Identification of double-stranded DNA in the cerebrospinal fluid of patients with acute neuromyelitis optica spectrum disorder

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Abstract

Neuromyelitis optica spectrum disorder (NMOSD) is an inflammatory disease of the central nervous system (CNS) that is characterized by severe myelitis and optic neuritis. Although accumulating evidence indicates the involvement of double-stranded DNA (dsDNA) in the pathogenesis of various autoimmune diseases, such as systemic lupus erythematosus, its implication in NMOSD remains to be elucidated. In this study, I aimed to assess whether dsDNA levels in the cerebrospinal fluid (CSF) are elevated in NMOSD patients. CSF samples were collected from 24 patients with NMOSD, 26 with multiple sclerosis (MS) and 42 with other neurological diseases (ONDs), and dsDNA concentrations were assessed. The levels of dsDNA in patients with NMOSD (mean: 0.10 ng/ml) were significantly higher than in the ONDs patients (mean: 0.02 ng/μl), and had a strong tendency to be higher than in patients with MS (mean: 0.04 ng/μl). CSF dsDNA levels were shown to decrease after treatment initiation. Moreover, CSF dsDNA levels positively correlated with CSF cell counts and myelin basic protein concentrations. These findings suggest the elevation of CSF dsDNA in association with the development of CNS inflammation, and further indicate its potentiality of a biomarker in assessing the disease activity of NMOSD.

Introduction

Neuromyelitis optica spectrum disorder (NMOSD) is an inflammatory disease of the central nervous system (CNS) that predominantly affects the optic nerves and spinal cord [1,2,3]. The prevalence of NMOSD ranges from 0.5 to 10 per 100,000 people [4,5,6]. In majority of patients with NMOSD, highly specific autoantibody, anti-aquaporin 4 antibody (AQP4-Ab), can be

detected within the serum [7,8]. Aquaporin 4 (AQP4) is known to be the most abundant water channel expressed on astrocytes in the CNS [9,10,11]. Furthermore, the essential role of AQP4-Ab in the pathogenesis of NMOSD has been demonstrated by both *in vitro* and *in vivo* models. AQP4-Ab cause astrocytic damage by inducing complement-dependent cytotoxicity [7,10,12]. On top of direct cytotoxic effect of AQP4-Ab, NMOSD pathology is also marked by fundamental involvement of neutrophils, eosinophils, and microglia [7].

Host-derived dsDNA is known to accelerate immune responses in certain circumstances, especially in autoimmunity. Despite the strictly regulated intracellular localization of dsDNA in nucleus and mitochondria, apoptosis, necrosis, and specific form of death, coined as NETosis, induce the extracellular release of self-derived dsDNA [13]. Several stimuli are reported to cause NETosis of neutrophils, and under certain conditions, e.g. in a reactive oxygen species (ROS)-rich environment, self-derived dsDNA exerts highly immunogenic activity [13,14].

Since astrocytic necrosis and invasion of neutrophils are the hallmarks of NMOSD lesions [7], it could be hypothesized that self-derived dsDNA is released into the CSF in large amount, and contributes to the augmentation of CNS inflammation. However, to the best of my knowledge, there has been no previous reports demonstrating the involvement of self-derived dsDNA in NMOSD patients.

In this study, I investigated whether dsDNA is increased in the CSF of patients with NMOSD. Interestingly, my results demonstrated that CSF dsDNA levels in patients with NMOSD were significantly higher than in the ONDs patients, and had a strong tendency to be higher than in patients with MS. The association of CSF dsDNA levels with NMOSD disease activity was also assessed.

Materials and Methods

Patient information

CSF samples were obtained from 24 patients with NMOSD, 26 with MS, and 42 with ONDs who had been admitted to Toyama University Hospital, Osaka University Hospital, or Kinki University Hospital during 2009 to 2019. All patients with NMOSD were AQP4-Ab positive and met the 2015 NMOSD diagnostic criteria [2]. MS was diagnosed according to the 2010 McDonald criteria [15]. Two patients with clinically isolated syndrome (CIS) were included in the MS group. Patients with ONDs (n=42) had chronic inflammatory demyelinating polyneuropathy (CIDP, n=13), Guillain-Barré syndrome (GBS, n=4), Miller Fisher syndrome (MFS, n=2), amyotrophic lateral sclerosis (ALS, n=12), Parkinson's disease (PD, n=7), or idiopathic normal pressure hydrocephalus (iNPH, n=4).

This study was approved by the ethics committee of Toyama University Hospital (approval No.: 29–32), Osaka University Hospital (approval No.: 12091-6), and Kinki University Hospital (approval No.: 25-138). Written informed consent was obtained from all the patients.

CSF collection and measurement of dsDNA levels

CSF samples were obtained from 24 NMOSD patients during the relapse phase, and 9 CSF samples were collected during the post-treatment (intravenous methylprednisolone (IVMP) treatment) phase. All 26 CSF samples from patients with MS were collected during the relapse phase. The CSF samples were centrifuged at 2,000 g at 4°C for 10 min, aliquoted, and stored at -80°C until analysis. DNA extractor SP kit (Wako, Osaka, Japan) was used to extract DNA from the CSF samples, following the manufacturer's protocol. The Qubit™ dsDNA HS Assay Kit

(Invitrogen, USA) was used to obtain highly selective dsDNA, which was then measured using a Qubit® 2.0 Fluorometer.

Statistical analysis

Statistical analysis was performed with a paired t-test for comparing paired data and a two-sample t-test for comparison between different groups, where Student's t-test was used if equal variances were assumed and Welch's t-test otherwise. Spearman's rank correlation coefficient was used to evaluate the statistical relationships between two variables. Statistical significance was set at $p < 0.05$.

Results

Clinical characteristics and CSF profiles of NMOSD, MS, and ONDs patients

The mean age of patients with NMOSD was 54.3 years, with a mean disease duration of 36.0 months, and the mean Expanded Disability Status Scale (EDSS) was 3.9. Lesions were found in the spinal cord (83%), brain (50%), and optic nerve (33%) in those patients. The mean age of patients with MS was 44.3 years, with a mean disease duration of 74.3 months, and the mean EDSS was 3.3. The lesions were found in the spinal cord (76.9%), brain (93.3%), and optic nerve (15.4%) in those patients. The mean age of patients with ONDs as a control group was 60.9 years and the mean disease duration was 25.3 months (Table 1).

CSF samples were obtained via lumbar puncture from patients with NMOSD (n=24), MS (n=26), and ONDs (n=42). CSF characteristics, including oligoclonal bands, cell count, protein levels, myelin basic protein (MBP) levels, and IgG index, are summarized in Table 2. The CSF cell counts

were significantly higher in patients with NMOSD (23.7 ±47.56 cells/mm^3) than in those with MS, while there were no significant differences in the levels of protein, MBP, or IgG index.

Table 1. Clinical characteristics of patients with NMOSD, MS and ONDs.

				P Value		
	NMOSD (n=24)	MS (n=26)	ONDs (n=42)	NMOSD versus ONDs	MS versus ONDs	NMOSD versus MS
Female/Male	17/7	15/11	16/26	0.0813	0.00254*	0.5592
Age, year; mean (range)	54.3 (27.4-77.3)	44.3 (24.3-76.7)	60.9 (21.0-83.3)	0.0324*	0.0002*	0.0174*
Disease duration, month; mean (range)	36.0 (0.0-274.0)	74.3 (0.0-359.0)	25.3 (0.0-371.0)	0.9733	0.0226*	0.0977
EDSS (range)	3.9 (2.0-7.5)	3.3 (1.5-5.5)				0.497
Location of lesion						
Spinal cord (%)	20/24 (83)	20/26 (76.9)				0.7966
Brain (%)	12/24 (50)	24/26 (93.3)				0.246
Optic nerve (%)	8/24 (33)	4/26 (15.4)				0.0294*
Length of spinal cord involvement, number of segments (range)	5.6 (1-13)	1.7 (1-3)				0.0273*
Treatment at CSF sampling (%)						
Oral prednisolone	6/24 (25)	4/26 (15.4)				0.0771
Immunosuppressant	0/24 (0)	0/26 (0)				
Interferon-β	0/24 (0)	5/26 (19.2)				
Fingolimod	0/24 (0)	3/26 (11.5)				
Dimethyl Fumarate	0/24 (0)	0/26 (0)				

NMOSD: neuromyelitis optica spectrum disorder, MS: multiple sclerosis,
ONDs: other neurological diseases, EDSS: Expanded Disability Status Scale

Table 2. CSF findings of patients with NMOSD, MS and ONDs.

CSF findings	NMOSD (n=24)	MS (n=26)	ONDs (n=42)	p-Value		
				NMOSD vs ONDs	MS vs ONDs	NMOSD vs MS
Positivity oligoclonal bands (%)	3/22 (14)	15/26 (58)	1/39 (3)	0.0915	<0.001*	0.0017*
Cell count (cells/mm^3)	23.7 (9.71)	3.4 (1.10)	1.2 (0.23)	<0.0001**	0.0329**	0.0081**
Protein (mg/dl)	49.2 (7.15)	37.9 (3.48)	77.2 (19.57)	0.0772	0.0016**	0.1989
MBP (pg/ml)	313.2 (123.29)	194.1 (69.38)	48.4 (15.91)	0.0112	0.0008**	0.704
IgG index	0.63 (0.032)	0.74 (0.11)	0.50 (0.01)	0.0009**	0.0139**	0.6178

Each value represents mean (SD), NMOSD: Neuromyelitis optica spectrum disorder, MS: Multiple Sclerosis, ONDs: other neurological diseases, CSF: Cerebrospinal fluid, MBP: myelin basic protein, dsDNA: double-stranded DNA

Increased CSF dsDNA levels in NMOSD patients

The dsDNA levels were 0.1 ± 0.14 ng/μl (mean ± SD) in NMOSD, 0.04 ± 0.03 ng/μl in MS, and 0.02 ± 0.01 ng/μl in ONDs. The levels of dsDNA in patients with NMOSD were significantly higher than those in patients with ONDs ($p < 0.01$), and had a strong tendency to be higher than in patients with MS ($p = 0.0513$) (Fig 1A). CSF samples were obtained from 7 patients with NMOSD after treatment, of which all patients received IVMP treatment, and 2 of the 7 patients received additional plasmapheresis treatment after IVMP. The levels of dsDNA in patients with NMOSD significantly decreased from 0.10 ± 0.14 at the relapse phase to 0.03 ± 0.02 after the treatments, and were then similar to the levels detected in ONDs patients (Fig 1B).

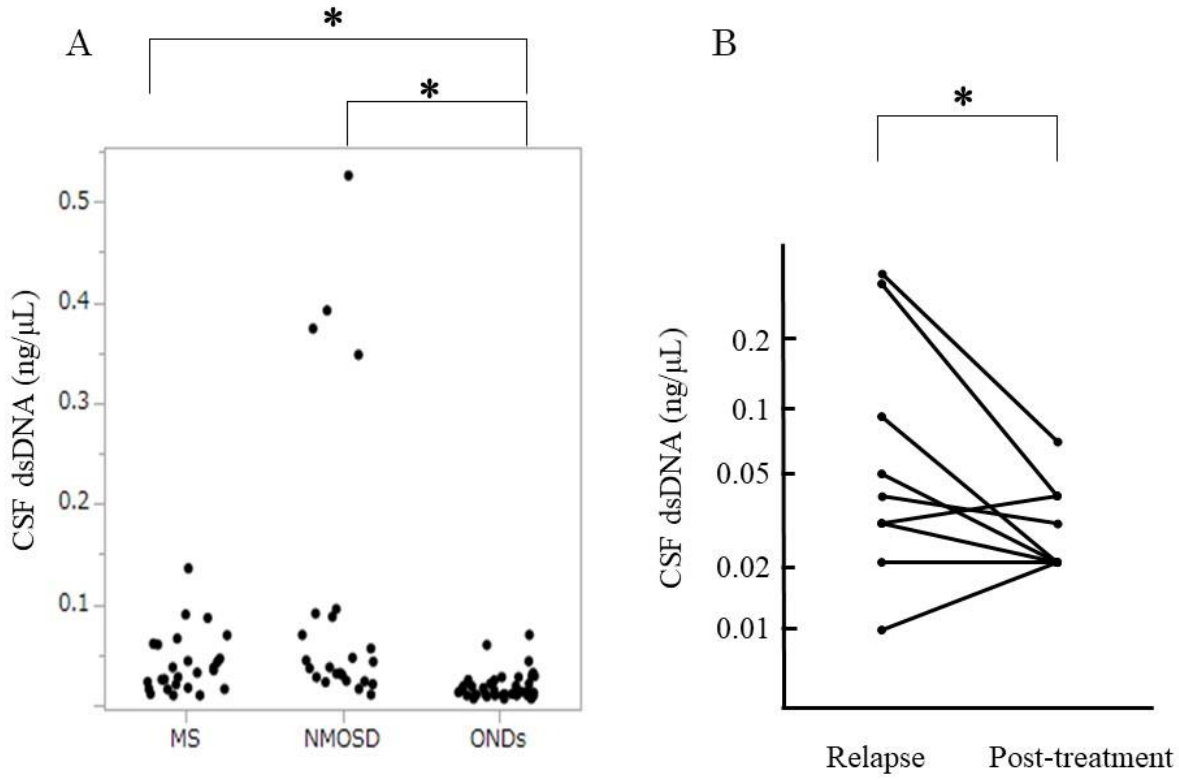

Fig. 1

Fig. 1. The elevation of CSF dsDNA levels in patients with NMOSD. (A) The levels of CSF dsDNA in patients with MS, NMOSD, and ONDs are shown. dsDNA concentration was significantly higher in NMOSD patients than in ONDs patients. Welch's t-test was used to compare differences between the groups. *P <0.05 (B) The levels of CSF dsDNA of patients with NMOSD in the acute phase were significantly decreased after the treatment. Paired t-test was used to compare paired data. *P <0.05

Correlation between the levels of CSF dsDNA and other variables in patients with NMOSD and MS

There was a significant positive correlation between CSF dsDNA levels and CSF cell counts (ρ=0.7717, p=0.0088), CSF protein levels (ρ=0.3860, p=0.0160), and CSF MBP (ρ=0.7357, p=0.0314) in NMOSD patients. However, no correlation was observed between CSF dsDNA levels and IgG index (ρ=0.0571, p=0.2886) (Fig 2). In patients with MS, there was no significant correlation between CSF dsDNA levels and CSF cell counts (ρ=0.1220, p=0.6935), protein levels (ρ=0.0813, p=0.4084), CSF MBP (ρ=0.3270, p=0.0643), and IgG index (ρ=0.1298, p=0.4828) (Fig 3).

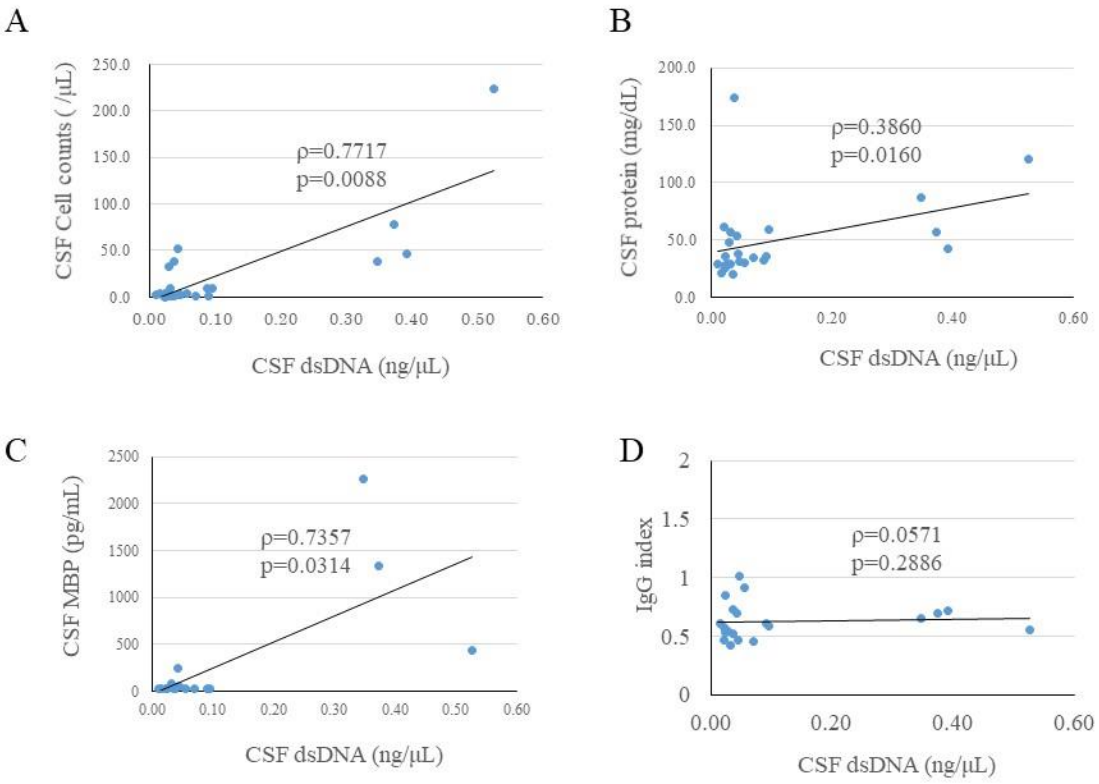

Fig. 2

Fig. 2. Correlations between CSF dsDNA levels and CSF variables in patients with NMOSD. (A, B, C) The levels of CSF dsDNA showed a Significant correlation with the CSF cell counts, CSF protein levels, and CSF MBP levels. (D) CSF dsDNA levels were not significantly correlated with IgG index. Spearman's rank correlation was used for statistical analysis.

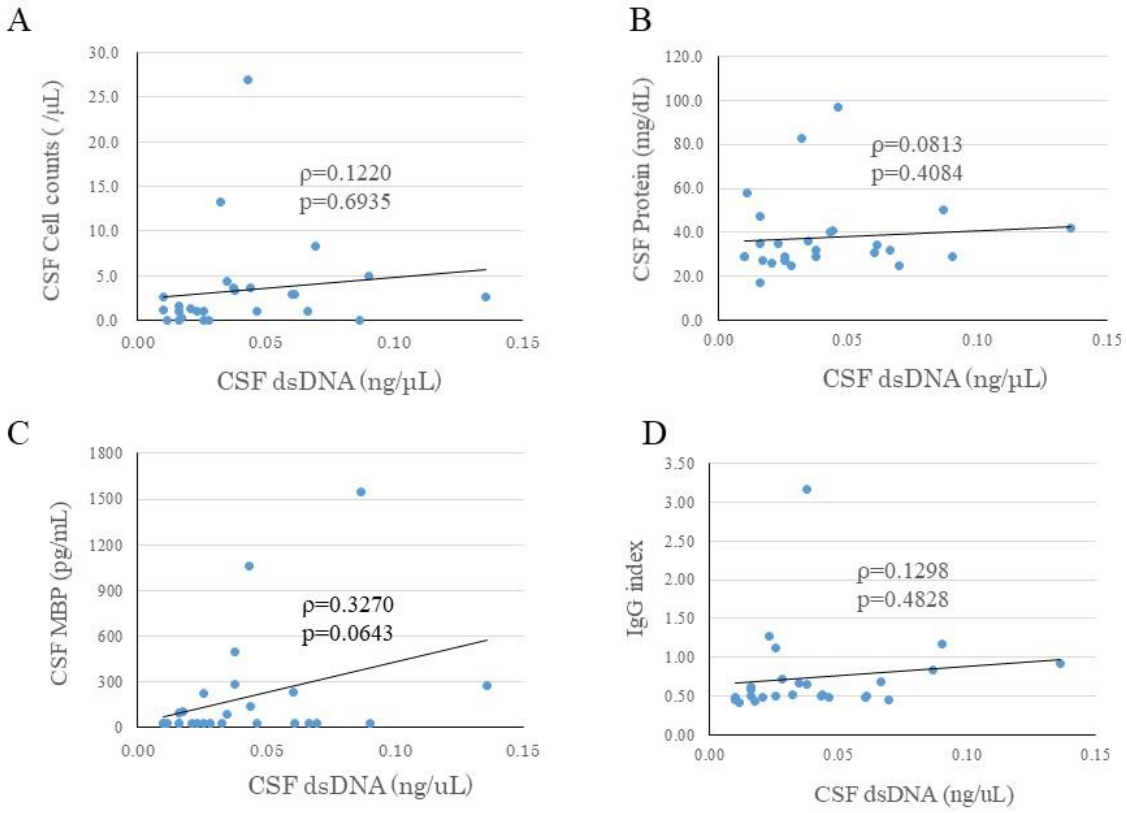

Fig. 3

Fig. 3. Correlations between CSF dsDNA levels and CSF variables in patients with MS.

CSF dsDNA levels were not significantly correlated with CSF cell counts, CSF protein levels, MBP levels, or IgG index. Spearman's rank correlation was used for statistical analysis.

Discussion

In this study, I showed that the levels of dsDNA in the CSF of patients with NMOSD at the acute phase were significantly increased compared to those of ONDs patients, and had a strong tendency to be higher than in patients with MS. Furthermore, elevated dsDNA levels decreased upon treatment initiation (Fig 1). Host-derived dsDNA is located in the nucleus and mitochondria in physiological conditions, but it will be extracellularly released from necrotic or apoptotic cells, thereby inducing inflammatory responses [13,16,17]. In the context of NMOSD pathogenesis, AQP4-Ab plays an essential role by inducing astrocytic necrosis. In vitro study by Kinoshita and colleagues demonstrated AQP4 binds specifically with AQP4-Ab in the sera of NMOSD patients, furthermore triggering downstream signaling which then causes cell damage [18]. Here, I demonstrated that CSF dsDNA concentration was positively correlated with CSF cell counts and CSF MBP levels in patients with NMOSD (Fig 2A and C). These findings suggest that increased CSF dsDNA might reflect the tissue destruction caused by CNS inflammatory processes at the acute stage of the disease.

Indeed, CSF findings in my study revealed approximately 5-fold increase of CSF cells compared to the normal range at NMOSD relapse phase. The significant increase of CSF cells was also observed in NMOSD when compared to that of MS or ONDs patients (Table 2). These data are consistent with a previous study of Jarius et al [19], and further indicate the active inflammatory processes proceeding at the acute phase of my NMOSD cohort.

Previous report showed increased CSF high-mobility group box 1 (HMGB1) levels in patients with NMOSD compared to MS or ONDs patients. HMGB1 is known to be involved in CNS inflammation and promotion of inflammatory cytokines release [20]. In addition, another study

also reported that levels of CSF-S100B, an astrocytic damage marker was increased in NMOSD patients [21].

It was previously reported that mitochondrial DNA (mtDNA) released from damaged astrocytes is increased in the CSF of patients with NMOSD compared to MS and ONDs patients [22]. mtDNA promotes innate immune responses through the production of interleukin (IL)-1β via Toll-like receptor 9 (TLR9) [22,23]. Moreover, a recent study showed that mtDNA activates monocytes and serves as a crucial molecule in augmenting innate responses in NMOSD [23]. Therefore, it is possible that dsDNA, in addition to mtDNA, also plays a crucial role in augmenting innate immune responses in NMOSD active lesions.

As for the source of CSF dsDNA detected in NMOSD, CSF infiltrating neutrophils might also contribute to the accumulation of extracellular host-derived dsDNA. Under inflammatory conditions, neutrophils are known to undergo unique type of cell death, coined as NETosis [14,24]. During NETosis, nuclear as well as granular membranes dissolve, and nuclear contents will be released into the cytoplasm [24]. To what extent CNS infiltrating neutrophils are involved in CSF dsDNA accumulation remains to be clarified in future studies.

To date, there have been no reports assessing the involvement of dsDNA in NMOSD. Here, I reported that dsDNA is released in CSF at significantly high level at the acute phase of NMOSD relapses. These findings suggest the elevation of CSF dsDNA in association with the development of CNS inflammation, and further indicate its potentiality of a biomarker in assessing the disease activity of NMOSD.

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