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A new myelin protein, TPPP/p25, reduced in demyelinated lesions is enriched in cerebrospinal fluid of multiple sclerosis

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ABSTRACT

Multiple sclerosis (MS) is a chronic inflammatory demyelinating disease with variable extent of remyelination coupled with the differentiation of oligodendrocytes, in which Tubulin Polymerization Promoting Protein/p25 (TPPP/p25) plays a crucial role. Previously we reported that the loss of TPPP/p25-positive oligodendrocytes in demyelinated lesions in the brain of MS patients could be a biomarker for MS [2]. In this work we tested the occurrence of TPPP/p25 in the cerebrospinal fluid (CSF) of MS patients, and by elaborating a sensitive assay for quantification of TPPP/p25 we showed that its level is significantly higher than in the case of non-MS patients. Patients with MS were diagnosed at the Department of Neurology, University of Szeged according to the clinical and laboratory diagnostic criteria of McDonald. In non-MS patients no significant pathological changes were detected on magnetic resonance imaging scans, while in MS patients multiple hyperintense T2 lesions in the white matter were detected. Kurtzke Expanded Disability Status Scale scores as well as IgG level and oligoclonal bands of MS patients were demonstrated. The sensitive assay elaborated in this study is based upon Western blot followed by chemiluminescent detection validated by human recombinant protein. The median TPPP/p25 contents in the CSF were 62.8 and 64.7 μg/L for patients with clinically isolated syndromes and relapsing remitting MS, respectively, while this value for non-MS patients was 27.9 μg/L. The enrichment of TPPP/p25 was independent of age, gender and the time period between lumbar puncture and relapse/shub. These data suggest that the TPPP/p25-based assay could be a powerful diagnostic test for MS patients.

1. Introduction

Multiple sclerosis (MS) is a chronic inflammatory disease of unknown origin causing lesions of the ensheathment of axons with myelin [1]. The self-repair mechanisms include remyelination of variable extent which is originated from the migration of the oligodendrocyte (OLG) precursor cells followed by their differentiation into mature OLGs. The depletion of the precursors and/or impaired differentiation could be mechanisms responsible for the lesions ([2] and references therein).

Magnetic resonance imaging (MRI) is an important tool for the diagnosis of MS [3]. The diagnostic McDonald criteria demonstrate the spatial and temporal dissemination of the disease by MRI even at early state of the disease [4]. Also cerebrospinal fluid (CSF) analysis is a useful diagnostic tool to define MS. The presence of oligoclonal bands (OCB) in the CSF is one of the most specific and sensitive tests in MS [5]. However, in certain cases it produces false negative result since some patients remain OCB-negative despite meeting typical criteria for diagnosis of MS. This could be due to the fact that demyelination in MS may occur independent of antibody, or CSF electrophoresis methods may be insensitive to detect local synthesis of antibody in all clinically definite cases of MS. Since a main constituting protein of myelin is the myelin basic protein (MBP), the quantification of its concentration in CSF was proposed to be a diagnostic indicator of myelin breakdown [6,7]. However, the CSF MBP values were found to be very much reduced before and after the acute attack periods and frequently became undetectable [8]. Intensive efforts are directed to search for additional CSF biomarkers in MS [9].

Tubulin Polymerization Promoting Protein/p25 (TPPP/p25), an interacting partner of MBP, is located predominantly in mature

Abbreviations: CSF, cerebrospinal fluid; EDSS, Kurtzke Expanded Disability Status Scale; MRI, magnetic resonance imaging; MS, multiple sclerosis; CIS MS, MS with clinically isolated syndromes; MBP, myelin basic protein; OCB, oligoclonal band; OD, other disease; OIND, other inflammatory neurological disease; OLG, oligodendrocyte; OND, other neurological disease; RR MS, relapsing–remitting MS; TPPP/p25, Tubulin Polymerization Promoting Protein/p25.

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OLGs and aggregates in oligodendroglial cytoplasmic inclusions in multiple system atrophy [10,11]. We suggested that its physiological function in OLGs could be the reorganization and stabilization of the microtubular ultrastructures in the course of ensheathment of axons [12]. We quantified the amount of TPPP/p25 in OLGs of human brain tissue from MS patient, and the demyelinated lesions revealed loss of TPPP/p25-positive OLGs within the MS plaques and increased number of TPPP/p25 immunoreactive OLGs in the normal appearing white matter [2]. These recent data suggested impaired differentiation, migration, and activation capacity of OLGs in later disease stages of MS [2]. These observations motivated us to test the appearance of TPPP/p25 in the CSF and serum samples of MS patients which might contribute to a better understanding of the demyelination of the ensheathment of axons.

2. Materials and methods

2.1. Standard protocol approvals, registrations, and patient consents

The study was approved by the Human Investigation Review Board of the University of Szeged, and written informed consent was obtained from each patient who participated in the study.

2.2. TPPP/p25 purification

Human recombinant TPPP/p25 possessing His-tag tail was expressed in *Escherichia coli* BL21 (DE3) cells and isolated on HIS-Select™ Cartridge (Sigma H8286) as described previously [10].

2.3. Protein determination

The protein concentration was measured by the Bradford method [13] using the Bio-Rad protein assay kit.

2.4. Western blot

CSF samples were stored at −70 °C. After thawing, 20 μl CSF sample was mixed with 5 μl sample loading buffer, and was loaded into Tris–Tricine sodium dodecyl sulfate–polyacrylamide gel [14]. The electrophoresis was followed by transfer onto a polyvinylidene fluoride membrane using wet transfer equipment (Sigma). After blocking, TPPP/p25 was detected by using a rat polyclonal anti-TPPP/p25 antibody [15] in 1:5000 dilution. Antibody binding was revealed by using anti-rat IgG coupled with peroxidase (dilution 1:10,000) (Sigma), ECL (enhanced chemiluminescence) Western Blotting Detection reagents (Amersham Biosciences) and Kodak X-Omat AR films. For quantification of the amount of TPPP/p25 in each CSF sample, a standard curve was obtained from the band intensities of the serially diluted samples of human recombinant TPPP/p25 (in the range of 25–250 μg/L, corresponding to 0.25–2.5 ng). The optical density of the immunoreactive bands was determined by ImageJ program, and was used as quantitative data. There was no chemiluminescent reaction, if the anti-TPPP/p25 antibody was omitted, or 5 μg human recombinant TPPP/p25 was added together with the anti-TPPP/p25 antibody.

2.5. Statistical analysis

All statistical analyses were performed with the help of the SPSS Statistics 17.0 software. Before statistical comparisons, we checked the distribution of data populations with the Shapiro–Wilk *W* test, and also performed the Levene's test for the analysis of the homogeneity of variances. The data population of age was distributed normally and equal variances were assumed, so we used independent *t* test and one-way ANOVA for that comparisons, respectively. All of the other examined data populations showed non-Gaussian distribution, so we used nonparametric statistics: the Mann–Whitney *U* test when two groups were compared, and the Kruskal–Wallis test, when there were more than two groups, followed by paired comparisons with the Mann–Whitney *U* test. The null hypothesis was rejected when the *p* level was lower than the value of 0.05 divided by the number of comparisons (in case of three comparisons: $p < 0.016$), and in such cases the differences were considered significant. In the event of Gaussian or of non-Gaussian distributions, data were presented as means (±SD) or medians (and interquartile range), respectively. Furthermore, where statistical analysis revealed significant differences between the groups showing Gaussian distribution, the 95% confidence interval (CI) of mean was also given. For correlation analyses, we used the nonparametric Spearman correlation coefficient.

3. Results

3.1. Patients

Thirty patients (6 men, 24 women; age: 37.9 ± 10.4 years (mean ± SD), duration of disease: 8.0 (3.0–67.5) months (median and interquartile range), disability score corresponding to Kurtzke Expanded Disability Status Scale (EDSS): 1.5 (1.0–2.0) (median and interquartile range)) with relapsing–remitting MS (RR MS) and 14 patients (3 men, 11 women; age: 35.9 ± 12.7 years, duration of disease: 2.5 (1–10.75) months, disability score (EDSS): 0.0 (0.0–0.0)) with clinically isolated syndromes (CIS MS) were included in this study. Patients with MS were diagnosed at the Department of Neurology, University of Szeged. The diagnosis of MS was confirmed according to the clinical and laboratory diagnostic criteria of McDonald [5] meaning that all the MS patients had typical MRI lesions and OCBs in CSF. In non-MS patients the MRI showed no significant pathological changes. At the time of spinal tap, the patients received neither steroid therapy nor vitamin supplementation. The control group comprised 32 individuals (10 men, 22 women; age: 36.9 ± 12.3 years), who underwent spinal tap for diagnostic purposes, but no autoimmune neurological disease was found in their cases. Fourteen patients suffer from other neurological diseases without inflammation (OND such as epilepsy, cavernoma or polyneuropathy), 12 patients suffer from other diseases without manifest neurological involvement (OD such as patients suspected of having subarachnoidal hemorrhage, but the CSF tapping indicate no bleeding) and 6 patients suffer from other inflammatory neurological diseases (OIND e.g. viral or bacterial meningitis).

3.2. TPPP/p25 is present in CSF

Since TPPP/p25 is located predominantly in mature OLGs and plays a crucial role in the myelination/re-myelination processes, we hypothesized its appearance in CSF of MS patients. To test this idea we elaborated a sensitive, specific assay based upon Western blot coupled with chemiluminescent detection (Fig. 1A). The calibration curve showed quantitative relationship for human recombinant TPPP/p25 at concentrations of 25–250 μg/L with a slope of 14.9 ± 0.84 (±SD), an intercept of −270 ± 121 (±SD) and an R^2 of 0.99 (Fig. 1B).

3.3. CSF TPPP/p25 is increased in MS compared to non-MS controls

Patients with MS divided into two groups denoted as CIS and RR patients with clinically isolated syndromes and relapsing remitting MS, respectively, were clinically characterized, and their CSF samples were used to evaluate TPPP/p25 level.

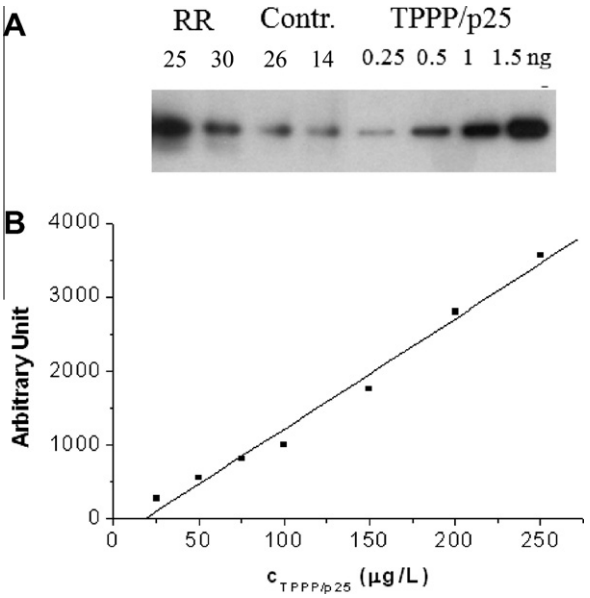

Fig. 1. Characteristics of the established Western blot assay to quantify TPPP/p25 level in CSF. (A) Representative Western blot of different CSF samples of patients (RR 25 and 30, see etable 1) and the corresponding controls (non-MS patients) (Control 26 and 14) using specific anti-TPPP/p25 antibody. (B) Calibration curve with human recombinant protein for quantification of TPPP/p25 content by chemiluminescent Western blot assay.

CSF TPPP/p25 contents of 44 patients with MS (30 RR and 14 CIS) and 32 patients with other clinical symptoms (OIND, OND and OD) obtained by Western blot are summarized in Fig. 2 as well as in Table 1 complemented with registered EDSS and local IgG synthesis (%) (cf. Supplementay Table 1). Among the three non-MS subgroups no significant difference in the TPPP/p25 concentration was found, for OD: 27.5 µg/L, for OND: 26.9 µg/L and for OIND: 29.9 µg/L (from the statistical analysis the median and the interquartile ranges are given in Supplementary Table 1). Then we determined the TPPP/p25 concentrations of both CIS and RR MS samples which were as follows: for CIS: 62.8 µg/L and for RR: 64.7 µg/L. As shown in Table 1, the TPPP/p25 concentration in the non-MS samples is 27.9 µg/L. The Kruskal–Wallis test revealed significant differences between the TPPP/p25 levels of CIS or RR and non-MS patients ($\chi^2 = 22.6$, df = 2, $p < 0.00005$). The p values obtained by the Mann–Whitney U test for group comparisons show no clear distinction between CIS and RR patients ($p = 0.51$), but significant differences were found between values of CIS and non-MS ($p < 0.005$) and RR and non-MS ($p < 0.000005$). The values of TPPP/p25 concentrations determined in the CSF samples are presented in Fig. 2. The lumbar puncture was carried out at different disease states (during the relapse or in remission), but the TPPP/p25 concentration was independent of the time period between lumbar puncture and relapse/shub. The values of the TPPP/p25 concentration in the CSF samples for the non-MS samples are below 50 µg/L except one; the median value is 27.9 µg/L. This value is around twofold lower than those for CIS and RR samples. In the case of CIS patients, the TPPP/p25 content spans a wide range while the TPPP/p25 levels of the RR patients were found to be mostly above the value established for the non-MS samples. It could be, therefore, concluded that patients above the 50 µg/L TPPP/p25 level in their CSF suffer from MS or are potentially compromised individuals; control examinations and/or treatments of these individuals are needed.

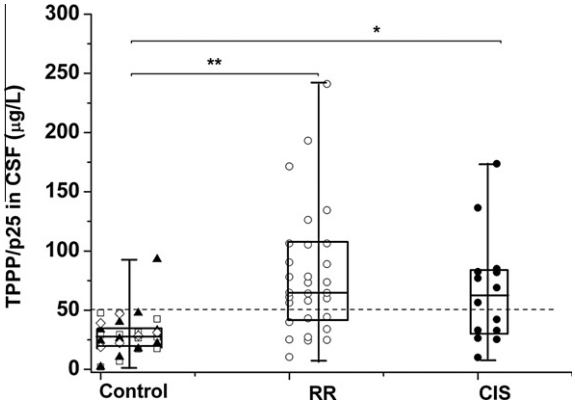

Fig. 2. Western blot to quantify TPPP/p25 level in CSF of MS and non-MS patients. TPPP/p25 level in CSF of patients quantified by Western blot using specific antibody against TPPP/p25 (32 control patients with other, non-MS disease (OND: □, OD: ▲, OIND: ◇), 30 RR patients, and 14 CIS patients). Box extends from the 25th to 75th percentile with the middle line representing the median. Whiskers indicate the full range of TPPP/p25 level. The p values were determined by Mann–Whitney U tests. $^{*}p < 0.005$; $^{**}p < 0.000005$. The dashed line corresponds to 50 µg/L.

4. Discussion

MS is an inflammatory demyelinating disease of the central nervous system and remyelination in MS ultimately fails. Although strategies to promote myelin repair are eagerly sought, mechanisms underlying remyelination *in vivo* have been elusive. TPPP/p25 is expressed mainly in OLGs in normal brain, however, it is enriched in pathological inclusions in the cases of Parkinson's disease and multiple system atrophy [10]. TPPP/p25 can induce aggregation of α-synuclein [16], displays high affinity to MBP, and colocalizes with it in myelin [17]. Recently, we have reported that TPPP/p25 depletion by siRNA or specific microRNA, mir-206, impeded the differentiation of progenitor OLGs, consequently, the development of projections required for the myelination/re-myelination [12]. TPPP/p25 as a microtubule-associated protein interacts with microtubular network affecting its stability and dynamics [18]. Thus we suggested that its physiological function in OLGs could be the reorganization and stabilization of the microtubular ultrastructures in the course of ensheathment of axons. Therefore, it can be expected that the de-myelination and/or defect of re-myelination of the axons is coupled with the enrichment of TPPP/p25 in the CSF of MS patients.

In this work we revealed that the concentration of TPPP/p25 in the CSF of MS patients is significantly increased as compared to that of the non-MS patients. As indicated in etable 1, the sensitive assay elaborated for quantification of TPPP/p25 for CSF samples showed that in the cases of the different non-MS patients, OND, OD and OIND, the TPPP/p25 concentration is below 50 µg/L, while this value was doubled in the case of MS patients. In our study in patients the EDSS scores varied between 0 and 7 (cf. etable 1), but no correlation was found between this score and the TPPP/p25 level. Although all the MS patients showed OCBs in CSF, no significant difference was found between the TPPP/p25 values in CIS and RR samples (cf. Table 1).

In the present study we demonstrated the enrichment of TPPP/p25 in MS CSF as compared to the non-MS ones as a characteristic feature of the clinical demyelinating disorders. Ohta and coworkers [7] published an ELISA for detection of MBP in MS CSF samples which was found to be more sensitive than the RIA assay elaborated previously [19]. However, the CSF MBP values were very much reduced before and after the acute attack periods and frequently became undetectable [8]. Comparing our present data obtained for TPPP/p25 level by Western blot assay with those obtained by RIA and ELISA for CSF MBP in the case of non-MS

Table 1
TPPP/p25 level in CSF and clinical data of patients with MS and non-MS.

	Non-MS	RR	CIS
Number of patients, *n*	32	30	14
Female, *n* (%)	20 (63%)	24 (80%)	11 (79%)
Age, year mean (*range*, *SD*)	36.9 (*19–65*, *12.3*)	37.9 (*18–55*, *10.4*) NS	35.9 (*18–55*, *12.7*) NS
Duration of sample storage, month median (*IQR*)	20.5 (*9.0–38.3*)	26.5 (*16.8–50.5*) NS	25.0 (*19.0–33.0*) NS
Total protein concentration, mg/ml median (*IQR*)	0.35 (*0.28–0.42*)	0.39 (*0.28–0.49*) NS	0.40 (*0.30–0.47*) NS
TPPP/p25 content in CSF, μg/L median (*IQR*)	27.9 (*19.9–34.9*)	64.7 (*42.3–105.7*)***	62.8 (*31.2–83.1*)**
% IgG level, median (*IQR*)	0.0	18.0 (*0.0–35.5*)***	5.0 (*0.0–33.3*)*
Steroid treatment, *n* median (*IQR*)	0.0	1.5 (*1.0–2.0*)***	1.0 (*0.0–1.0*)**
EDSS median (*IQR*)	NA	1.5 (*1.0–2.0*)	0.0 (*0.0–0.0*)

M, male; F, female; IQR, interquartile range, NA, not applicable, NS, not significant. *p*-values (Mann-Whitney test) for comparison between CIS and non-MS or RR and non-MS: ***$p < 0.00005$, ** $p < 0.005$, *$p < 0.05$. Significant difference was found between RR and CIS in the steroid treatment ($p < 0.005$) and in the EDSS scores ($p < 0.00005$). The other comparisons did not show significant differences.

samples, it is clear that the TPPP/p25 content is higher with about two orders of magnitude. It is interesting to note that in the case of the MS patients the CSF TPPP/p25 content varied in a narrower range (10–240 μg/L) as compared to that of the CSF MBP (0.03–20 μg/L). In addition, correlation study published for the RIA and ELISA showed that the former gave higher values than the latter at low concentrations while the inverse relationship was found at higher concentrations [7]. This obscurity could be due to the difficulty to quantify the very low MBP level. In fact, our Western blot analysis to quantify MBP content in the MS CSFs using commercial monoclonal antibody was not successful (data not shown).

As MS is a central nervous system disease, most of the diagnostic methods and biomarker research is focused on the analysis of CSF and serum/plasma. In this work we also assayed TPPP/p25 concentration in the sera of 7 MS and 6 non-MS patients, no significant difference was found between the patients and controls (data not shown). It should be, however, added that the extreme high concentration of the albumin/globulins and the presence of hemoglobin in the serum samples made the determination unreliable. At the same time, our data indicated the applicability of CSF samples, and TPPP/p25 enrichment in the CSF appeared to be a potential biomarker of MS. Nevertheless, analysis in larger group of patients suffering from MS or other neurological disorders will be helpful to demonstrate the specificity and sensitivity of our TPPP/p25 assay.

Previously, demyelinated lesions revealed loss of TPPP/p25-positive OLGs within the plaques in MS patients. In this work we provided evidence for the enrichment of TPPP/p25 in the CSF of MS patients as compared to non-MS ones by the assay for quantification of TPPP/p25 in CSF samples. The chemiluminescent Western blot assay is a sensitive diagnostic indicator of myelin breakdown in the central nervous system since it can distinguish CSF of MS patient from that of non-MS one; moreover, the TPPP/p25 content is independent of the age, gender and the time period between lumbar puncture and relapse/shub. Our investigations suggest the CSF-TPPP/p25 level could become diagnostic marker of MS and other demyelination-related diseases.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.04.130.

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