy correlated with the CSF-GFAP ($p = 0.545$, $p = 001$). (d) However, there was no correlation between the CSF-GFAP levels and EDSS during remission.

who were positive for anti-MOG IgG, 40 multiple sclerosis patients, 26 patients with autoimmune encephalitis, and a group of 195 patients with "other inflammatory neurological disorders". All samples were tested for GFAP, AQP4, and MOG autoantibodies via an indirect immunofluorescence assay as previously described (Long et al., 2018). Patients with two or more of the above-mentioned antibody types in CSF were excluded. In 32 patients with GFAP-A, two patients experienced stereotactic brain biopsy before treatment and two patients undertaken that after treatment. We also performed repeated lumbar puncture in 15 patients with GFAP-A to obtain CSF samples after immunotherapy with high-dose intravenous methylprednisolone (IVMP), of which 9 patients were performed two rounds of testing for CSF-GFAP.

The study protocol was approved by the Ethics Committee of the Second Affiliated Hospital of Guangzhou Medical University, China, and all patients provided informed consent to participate in the study.

2.2 Measurement of GFAP, AQP4, and MOG in CSF

GFAP (NS830, Millipore, Billerica, MA, USA), AQP4 (AE55912HU, Abebio, Wuhan City, China), and MOG (AE33186HE, Abebio) concentrations were assessed using commercially available sandwich enzyme-linked immunosorbent assay (ELISA) kits in accordance with the manufacturers' instructions. All CSF samples were initially assessed using a 1:1 dilution. Any result greater than the maximum detection concentration may not be accurate, and samples yielding such results were diluted with assay buffer until results fell within an appropriate range. The detection assays used to determine the levels of the three proteins were similar, and can be summarized as follows: 1) prepare all reagents, samples, and standards in accordance with the manufacturer's instructions; 2) add 100 μl of standard or sample to each well, then incubate with agitation for 2 h at room temperature; 3) add 100 μl of prepared biotin antibody to each well, then incubate with agitation for 1 h at room temperature; 4) add 100 μl of horse radish peroxidase-streptavidin to each well, then incubate with agitation for 1 h at room temperature; 5) add 100 μl of TMB solution then incubate for 5–30 min while monitoring color development; 6) add 50 or 100 μl of Stop Solution then read absorbance at 450 nm immediately. The lower limits of sensitivity of the ELISAs were 1.5 ng/ml for GFAP, 0.1 ng/ml for AQP4, and 39 pg/ml for MOG.

2.3 Statistical analysis

All the results were statistically analyzed using Statistical Package for Social Sciences 16.0 software. Continuous data were compared using the nonparametric Mann-Whitney *U* test. In correlational

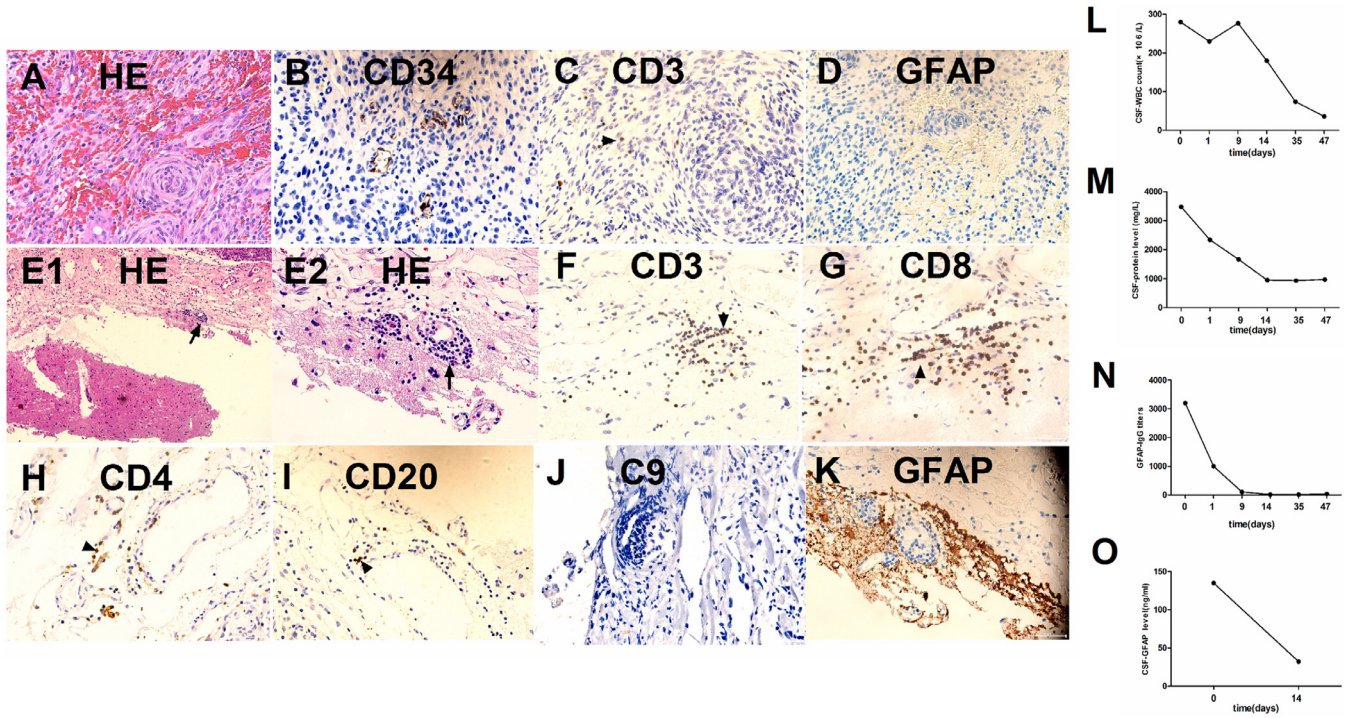

Fig. 3. A GFAP astrocytopathy patient with meningioma resection after treatment of long-term oral steroid and mycophenolate mofetil. A-K: Pathological features of meningioma and pia matter. A: hematoxylin and eosin staining showed meningioma with hemorrhage. B: Positive staining of CD34 could be found in vascular endothelial cell in neoplasm. C: Scattered $CD3^+$T cells in the neoplasm tissue. D: No positive GFAP expression in the neoplasm tissue.E1 and E2(a larger image of E1) showed perivascular inflammatory cell infiltration in pia matter. F: $CD3^+$T cells around the microvessel. T cells included $CD8^+$T cells(G) and $CD4^+$T cells(H). Few B cells with CD20 staining also could be seen(I), No C9 complement deposition in the inflammatory area(J) and relatively persevered GFAP around in pia matter(K). After treatment, decreased white blood cells counts (L), protein levels (M), GFAP-IgG titers (O), and GFAP protein levels (P) in CSF from exacerbation stage to remission.

analyses, Pearson's correlations were calculated with Bonferroni correction. Two-tailed p values < 0.05 were considered statistically significant.

3. Results

Demographic data and CSF GFAP, AQP4, and MOG levels in the different groups are shown in Table 1. In the 32 patients with GFAP-A the median age was 44 years (range 14–86 years), and 19 (59.4%) were female. The CSF GFAP levels in the GFAP-A group during acute exacerbation (0–135.0 ng/ml) were higher than the levels in the MOG encephalomyelitis (0–36.0 ng/ml, $p < 0.0001$), multiple sclerosis (0–9.3 ng/ml, $p < 0.0001$), autoimmune encephalitis (2.0–69.0 ng/ml, $p < 0.0001$) and "other inflammatory neurological disorders" (2.0–68.9 5.0 ng/ml, $p < 0.0001$) groups. CSF GFAP was a little higher in the GFAP-A group than in the anti-AQP4 IgG NMOSD-positive (P-NMOSD) group ($p = 0.012$), but there was no significant difference between the GFAP-A group and the anti-AQP4 IgG NMOSD-negative (N−NMOSD) group ($p = 0.124$). There were no significant differences in CSF MOG or AQP4 levels between the GFAP-A group and other groups (Table 1 and Fig. 1).

To ascertain whether intravenous methylprednisolone (IVMP) affects CSF GFAP level in the active stage, GFAP was assessed before and after one or two courses of IVMP in 9 patients who had never received any therapy prior to IVMP. Paired CSF samples were obtained shortly before IVMP and approximately 1 day to 90 days after IVMP. There was a significant decrease after IVMP treatment (66.57 ± 51.74 ng/l vs. 13.85 ± 19.80 ng/l; $p = 0.011$). With the exception of one child in whom there was an increase, GFAP levels decreased markedly after IVMP in all cases, to near the control levels (Fig. 2). Pathological examination revealed that two patients without treatment had marked brain inflammatory response and GFAP loss, accompanying with increased CSF-GFAP . However, although typical meningitis and encephalitis could be observed in the two patients with treatment, GFAP normally expressed in the brain and decreased GFAP levels in CSF could be detected (Fig. 3).

To ascertain whether CSF GFAP levels differed in patients with and without encephalitis, 32 patients with anti-GFAP IgG were further analyzed. First, all 32 patients were divided into those with encephalitis ($n = 21$) and without encephalitis ($n = 11$). CSF GFAP levels differed significantly in the two groups ($p = 0.016$). CSF GFAP levels in patients with GFAP-A were then compared with those of patients with autoimmune encephalitis, and GFAP-A patients also had higher GFAP levels than those patients. There were no significant differences in CSF AQP4 or CSF MOG levels between these groups.

To ascertain whether CSF GFAP levels differed in patients with and without myelitis, 32 patients were divided into those with myelitis ($n = 14$) and those without myelitis ($n = 18$). There were no significant differences in CSF GFAP ($p = 0.927$), CSF AQP4 ($p = 0.372$), or CSF MOG ($p = 0.174$) between these two groups.

In patients with GFAP-A, CSF-GFAP was correlated with Expanded Disability Status Scale (EDSS) score during attack ($r = 0.545$, $p = 0.001$). In follow-up studies of EDSS in the GFAP-A group however, CSF GFAP levels were not correlated with EDSS score at 6 months.

4. Discussion

Anti-GFAP IgG-related disorders and anti-AQP4 IgG-related disorders both belong to the astrocytopathy spectrum because they both involve specific IgGs targeting astrocytes and they both involve the brain, spinal cord, and optic nerve. However, many clinical differences have been demonstrated in previous studies (Sechi et al., 2018; Yang et al., 2018). Compared with AQP4 astrocytopathy, GFAP-A is more prevalent in males, and it is more likely to involve fever, headache,

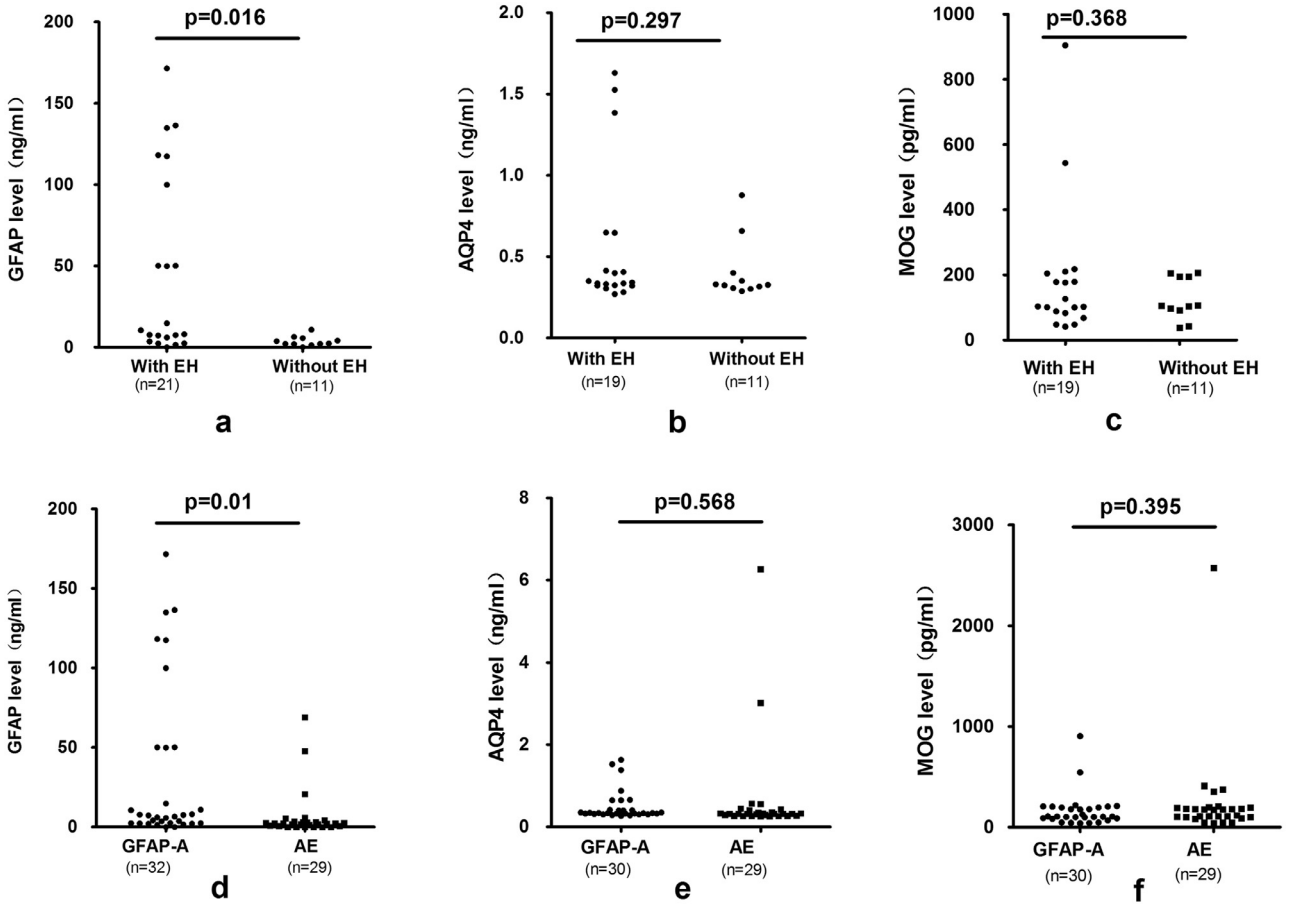

Fig. 4. CSF levels of glial fibrillary acidic protein (GFAP), aquaporin 4, and aquaporin 4 (AQP4), and myelin oligodendrocyte glycoprotein (MOG) in different subgroup a: GFAP astrocytopathy patients with encephalopathy(EH) had significantly higher levels of CSF-GFAP than those without EH ($p = 0.016$).The CSF-AQP4 (b) levels and MOG(b) levels were no significant difference between GFAP astrocytopathy with EH and without EH ($p > 0.05$).d: GFAP astrocytopathy patients had significantly higher levels of CSF-GFAP than autoimmune encephalitis patients ($p = 0.01$). The CSF-AQP4 (e) levels and MOG(f) levels were no significant difference between GFAP astrocytopathy and autoimmune encephalitis patients ($p > 0.05$).

psychosis, neuronal antibody, an abnormal CSF white blood cell count, a higher CSF protein level, a more “radial pattern” of abnormality with enhancement, and meningeal abnormality, suggesting that the two conditions may involve different immune mechanisms.

Previous studies suggest that anti-AQP4 IgG has a critical role in NMOSD, and that it may be involved in the pathomechanism of NMOSD and astrocyte dysfunction or damage (Hinson et al., 2007). It has been demonstrated that anti-AQP4 IgG from patient serum binds to extracellular domains of AQP4 with activated complement, and it reportedly induces endocytosis and degradation of AQP4 in vitro and in animal studies *in vivo* (Hinson et al., 2007; Herwerth et al., 2016; Hinson *et al.*, 2017). AQP4 antigen and astrocyte loss in brain lesions is an early feature of NMOSD(). Furthermore, CSF GFAP levels were reportedly highly elevated during the acute phase of NMOSD compared with those of patients with control disorders, and soon after corticosteroid therapy clinical improvement ensued and CSF GFAP levels returned to nearly normal levels (Takano et al., 2008; Misu et al., 2009; Takano et al., 2010; Uzawa et al., 2013).

GFAP-A pathology can reportedly involve periangitis, gliosis, and T and B cell infiltration, despite intact blood vessels in the brain parenchyma (Caselli et al., 1999; Flanagan et al., 2017; Long et al., 2018). Whether astrocyte damage was involved was not clear in previous studies. In 5 Chinese patients with GFAP-A who underwent pathological examination via stereotactic biopsy, astrocyte loss was heterogeneous (Long et al., 2018; Shu et al., 2018). It may be that (1) astrocyte injury is a secondary phenomenon indicating some GFAP-A without astrocyte loss because of mild inflammation; (2) owing to the limitations of stereotactic biopsy, it could not reflect all pathological features; (3) there was remission after the treatment because GFAP-A is associated with a good response to steroid therapy. Therefore, in the present study the use of CSF GFAP detection was considered essential when seeking evidence of astrocyte injury in GFAP-A.

Several facts were confirmed in the present study. First, similar to NMOSD, CSF GFAP levels were highly elevated during the acute phase of GFAP-A compared with those in patients with anti-MOG IgG-related encephalomyelitis, multiple sclerosis, autoimmune encephalitis, and other inflammatory neurological disorders. Therefore, the results support previous pathological examination-based reports of GFAP loss in GFAP-A. Possibly due to the very small number of patients in the N−NMOSD group, there was no significant difference between N−NMOSD and GFAP-A patients as determined via nonparametric tests. Second, the study suggests that CSF GFAP level may be markedly affected by steroid treatment, which is concordant with the clinical observation that patients’ symptoms responded well to steroid therapy. It may also explain why some patients only exhibited slight loss of GFAP or localized normal expression in pathological examinations, i.e., because they received steroid treatment prior to biopsy. Notably, a typical patient with a history of long-term oral steroid and mycophenolate mofetil use exhibited very stable status, low anti-GFAP IgG titers, and low CSF GFAP levels, we detected marked inflammation in pia mater but relatively preserved GFAP around the meninges (Fig. 3).

AQP4 astrocytopathy was induced by anti-AQP4 IgG, resulting in humoral immunity around the lesion (Lucchinetti et al., 2014). There were pronounced losses of AQP4 and GFAP within the lesions,

accompanying pronounced perivascular deposition of immunoglobulins and complement in active lesions (Lucchinetti et al., 2014). At present however, data pertaining to GFAP-A is limited and little is known about its involvement in the pathogenesis of astrocyte damage. Because GFAP is an intracellular protein, the antigen is not expressed outside the cell and is thus not modulated or down-regulated on the cell surface, as is the case for AQP4 in NMOSD. Although CD138-rich cells secreting IgG in lesions have previously been reported, there was no obvious deposition of immunoglobulins and complement in biopsy cases. Therefore, there was apparently an absence of a humoral immunity response in GFAP-A, indicating that anti-GFAP IgG may be a bystander. How astrocyte damage occurs in GFAP-A remains unclear.

Neoplasm and viral infection may be associated with GFAP-A (Flanagan et al., 2017; Iorio et al., 2018; Long et al., 2018). In a report from the Mayo clinic describing an ovarian teratoma from a patient with GFAP-A, the tumor tissue expressed GFAP. This indicates that ectopic expression of nerve tissue can contribute to triggering an immune response. It has also been previously reported that GFAP-derived peptides can induce cytotoxic T cell attack in inflamed meningeal astrocytes via ambient interferon γ (Sasaki *et al.*, 2014; Fang *et al.*,2016). Rats injected with rat cerebral homogenate exhibited marked CD3-positive T cell responses around the lesions (Park et al., 2014). In the present pathological examination, CD4-positive and CD8-positive T cells were detected around the inflamed meningeal area and brain. Therefore, T cell immune responses may be the main cause of astrocyte damage. However, no staining of GFAP could be certified in neoplasm tissue in the case with meningioma. Thus the relationship between neoplasm and GFAP-A is unclear and requires further study.

In conclusion, though it has been suggested that anti-GFAP antibody does not induce disease in the manner that anti-AQP4 IgG does, pathological examination of patients with GFAP-A revealed astrocyte damage and GFAP in CSF in the present study. CSF GFAP level was associated with IVMP treatment at the acute stage; therefore, CSF-GFAP may be a sensitive biomarker that is indicative of the effects of therapy at the acute stage. The main mechanism involved in astrocyte injury remains unknown, but it may be a result of a T cell immune response.

Potential conflicts of interest

The authors have declared that no competing interests exist.

Acknowledgments

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