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Interleukin-36 alpha levels are elevated in the serum and cerebrospinal fluid of patients with neuromyelitis optica spectrum disorder and correlate with disease activity

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ABSTRACT

Neuromyelitis optica spectrum disorder (NMOSD) is an autoimmune inflammatory neurological disease characterized by longitudinally extensive transverse myelitis (LETM) and optic neuritis. Interleukin (IL)-36 is a novel cytokine of the IL-1 family that is involved in the development of inflammatory diseases. The aim of this study was to investigate the role of IL-36α in NMOSD. We retrospectively collected 73 patients, who fulfilled the 2015 criteria for NMOSD diagnosis and were admitted to the Department of Neurology of the First Hospital of Jilin University from 2015 to 2016. Fifty age and gender matched patients with non-inflammatory neurological disorders (ONNDs) were collected in the same period and served as controls. Neurological function was evaluated by the expanded disability status scale (EDSS). All participants were assessed for the annual relapse rate (ARR). Blood and cerebrospinal fluid (CSF) samples were obtained and the levels of IL-36α in the serum and CSF were analyzed by enzyme-linked immunosorbent assay (ELISA). IL-36α levels in serum and CSF were found to be significantly increased in patients with NMOSD compared to those in the controls. Furthermore, IL-36α levels in both serum and CSF were positively correlated with the EDSS score. CSF IL-36α levels were positively correlated with CSF leukocyte counts, protein concentration and immunoglobulin IgG. Our results suggest that IL-36α may be a novel biomarker for monitoring disease severity in NMOSD.

1. Introduction

Neuromyelitis optica spectrum disorder (NMOSD) is an autoimmune neuro-inflammatory disease characterized by longitudinally extensive transverse myelitis (LETM) and optic neuritis (Jasiak-Zatonska et al., 2016; Wingerchuk et al., 2015; Kleiter and Gold, 2016). The discovery of the disease-specific NMO-immunoglobulin G (NMO-IgG) and its effects on astrocytic aquaporin-4 (AQP4) water channel protein has revolutionized our understanding of NMOSD (Lennon et al., 2004; Papadopoulos and Verkman, 2012).

It has been reported that levels of inflammatory cytokines such as interleukin (IL)-6, IL-17, and IL-35 are elevated in NMOSD patients (Matsushita et al., 2013; Zhang et al., 2016), suggesting the involvement of inflammatory cytokines in the pathogenesis of NMOSD. IL-36 is a novel inflammatory cytokine that belongs to the IL-1 family and plays an essential role in both acute and chronic inflammatory processes. Three different forms of IL-36 have been identified so far: IL-36α (IL-1F6), β (IL-1F8) and γ (IL-1F9) (Busfield et al., 2000; Kumar et al., 2000). IL-36α is a 17 kDa protein expressed mainly in monocytes, T and B lymphocytes as well as keratinocytes. Moreover, IL-36α levels were reported to be elevated in autoimmune diseases such as psoriasis, rheumatoid arthritis (RA) and primary Sjögren's syndrome, and thus, it may play a pro-inflammatory role in these diseases (Blumberg et al., 2007; Frey et al., 2013; Ciccia et al., 2015). In addition, IL-36α was able

Abbreviations: NMOSD, Neuromyelitis optica spectrum disorder; LETM, Longitudinally extensive transverse myelitis; IL, Interleukin; ONNDs, Non-inflammatory neurological disorders; EDSS, Expanded disability status scale; ARR, Annual relapse rate; CSF, Cerebrospinal fluid; ELISA, Enzyme-linked immunosorbent assay; IL, Interleukin; NMO-IgG, NMO-immunoglobulin G; AQP4, Aquaporin-4; RA, Rheumatoid arthritis; OCB, Oligoclonal band; MOG-Ab, Myelin oligodendrocyte glycoprotein antibody; NF-κB, Nuclear factor kappa B; MAPK, Mitogen-activated protein kinase; PBMC, Peripheral blood mononuclear cells; Th, T helper; DC, Dendritic cell; BMDC, Bone marrow-derived DC; IFN, Interferon; PBS, Phosphate-buffered saline

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to induce IL-6 and IL-8 production in synovial fibroblasts. Nevertheless, the role of IL-36α in NMOSD has not been reported. In the present study, we aimed to investigate the role of the IL-36α in NMOSD.

2. Materials and methods

2.1. Patient information

Seventy-three patients who fulfilled the 2015 diagnostic criteria for NMOSD were recruited from January 2015 to August 2016 in the Department of Neurology and Neuroscience Center of the First Hospital of Jilin University. Meanwhile, fifty age and gender matched patients with ONNDs were collected in the same period and served as controls. The ONNDs refer to diseases such as tension type headache, subacute combined degeneration of the spinal cord, vascular diseases of the spinal cord, motor neuron disease and Wernicke–Korsakoff syndrome. All the study procedures were approved by the ethics committee of the Neurology and Neuroscience Center, the First Hospital of Jilin University, and the informed consent forms were signed by all participants.

2.2. Sample collection

All the blood and CSF samples were obtained before immunosuppressive medication and high-dose intravenous methylprednisolone during acute exacerbations. The blood samples were collected into a 5 ml tube without adding anticoagulant. Afterwards, the samples were centrifuged using 3000 rpm at 4 °C for 5 min. And then, the supernatant was collected into a new tube, aliquoted and stored at −80 °C until use. The CSF samples were collected, aliquoted and stored at −80 °C until use.

2.3. Detection of anti-AQP4-Ab

All samples were examined for AQP4-IgG using a commercial cell-based assay (Euroimmun, Lübeck, Germany) as previously reported (Fig. 1A-B) (Jarius et al., 2010). Briefly, HEK 293 cells transfected with AQP4 M1 were seeded on the slides, and patient serum (30 µl) was applied to the slides and incubated for 30 min at room temperature. The slides were washed with 1x phosphate-buffered saline (PBS)-Tween 20 for 5 min. After that, 25 µl fluorescently labeled anti-human globulin was added to each reaction field and incubated for 30 min at room temperature. The slides were washed for 5 min with 1x PBS-Tween 20. The slides were mounted onto cover glasses using embedding medium after they had been dried. The fluorescence was evaluated under the microscope.

Table 1
Clinical features of NMOSD and ONNDs groups.

Clinical features	NMOSD (73)	ONNDs (50)
Age (years)	51	52
Gender (F:M)	59:14	40:10
Disease duration at sampling (years)	1	–
EDSS score	3.5	–
ARR	1	–
Leukocyte counts in CSF	10	–
Protein concentration in CSF	0.48	–
Immunoglobulin IgG in CSF	47.45	–
AQP4-Ab positive (n)	68	–
MOG-Ab positive (n)	1	–
OCB positive (n)	13	–
AQP4-Ab and OCB positive (n)	12	–
AQP4-Ab and MOG-Ab positive (n)	1	–
spinal cord lesion length (< 3)	14	–
spinal cord lesion length (≥ 3)	57	–

Data are expressed as median. EDSS: Expanded disability status scale. Standard CSF cell count is 0–8 × 10^6/L; Protein is 0.15-0.45 g/L; Immunoglobulin IgG is 0–34 mg/L.

2.4. Myelin oligodendrocyte glycoprotein antibody (MOG-Ab) assays

MOG-Ab were tested by a commercial cell-based assay (Euroimmun, Lübeck, Germany). Full-length human MOG M1 transfected HEK 293 cells were used in the anti-MOG immune-fluorescence test.

2.5. Measurement of IL-36α levels

The levels of IL-36α in serum and CSF were analyzed using a commercially available enzyme-linked immunosorbent assay (ELISA) (Cusabio Biotech, Wuhan, China) according to the manufacturer's instructions.

2.6. Disease severity assessment

The symptoms of all patients were evaluated according to the clinical manifestation. The EDSS scores were evaluated separately by two neurologists who had passed the Neurostatus e-Test and were qualified to perform EDSS assessments. ARR was assessed according to the medical records and the follow-up information.

2.7. Statistical analysis

The normality test was analyzed using Shapiro-Wilk test. Mann-Whitney U tests were used to compare the differences between groups. The correlations between IL-36α levels and clinical parameters were

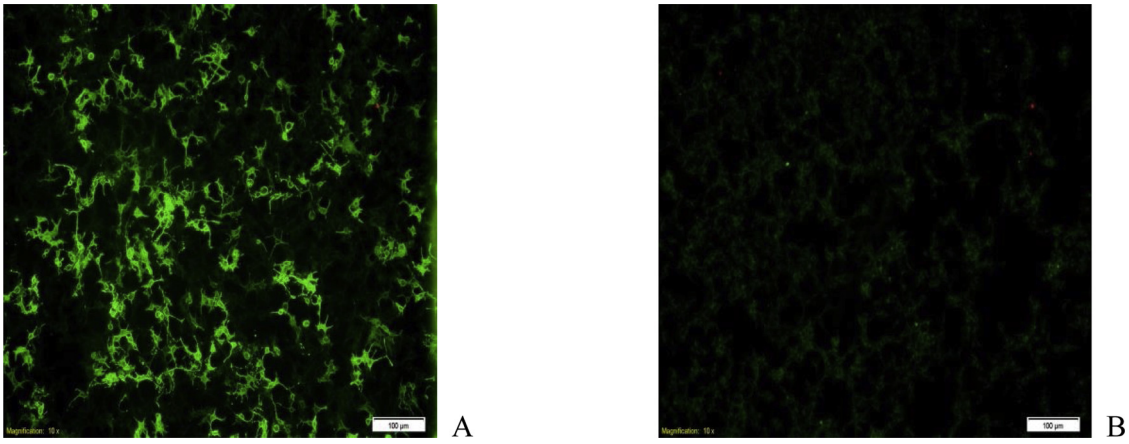

Fig. 1. (A) AQP4-Ab positive fluorescence images. (B) AQP4-Ab negative fluorescence images.

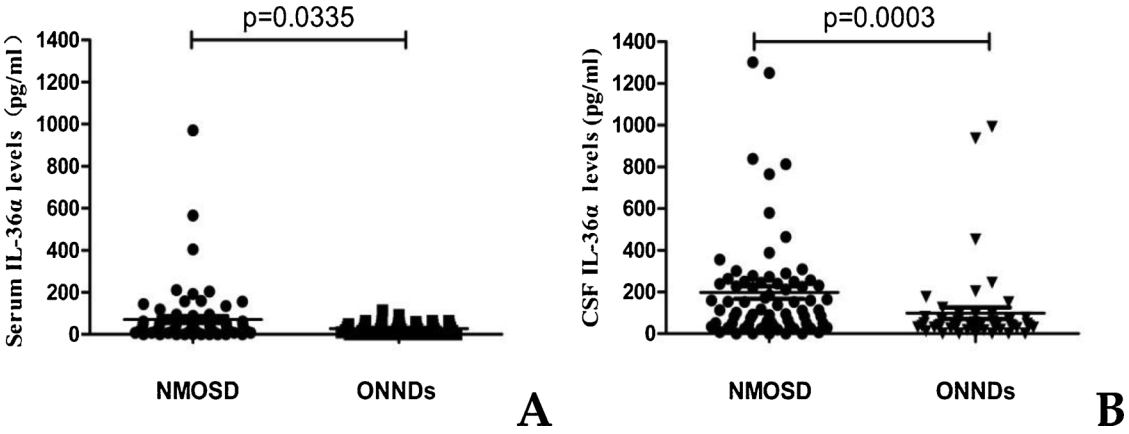

Fig. 2. Serum and CSF IL-36α levels in NMOSD patients and the controls with ONNDs.

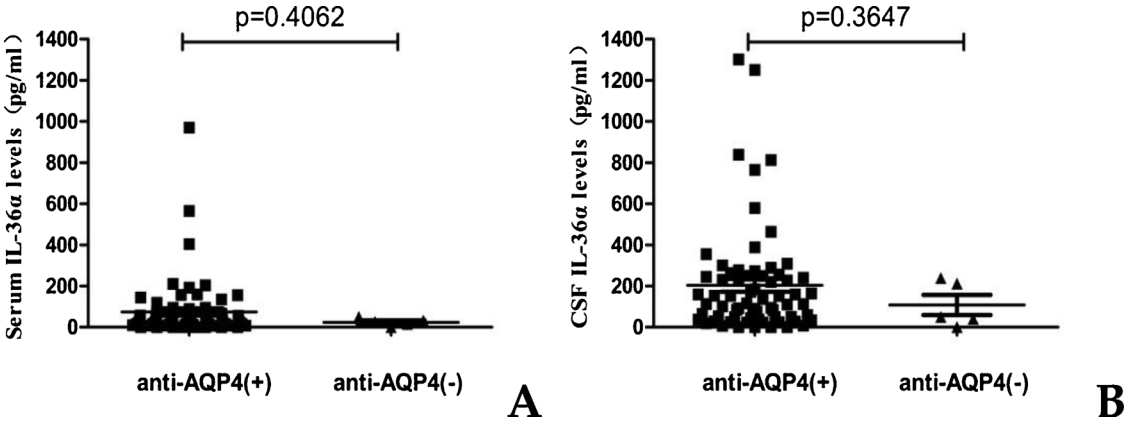

Fig. 3. Serum and CSF IL-36α levels in the AQP4-Ab positive NMOSD group and the AQP4-Ab negative NMOSD group.

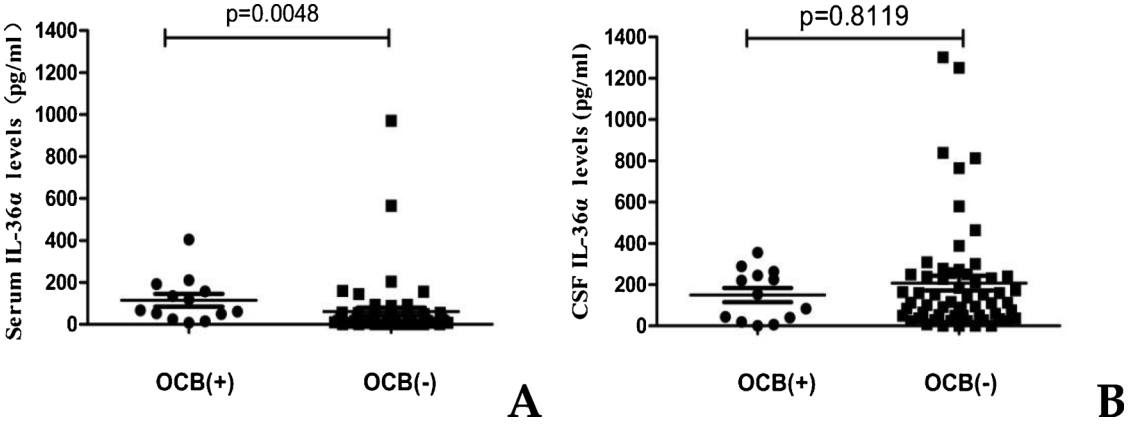

Fig. 4. Serum and CSF IL-36α levels in the OCB positive NMOSD group and the OCB negative NMOSD group.

analyzed using the non-parametric Spearman's *rho* test. The data were analyzed using SPSS Statistics 20.0 software (SPSS, Inc., Chicago, IL, USA). A p-value less than 0.05 was considered statistically significant.

3. Results

3.1. Clinical features of patients with NMOSD

A total of 73 NMOSD patients were enrolled in this study, of which 14 were male and 59 were female (1:4.2), and the median age at onset was 51 years (Table 1). The median disease duration at sampling was 1 years. The median expanded disability status scale (EDSS) score of NMOSD patients was 3.5. The median annual relapse rate (ARR) was 1. The median CSF leukocyte counts, protein concentration and immunoglobulin IgG in NMOSD patients was 10×10^6/L, 0.48 g/L and 47.45 mg/L respectively. Among the 73 patients, 68 were found aquaporin-4 antibody (AQP4-Ab) positive (93.1%), 13 were oligoclonal bands (OCB) positive (17.8%), only 1 was myelin oligodendrocyte glycoprotein antibody (MOG-Ab) positive (1.4%), only 1 was AQP4-Ab and MOG-Ab double positive (1.4%), and 5 were AQP4-Ab and MOG-Ab double negative (6.8%). Among the 73 patients, 71 were suffered from spinal cord lesions (97.2%), which we divided into two groups: < 3 spinal cord segments (19.7%) and ≥3 spinal cord segments (80.3%). There were no gender and age differences between NMOSD patients

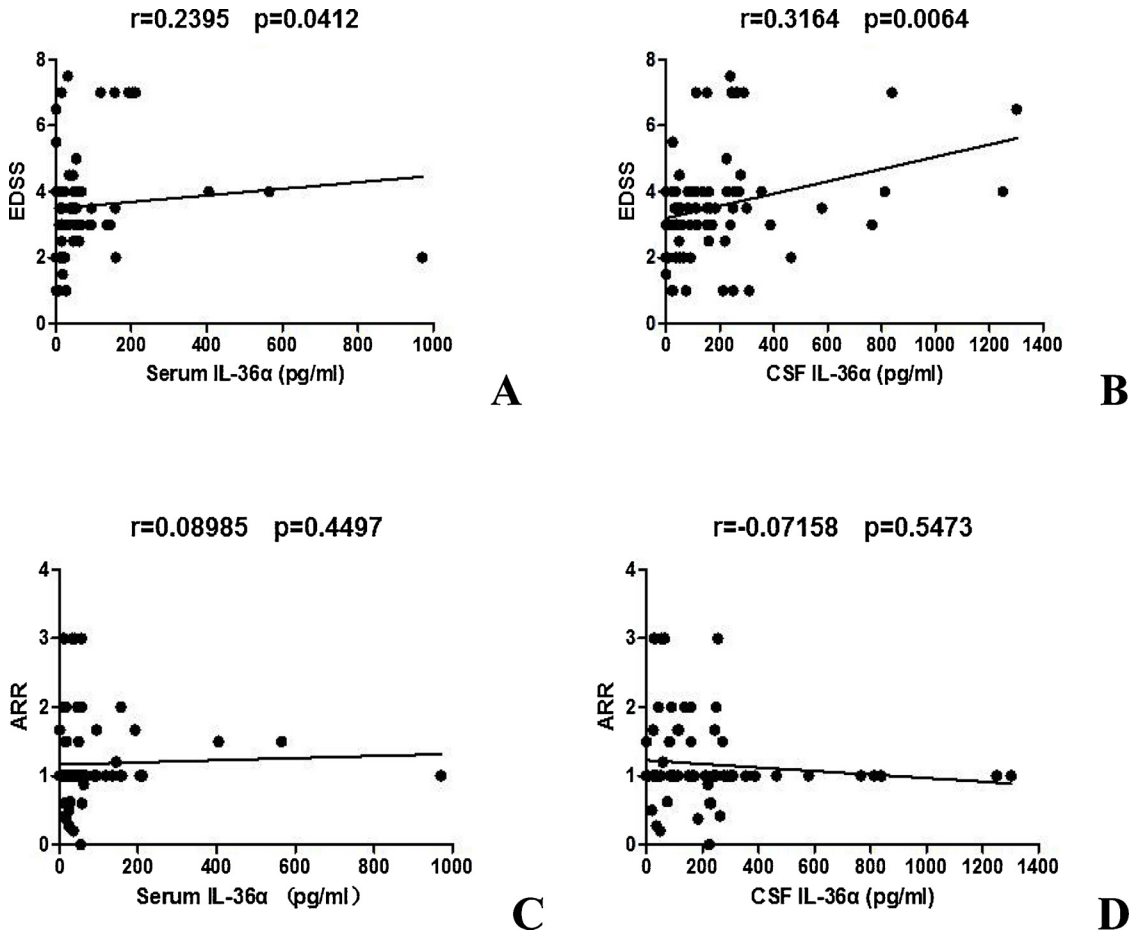

Fig. 5. Correlation between serum and CSF IL-36α levels and EDSS score and ARR, respectively, in patients with NMOSD. (A) Correlation between serum IL-36α levels and EDSS score in patients with NMOSD. (B) Correlation between CSF IL-36α levels and EDSS score in patients with NMOSD. (C) Correlation between serum IL-36α levels and ARR in patients with NMOSD. (D) Correlation between CSF IL-36α levels and ARR in patients with NMOSD.

and the controls with ONNDs (data not shown).

3.2. Increased IL-36α in serum and CSF in NMOSD

Our results showed that the levels of IL-36α in both serum and CSF were significantly increased in patients with NMOSD compared to those in the controls with ONNDs (Fig. 2). However, no statistical differences regarding the levels of IL-36α in serum and CSF were found between the AQP4-Ab positive NMOSD group and the AQP4-Ab negative NMOSD group (Fig. 3). Moreover, the serum levels of IL-36α were found significantly increased in the OCB positive NMOSD group than in the OCB negative NMOSD group (Fig. 4). While, the CSF levels of IL-36α showed no statistical differences between the OCB + NMOSD group and the OCB- NMOSD group.

3.3. Correlation between levels of IL-36α and clinical features

Our results showed that the levels of IL-36α in both serum and CSF were positively correlated with EDSS score (Fig. 5A-B). However, no significant correlation was observed between the levels of IL-36α in serum and CSF and the ARR (Fig. 5C-D). Moreover, Correlation between CSF leukocyte counts, protein concentration, immunoglobulin IgG and affected total spinal cord lesion length and CSF IL-36α levels were significant ($p < 0.05$, $p < 0.01$, $p < 0.01$ and $p < 0.005$ respectively). However, no significant differences between CSF leukocyte counts, protein concentration and immunoglobulin IgG and serum IL-36α levels. In addition, none of age, gender and disease duration were significantly correlated with the serum and CSF levels of IL-36α in NMOSD patients.

4. Discussion

In this study, we demonstrated for the first time that serum and CSF levels of IL-36α were significantly higher in NMOSD patients than in patients with ONNDs and correlated with the disease severity, indicating that IL-36α may play an inflammatory role and may be involved in NMOSD pathogenesis.

IL-36α is a 17 kDa protein belongs to the IL-1 cytokine family and expressed mainly in monocytes, T and B lymphocytes as well as keratinocytes. IL-36α has been previously proved to exert pro-inflammatory effects via nuclear factor kappa B (NF-κB) and mitogen-activated protein kinase (MAPK) signaling pathway and contributes to the pathogenesis of psoriasis, RA and primary Sjögren's syndrome (Blumberg et al., 2007; Frey et al., 2013; Ciccia et al., 2015). However, the role of IL-36α in NMOSD has not been reported. In our study, the levels of IL-36α were significantly increased in the serum and CSF of NMOSD patients, suggesting a potential role of IL-36α in disease pathogenesis. The similarity of the cytokines milieu in the CSF of NMOSD patients and synovial fluid of RA patients indicates a similar inflammatory cascade occurs. Moreover, IL-36α may enhance the effect of IL-17 A and TNF-α locally, thus contributing to the pathogenesis of psoriasis (Carrier et al., 2011). In addition, IL-36α can stimulate peripheral blood mononuclear cells (PBMC) to produce pro-inflammatory molecules such as IL-6 and CXCL8 in SLE patients. NMOSD is recognized as a B cell-mediated humoral immune disease. However, accumulating evidence suggests that T helper (Th) 17 cell-mediated immunity may be involved in the

pathogenesis of NMOSD (Barros et al., 2016; Li et al., 2011; Axtell et al., 2011). Previous reports have been showed that CSF levels of IL-6 and IL-17 A, which prefer B-cell activation, survival, and AQP4-IgG production, are elevated in NMOSD patients. Accordingly, we speculate that IL-36α may act via similar mechanisms by triggering the production of pro-inflammatory cytokines in NMOSD.

DCs play an important role in inducing Th cell differentiation (Liu et al., 2001). Vigne et al (Vigne et al., 2011) showed that both murine bone marrow-derived DCs (BMDC) and CD4 + T lymphocytes can express IL-36 receptor (IL-36R) and respond to IL-36α. Upon binding to its receptor, IL-36α can activate the NF-κB and MAPK signaling pathway and induce the production of pro-inflammatory cytokines such as IL-6, TNF-α and IL-23 by BMDC and interferon (IFN)-γ, IL-4, and IL-17 by CD4 + T cells (Towne et al., 2004). Therefore, the increased IL-36α may lead to persistent inflammation by influence the immune cells in NMOSD.

The degree of disease severity of NMOSD was positively correlated with EDSS score, CSF leukocyte counts, protein concentration and immunoglobulin IgG. Correlation between EDSS score and serum IL-36α level is barely significant ($p = 0.04$) considering the presence of several outliers. Actually, correlation between EDSS score and serum IL-36α level is still statistically significant after omitting outliers data ($p = 0.019$). However, IL-36α does not correlate with the ARR, probably due to the relatively small sample size used in our study. Accordingly, IL-36α may be a novel biomarker for monitoring the severity of NMOSD.

The anti-IL-6 receptor antibody drug tocilizumab has been reported to have a protective effect against NMOSD (Araki et al., 2014; Ringelstein et al., 2015), indicating that blocking pro-inflammatory cytokine signaling may be beneficial for NMOSD patients. Therefore, it can be speculated that IL-36α alone or together with other pro-inflammatory cytokines molecules may represent a novel therapeutic target for treating NMOSD.

In conclusion, Our results showed that the levels of IL-36α in both serum and CSF were positively correlated with EDSS score (Fig. 2A-B). Our data suggest that IL-36α-mediated inflammation may be involved in NMOSD, and IL-36α may be a novel biomarker for monitoring the severity of NMOSD. More studies are required to define the role of IL-36α in the pathogenesis and progression of NMOSD.

Conflicts of interest

The authors report no conflicts of interest regarding the publication of this paper.

Author contributions

Experimental design: Tao Jin and Jie Zhu; Writing the manuscript and Implementation the experiment: Yangyang Song and Mingqin Zhu; Data analysis: Caiyun Liu, Chao Zheng and Yang Zhou.

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