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学位論文

Detection of NUCB2/nesfatin-1 in cerebrospinal fluid of  
multiple sclerosis patients

(多発性硬化症患者の脳脊髄液における NUCB2/nesfatin-1 の検出)

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## 論 文 内 容 要 旨

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学位論文題名	Detection of NUCB2/nesfatin-1 in cerebrospinal fluid of multiple sclerosis patients. 多発性硬化症患者の脳脊髄液における NUCB2/nesfatin-1 の検出
<p>多発性硬化症(multiple sclerosis :MS)は中枢神経の炎症性脱髄疾患であり多彩な神経症状を呈する。自己免疫が関与した炎症が脱髄を引き起こしていると考えられているが詳細な病態機序は不明である。現時点で診断のための特異的なマーカーは存在せず、診断においては脱髄病変に基づく神経症状が空間的多発性と時間的多発性を示すという症状の特徴が基準となる。特に日本では非典型例の割合が多く、診断や治療に困難が伴う場合も多い。</p> <p>Nesfatin-1 は分泌タンパク質 nucleobindin2 の N 末端の 82 個のアミノ酸であり当初、摂食抑制作用を持つペプチドとして報告された。しかし近年、血圧調整やストレス、糖代謝、炎症との関与が指摘されている。しかし、神経炎症性疾患において NUCB2/nesfatin-1 を測定した報告はまだなく今回、多発性硬化症患者の髄液中の NUCB2/nesfatin-1 の検出を試み、診断マーカーとしても可能性を検討した。</p> <p>髄液のサンプルとして 24 名の MS 患者(男 7 女 17)、10 名のコントロール患者(頭痛、転換性ヒステリー：男 4 女 6)を用いた。ウエスタンブロット法を用いて多発性硬化症患者の髄液から NUCB2/nesfatin-1 の存在を確認し ELISA 法にて髄液中 NUCB2/nesfatin-1 濃度を測定した。併せて髄液中の炎症性サイトカイン (TGF-<math>\alpha</math>,IL-1<math>\beta</math>) と抗炎症性サイトカイン (IL-10,TGF-<math>\beta</math>) を測定した。</p> <p>MS において髄液中の NUCB2/nesfatin-1 は正常コントロールと比較して有意に上昇を認めた。血清と髄液中の nesfatin-1 濃度には相関を認めず MS の病型間でも有意差を認めなかったが、ステロイドパルス治療中の患者髄液では NUCB2/nesfatin-1 を検出することができなかった。</p> <p>髄液中のサイトカイン濃度測定においては炎症性サイトカインである IL-1<math>\beta</math> においてのみコントロールと比して有意に低下を認めた(<math>p &lt; 0.05</math>)。</p> <p>NUCB2/nesfatin-1 は外傷後の脳損傷において炎症に対して抑制的に働くという報告があり、今回得られた MS 患者髄液で IL-1<math>\beta</math> が減少していたことも併せて NUCB2/nesfatin-1 の上昇は炎症の結果というよりは抗炎症作用として働いている可能性も指摘される。</p> <p>MS の髄液中の NUCB2/nesfatin-1 を測定した報告はなく、今後、診断のマーカーや症例を積み重ねることにより、病勢の判定、予後の予測などに用いることができる可能性がある。</p>	

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## ABSTRACT

NUCB2/nesfatin-1 was originally discovered as an anorexigenic peptide. However, recent studies revealed various additional functions including the regulation of inflammation. However, there are no studies that investigated the involvement of NUCB2/nesfatin-1 in neuroinflammatory diseases. Here, we aimed to investigate the involvement of NUCB2/nesfatin-1 in a representative neuroinflammatory disease, multiple sclerosis (MS). Cerebrospinal fluids (CSF) were collected from 24 MS patients and 10 control subjects and NUCB2/nesfatin-1, proinflammatory cytokines (TNF- $\alpha$ , IL-1 $\beta$ ) and anti-inflammatory cytokines (IL-10, TGF- $\beta$ ) levels were measured by using ELISA assay. Also the expression of NUCB2/nesfatin-1 in the CSF of MS patient was investigated by western blot analysis. Expression of NUCB2/nesfatin-1 was confirmed in the CSF of the MS patient by western blot analysis. NUCB2/nesfatin-1 levels were significantly higher in the CSF of the MS patients. Among the measured cytokines, only IL-1 $\beta$  was lower in the CSF of the MS patients. We report for the first time increased NUCB2/nesfatin-1 levels in the CSF of MS patients.

**Keywords:** NUCB2/nesfatin-1, Multiple sclerosis, Inflammation

## Abbreviation:

MS: Multiple Sclerosis CSF: Cerebrospinal fluids CNS: Central Nervous System

TNF: Tumor Necrosis Factor NUCB2: Nucleobindin2

IL: Interleukin TGF- $\beta$ : Transforming Growth Factor- $\beta$

SDS: Sodium dodecylsulfate PBS: Phosphate-Buffered Saline

DIS: Disseminated in Space MRI: Magnetic Resonance Imaging

## Introduction

Multiple sclerosis (MS) is an inflammatory disease affecting the central nervous system (CNS) of more than 2 million people worldwide [1]. Its symptoms vary from physical to mental such as visual problems, depression, anxiety and fatigue [2].

Although detailed pathological mechanisms still remain unclear, the key factor is considered to be dysfunction of the immune system associated with CNS inflammation and demyelination which ultimately leads to axonal damage and destruction of the neural network [3]. Recent studies have revealed the possible interference of inflammation related molecules and cytokines such as interleukin (IL)-1 $\beta$ , IL-6, IL-8, and tumor necrosis factor (TNF) in the induction of demyelination [4-6].

The major challenge of diagnosing MS is that clinical symptoms and results of imaging examinations vary substantially between patients [7].

As mentioned above, because neural inflammation is the key factor for developing MS, proinflammation and anti-inflammation cytokines in the cerebrospinal fluid (CSF) may be associated with the disease and may be useful as biomarkers to diagnose MS. In fact, recent studies reported the elevation of proinflammatory molecules in MS patients [4-6, 8].

On the other hand, nesfatin-1 is an 82-amino-acid peptide derived from NEFA/nucleobindin2 (NUCB2) which is expressed in both the peripheral and CNS [9]. NUCB2/nesfatin-1 was originally identified as an endogenous anorexigenic peptide but recent studies have indicated various additional functions aside from feeding regulation [9-16]. Among such newly discovered functions of NUCB2/nesfatin-1, some studies indicated possible involvement in anti-inflammation mechanism [17-19]. Stengel et al., reported that Lipopolysaccharide administration in rats showed increase in circulating NUCB2/nesfatin-1 level [20] Also, recent

studies reported the change of serum or CSF NUCB2/nesfatin-1 levels in neurological diseases such as epilepsy and depression [21,22].

In this study, we collected the CSF from MS patients and examined the level of NUCB2/nesfatin-1 together with proinflammatory and anti-inflammatory cytokines. Our present results may indicate the potentials of NUCB2/nesfatin-1 as a biomarker for diagnosing MS. Also, the results of inflammation related cytokine levels provide insight into the pathological mechanism and the involvement of inflammation in developing MS.

## **Materials and methods**

### *MS patient samples*

CSF samples were collected from 24 MS patients (7 male and 17 female) admitted to the Department of Neurology in Matsumura General Hospital. As control, 10 CSF samples were collected from patients affected by non-inflammatory neurological diseases (patients with strong headache or conversion disorder: 4 males and 6 females). The study was approved by the ethics committee of Fukushima Medical University, School of Medicine and Matsumura General Hospital, according to the Declaration of Helsinki. All patients were provided written informed consent to this study. MS diagnosis was made according to 2017 McDonald Criteria for Diagnosis of Multiple Sclerosis, which is based on the combinations of clinical presentation and additional data (Table) [7].

### *Western blot analysis*

Twenty  $\mu$ l of CSF samples dissolved in 2 X SDS sample buffer was loaded onto a 10% polyacrylamide gel (156103, Bio-Rad, CA, USA). The samples were separated at 20 mA. Proteins were transferred from the gel onto a PVDF membrane at 118 mA for 60 min with transfer buffer. The membrane was washed several times with PBS containing 0.05% of Tween-20 (PBST) and blocked with 5% skim milk in PBST for 60 min at room temperature. Then the membrane was incubated with a rabbit polyclonal NUCB2 antibody (1:1000, N9414, Sigma-Aldrich, MO, USA) for overnight at 4°C. The antibody was diluted in blocking solution. The membrane was washed with PBST and incubated with HRP-conjugated secondary antibodies against rabbit IgG (1:1000, Vector Laboratories, CA, USA) for 60 min at room temperature. Then the membrane was washed several times and peroxidase activity was detected.

### *ELISA assay*

Levels of NUCB2/nesfatin-1 (Abcam, Cambridge, UK), IL-1 $\beta$ , TNF $\alpha$ , IL-10 and TGF- $\beta$  (Biolegend, San Diego, CA, USA) in CSF and serum were measured by commercially available ELISA assay kit.

### *Statistical Analysis*

All data are presented as mean. Statistical analysis was made by student's *t*-test and one-way ANOVA followed by Tukey test for Fig 2.  $P < 0.05$  was considered significant.

## Results

### *NUCB2/nesfatin-1 levels of CSF from MS patients.*

The mean ages were  $43.2 \pm 1.8$  for the MS patients and  $36.5 \pm 5.1$  for the control subjects.

As shown in Fig. 1 (above), expression of NUCB2/nesfatin-1 was confirmed in western blot analysis. The levels of NUCB2/nesfatin-1 were 38.2 and 138.3 pg/ml in the control subjects and MS patients, respectively.

Analysis of the CSF showed significantly higher levels of NUCB2/nesfatin-1 in the MS patients (Fig. 1;  $p=0.02$ ) than in the control subjects. Serum and CSF levels of NUCB2/nesfatin-1 had no significant correlations ( $r = -0.6$ ,  $P = 0.09$ ).

When comparing the levels of NUCB2/nesfatin-1 in control subjects, patients with primary progressive MS and relapsing remitting MS, average NUCB2/nesfatin-1 level was significantly higher in CSF of relapsing remitting MS patients (Fig2). There were no differences in levels of NUCB2/nesfatin-1 among each phenotypes of MS (Clinically isolated syndrome, relapsing-remitting MS, Primary progressive MS, Secondary progressive MS).

There were 2 subjects under steroid pulse treatment when CSFs were collected (which were excluded from results shown in Figs 1 and 2). These two subjects showed low levels of NUCB2/nesfatin-1 that were undetectable by the assay kit we have used in this study and therefore these data are omitted from this study.



*Levels of proinflammatory cytokines in CSF of MS patients.*

We have measured the levels of IL-1 $\beta$  and TNF $\alpha$  as proinflammatory cytokines in the CSF. TNF $\alpha$  levels were not significantly different between the MS patients and control subjects (Fig. 3 above right). However, to our surprise, IL-1 $\beta$  level of the CSF from the MS patients was significantly lower than that of the control subjects (Fig. 3 above left).

*Levels of anti-inflammatory cytokines in CSF of MS patients.*

We also measured the levels of IL-10 and TGF- $\beta$  as anti-inflammatory cytokines in the CSF. Both IL-10 and TGF- $\beta$  had no significant difference between the MS patients and control subjects (Fig. 3 below).

## **Discussion**

In this study, we have shown increased NUCB2/nesfatin-1 level in the CSF of MS patients. Although the number of subjects is relatively small, to the best of our knowledge, this is the first study to evaluate NUCB2/nesfatin-1 levels in the CSF of human patients with neuroinflammatory disease.

It is considered that some form of immunological reaction may initiate neuroinflammation that ultimately leads to demyelination and neurodegeneration in the CNS [2, 23, 24]. Recently, many studies have been performed to evaluate the interference of inflammatory factors in the CSF of MS patients [4-6]. In these studies, proinflammatory conditions of the CSF were shown to be a critical factor that induces and maintains the disease. Proinflammatory cytokines such as IL-6 and IL-8 are reported to be related to long-term disease activity and progression of

symptoms of MS [5]. It is thus obvious that existence of inflammation and inflammatory cytokines in the CSF are the key factors to understand the mechanism of developing MS.

NUCB2/nesfatin-1, which was originally discovered as an anorexigenic peptide is now considered to have various functions in both the CNS and peripheral tissues [9-16]. Although the receptor for NUCB2/nesfatin-1 is not yet discovered, study using the autoradiography indicated the existence of receptor in pancreas, kidney, liver and CNS in rats [25]. These new functions include regulations of blood pressure, glucose homeostasis and even cardiac performance [11-16]. In addition, regulation of inflammation by NUCB2/nesfatin-1 has been reported. Naseroleslami et al. reported that NUCB2/nesfatin-1 in cardiomyocytes attenuates myocardial infarction by reducing proinflammatory cytokines [17]. Also, Jiang et al. showed that NUCB2/nesfatin-1 can ameliorate osteoarthritis by suppressing inflammation [19]. These reports indicate the ability of NUCB2/nesfatin-1 to suppress inflammation. In the present study, we found that NUCB2/nesfatin-1 level in the CSF is increased in MS patients. The results showing NUCB2/nesfatin-1 level in the CSF of patients under steroid pulse treatment was undetectably low in assay kit we have used in this study, further support the involvement of NUCB2/nesfatin-1 on inflammation. Because exogenously applied NUCB2/nesfatin-1 is reported to suppress inflammation of post traumatic brain injury by suppressing NF- $\kappa$ B inflammatory pathway and reducing the inflammatory cytokine levels such as TNF- $\alpha$  and IL-1 $\beta$  [26], increase of NUCB2/nesfatin-1 which was found in this study is likely to reflect the anti-inflammatory response rather than result of neuroinflammation itself. Clinically, we found no relation with expanded disability status scale (EDSS) and NUCB2/nesfatin-1 level, it may be important to investigate further within relapse and remission period. Further study is required to elucidate the detailed mechanism.

To our surprise, a proinflammatory cytokine, IL-1 $\beta$ , was found to be significantly reduced in the CSF of MS patients. Because of ongoing inflammation in MS patients, the reduction of proinflammatory cytokine IL-1 $\beta$  was unexpected. In the past, Rossi et al. reported the detection of IL-1 $\beta$  in the CSF of MS patients only in the remission phase [4]. The present study analyzed CSF samples from both primary and remitted MS patients but IL-1 $\beta$  level was clearly and significantly reduced in both patients. This may be explained by the elevation of NUCB2/nesfatin-1 level in the CSF. Jiang et al. reported that NUCB2/nesfatin-1 can suppress IL-1 $\beta$ -induced inflammation [19]. Therefore NUCB2/nesfatin-1 and IL-1 $\beta$  levels may have some functional connection in the CSF of MS patients. The study using knock-out mice maybe useful to investigate these functions. So far, reports on NUCB2/nesfatin-1 knock out mice in very limited, but there is no report of immunological disorder. Further study is required.

Up to present, diagnosis of MS is based on symptoms and brain images obtained from MRI. In addition, oligoclonal bands of IgG on electrophoresis of the CSF are an effective marker for its diagnosis [7]. There are reports of other possible CSF biomarkers to diagnose MS. Recently, CSF proteome study was performed using the CSF from the MS patients [27]. This study indicated possible biomarker such as apolipoprotein-C-I, A-II and augurin but these factors are still under investigation for clinical use and discovery of a promising new biomarker is expected. However, it would be ideal if we could detect the change of NUCB2/nesfatin-1 level in blood or urine sample.

As a limitation of this study, the number of participants of this study is small. In this study, it was difficult to diagnose relapsing remitting patients and progressive patient due to the limited observation period. Also, investigation of NUCB2/nesfatin-1 levels within the relapsing period (early, peak and end phase) is important considering the possible use as biomarker or even treatment. In addition, NUCB2/nesfatin-1 level in brain is known to

have diurnal rhythm [28] and therefore it is important to determine an ideal timing within a day for obtaining CSF. Further investigation is required. However, our present data show for the first time that level of NUCB2/nesfatin-1 in CSF may have a potential to be an effective biomarker for diagnosis of MS.

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### **Conflicts of interests**

The authors declare no competing financial or non-financial interests.

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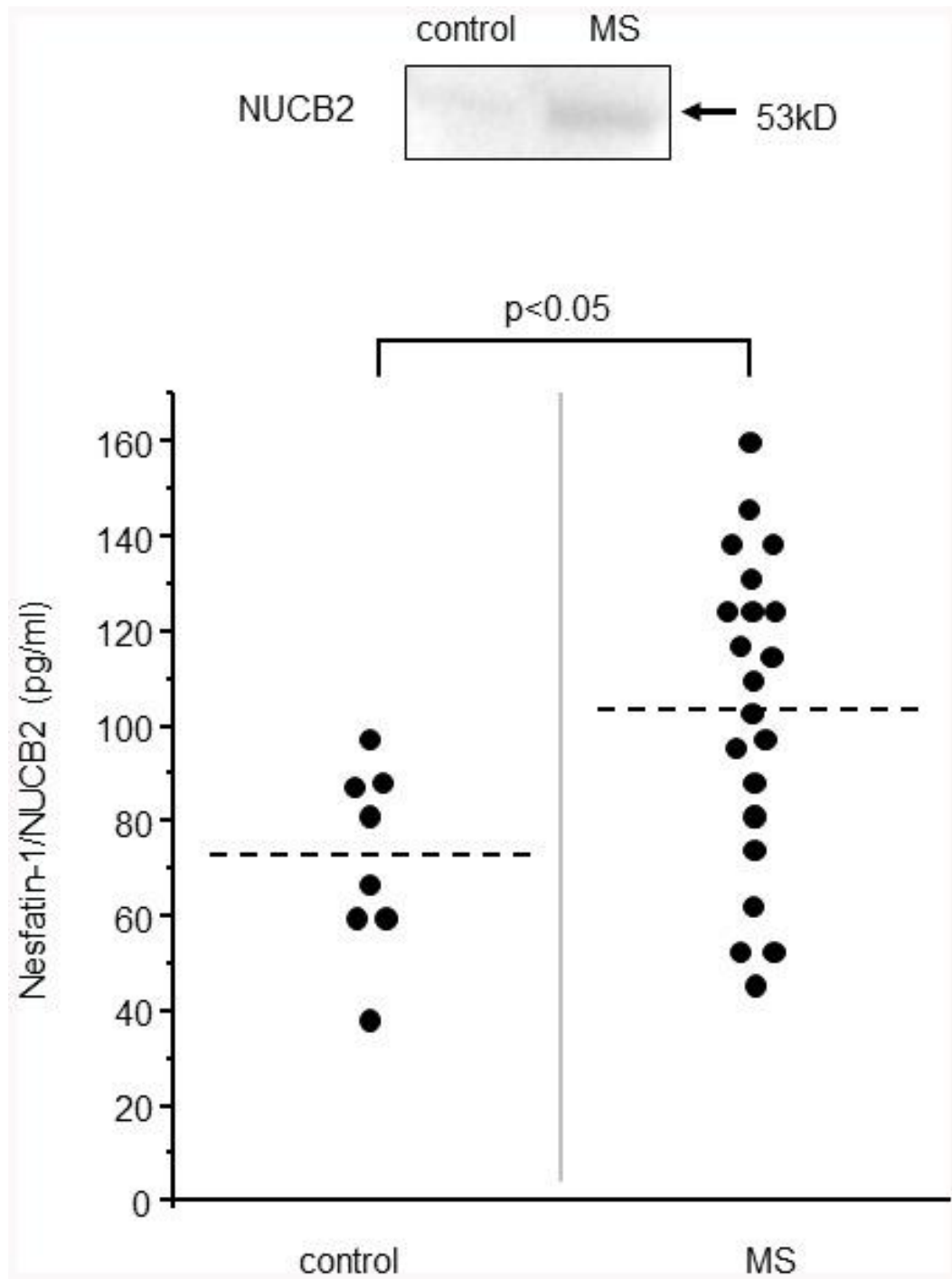
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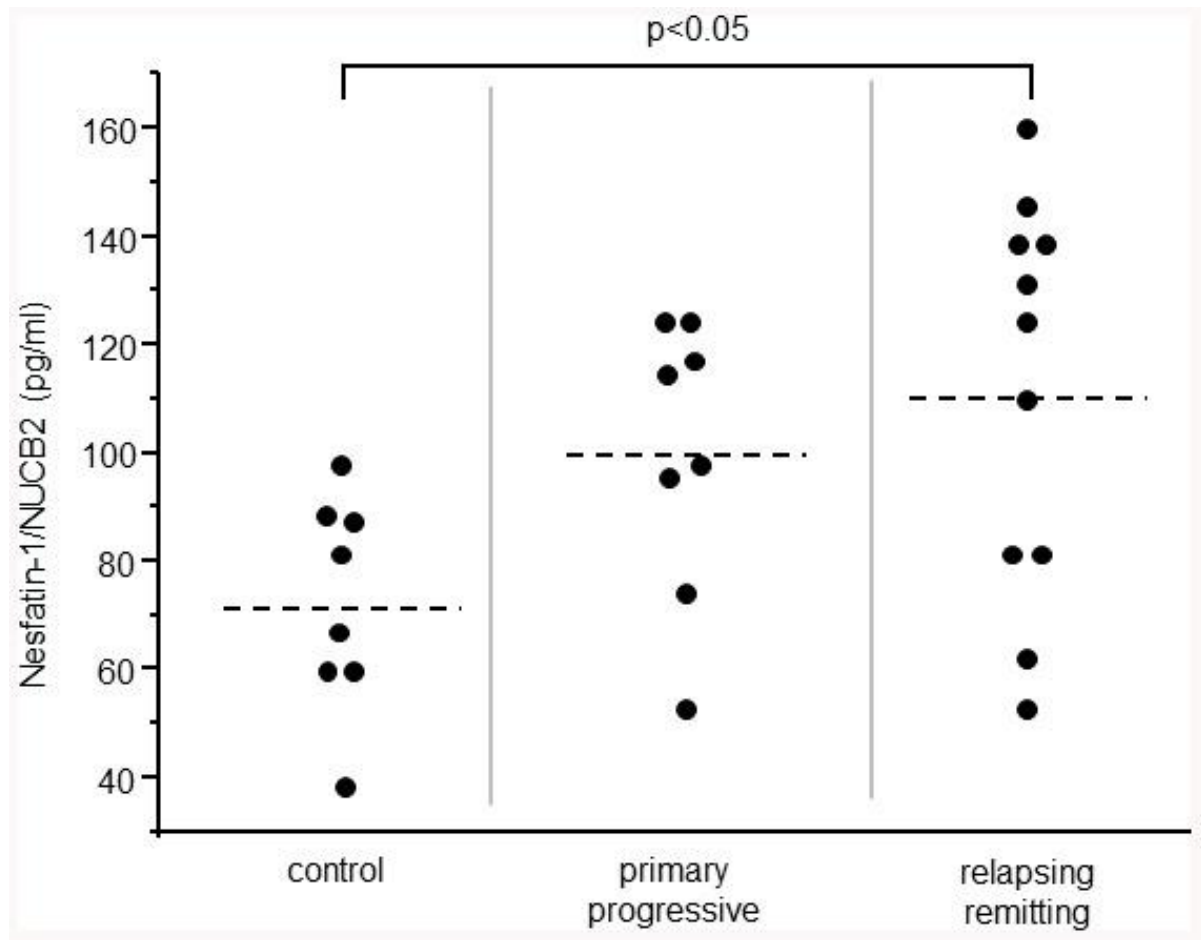


**Table 1. Diagnostic criteria for MS**

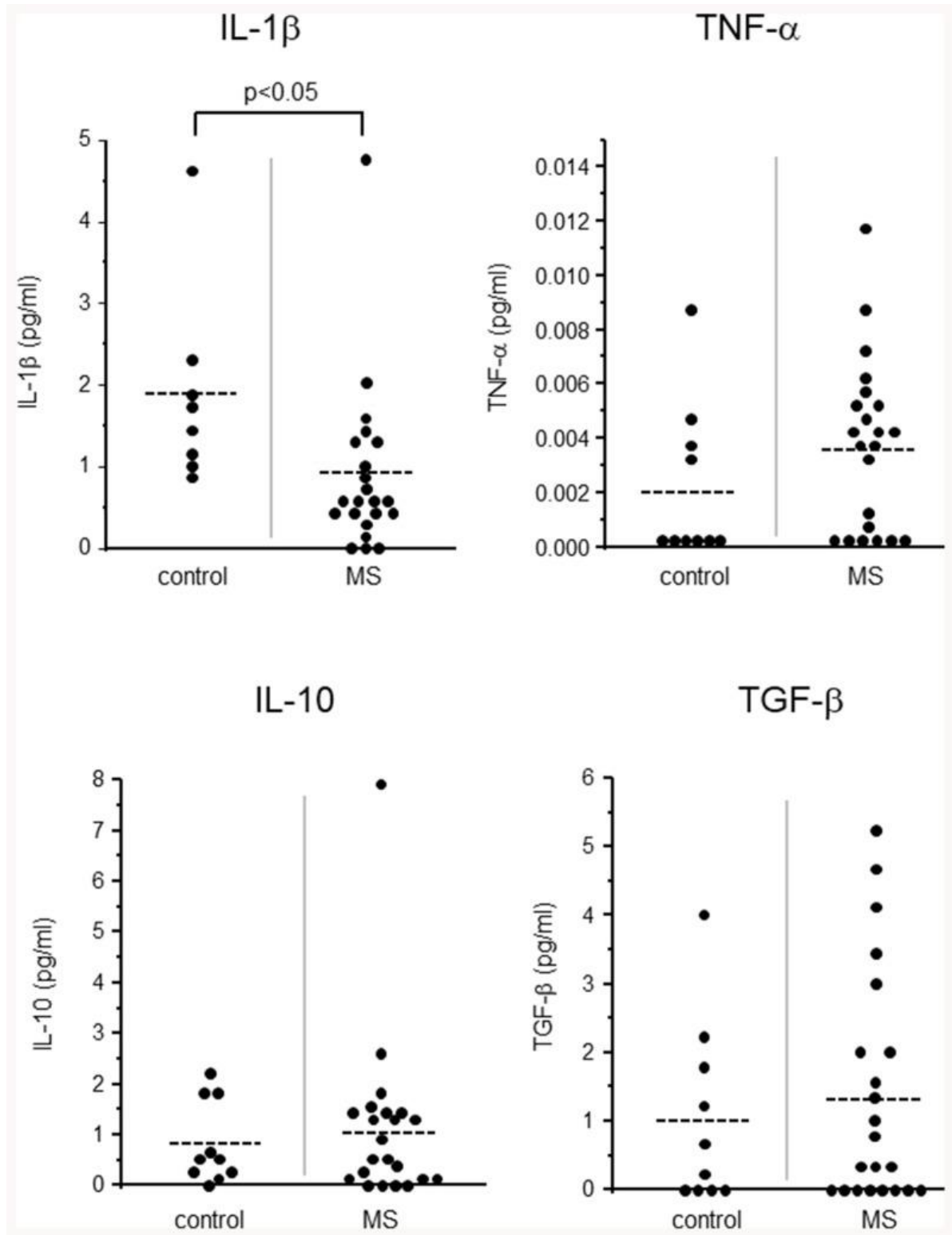
<b>Clinical Presentation</b>	<b>Additional Data Needed for Diagnosis</b>
$\geq 2$ clinical attacks and objective evidence of 1 lesion	Disseminated in space (DIS): an additional attack implicating a different CNS site OR by MRI ( $\geq$ new lesions on follow-up imaging both gadolinium-enhancing and non-enhancing lesions on single MRI.)
1 clinical attack and objective clinical evidence of $\geq 2$ lesions	Disseminated in time (DIT): an additional clinical attack implicating a different CNS site OR by MRI ( $\geq$ 1 symptomatic or asymptomatic lesion in $\geq 2$ areas including cortical/juxtacortical, periventricular, infratentorial, or spinal.) <b>OR</b> CSF-specific oligoclonal bands
1 clinical attack and objective evidence of 1 lesion	DIS: an additional clinical attack implicating a different CNS site OR by MRI ( $\geq$ new lesions on follow-up imaging both gadolinium-enhancing and non-enhancing lesions on single MRI.) <b>OR</b> DIT: an additional clinical attack OR by MRI ( $\geq$ 1 symptomatic or asymptomatic lesion in $\geq 2$ areas including cortical/juxtacortical, periventricular, infratentorial, or spinal.) <b>OR</b> CSF-specific oligoclonal bands



**Figure 1.** NUCB2/nesfatin-1 in the CSF of the MS patients. The expression of NUCB2/nesfatin-1 in the CSF of MS patient confirmed by western blot analysis (above). The NUCB2/nesfatin-1 levels in the control (n=10) and MS patients (n=24).



**Figure 2.** NUCB2/nesfatin-1 in the CSF of the control subjects, patients with primary progressive MS and relapsing remitting MS.



**Figure 3.** Levels of proinflammatory cytokines (IL-1 $\beta$ , TNF- $\alpha$ ; above) and anti-inflammatory cytokines (IL-10, TGF- $\beta$ ; below).