Contribution of Cerebrospinal Fluid Thymosin β4 Levels to the Clinical Differentiation of Creutzfeldt-Jakob Disease

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Objective: To assess thymosin β4 specificity as relevant to the diagnosis of Creutzfeldt-Jakob disease (CJD).

Design: A matrix-assisted laser desorption ionization time-of-flight mass spectrometry protein profiling analysis was applied to several neurological disorders that are known to lead to dementia. The relative peak area (percentage of area) of the thymosin β4 MS signal was taken into account.

Setting: National Research Council, Cosenza, Italy.

Patients: Cerebrospinal fluid analysis was performed on 21 patients with neuropathologically confirmed CJD; 15 patients with frontotemporal dementia; 18 patients with probable Alzheimer disease; and 9 patients with a rapid-onset progressive dementia. A non–cognitively impaired control group consisted of 25 individuals without CJD or dementia.

Results: The thymosin β4 cerebrospinal fluid levels appeared to be markedly increased in CJD samples compared with frontotemporal cases (P = 10^{-7}) and patients with Alzheimer disease (P = 10^{-7}). A lower significance was observed vs the group with rapid-onset progressive dementia (P = .0004). Thus, at a cutoff value of 1.2% of the thymosin β4 relative peak area, we estimated 100% sensitivity with 98.5% specificity.

Conclusion: These findings indicate that cerebrospinal fluid levels of thymosin β4 protein measured by matrix-assisted laser desorption ionization time-of-flight mass spectrometry may effectively contribute to discriminate CJD from other forms of dementia.

to define the clinical value of this biomarker, we extended the study to a number of other conditions that lead to dementia.

METHODS

SUBJECTS

In detail, patients included were 21 with pathologically proven CJD (7 with the genetic form E200K and 14 with the sporadic form); 15 with frontotemporal dementia (FTD); and 18 with probable Alzheimer disease (AD), all after clinical, neuropsychological, and neuroimaging evaluations according to the Neary et al criteria and National Institute of Neurological and Communicative Disorders and Stroke–Alzheimer’s Disease and Related Disorders Association criteria, respectively. Nine had rapid-onset progressive dementia, comprising 6 patients with T2-weighted and fluid-attenuated inversion recovery mediostriatal hyperintensities without atrophy whose diagnosis of limbic encephalitis was made on the features of limbic signs and symptoms (ie, seizures of temporal semiology, disturbance of episodic memory, affective disturbances with prominent mood lability, or disinhibition), 1 with Hashimoto encephalitis, 1 with acute demyelinating encephalitis, and 1 with stiff-person syndrome. A non–cognitively impaired control (NCIC) group consisted of 25 subjects including patients scanned for idiopathic intracranial hypertension and patients with various peripheral nervous system disorders. Furthermore, none of the control group had dementia, past or current acute or subacute central nervous system diseases, or other neurodegenerative disorders. The CSF samples were obtained by lumbar puncture during the care of patients. Once the samples were collected, they were immediately put on ice and visually inspected to assess blood contamination. All CSF samples appeared to be free of blood contamination; thus, the samples were centrifuged at 13,000 rpm for 10 minutes at 4°C to remove insoluble material. Finally, all the samples were divided into aliquots after the addition of a protease inhibitor cocktail (1 mM leupeptin, 1 mM aprotinin, 0.2 mM phenylmethanesulfonyl fluoride, and 2 mM sodium orthovanadate) and stored at −80°C. Some aliquots of the frozen samples were successively shipped to our laboratory on dry ice and stored at −80°C until further use. Control patients and patients with dementia were selected for the study so that all the CSF samples were comparable in approximate storage time. For each CSF sample, a written informed consent was obtained from the patients under a protocol approved by an institutional review board.

SAMPLE PREPARATION AND MALDI-TOF MS PROFILING

Protein content in the CSF samples was measured by a protein assay (Bio-Rad Laboratories) according to the manufacturer’s instruction. Tau protein concentration was estimated by enzyme-linked immunosorbent assay (Innotest hTAU-Ag; Innogenetics) according to the manufacturer’s instructions. A 14-3-3-protein assay was carried out using Western blotting, as previously reported, and included on each run as internal standards CSF samples both positive and negative for 14-3-3. Moreover, we included CSF samples from patients who did not have CJD but showed a weak positivity for 14-3-3. For the MALDI-TOF protein profiling analysis, an aliquot of each CSF sample, containing approximately 5 µg of total protein, was subjected to a de-salting/concentration step over a ZipTip C18 (Millipore Corporation). The eluted samples were typically mixed at a 1:1 volume to volume ratio with 5 mg/mL of α-cyano-4-hydroxycinnamic acid in acetonitrile, 50%, and trifluoroacetic acid, 0.1%; 1 µL of this solution was deposited onto stainless-steel target surfaces and allowed to dry at room temperature. Peptide/protein profiles were analyzed using a Voyager DE PRO MALDI-TOF mass spectrometer (PerSeptive Biosystems) equipped with a 337-nm nitrogen laser and delayed-extraction technology. Separate spectra were obtained for a restricted mass-to-charge range of 1000 to 25,000 Da in linear mode geometry by applying an acceleration voltage of 25 kV, and the delayed extraction was maintained at 250 nanoseconds to give an appropriate time lag focusing after each laser shot. Each individual spectrum, representing 5 accumulated subspectra, was obtained using 500 laser shots. The acquired raw spectra were then processed for automated advanced baseline correction and noise filter with Voyager Data Explorer software, version 4.1 (PerSeptive Biosystems). Finally, the peak area of thymosin β4 signal at 4963.7 mass to charge, derived from the total peak list, was normalized as a percentage of the total peak area (individual peak area percentage/total area of peaks). To evaluate intraexperimental variation, each sample was assayed in duplicate; for interexperimental variation, some samples were analyzed on separate days. Thus, the coefficient of variation was calculated. Data from the CJD and NCIC groups were in part the same used in our previous article.

DATA ANALYSIS

The relative peak area (percentage of area) of the thymosin β4 M5 signal assayed in duplicate, organized in a data set, was analyzed among patients applying the t test. The P values <.05 were considered significant. The diagnostic accuracy of the thymosin β4 biomarker was assessed using a receiver operating characteristic curve analysis. A previously determined optimal cutoff of 1.2% of the area was considered. The sensitivity, specificity, positive predictive value, negative predictive value, and efficiency were calculated. The area under the curve was determined using the Mann-Whitney U value divided by the number of subjects in each group (ie, area under the curve = U/n1 × n2, where n1 corresponded to the CJD group and n2, to the NCIC and dementia groups).

RESULTS

Results are summarized in Table 1 and Figure 1. A good reproducibility in the measurement of the thymosin β4 relative peak area (percentage of area) was observed for each sample both within a single assay and between the assays (coefficient of variation ranging between 5%-10%). The percentage of area of the thymosin β4 MS signal significantly increased in the patients with CJD compared with all the other patients. The highest significance was in comparison with the NCIC group (P = 10−19); nevertheless, an extremely significant difference was also observed in comparison with the FTD and AD groups (P = 10−7). Compared with the rapid-onset progressive dementia group, the thymosin β4 level was also elevated even if the significance was reduced (P = .0004). One of the patients with limbic encephalitis demonstrated a high thymosin β4 level. Receiver operating characteristic curve analysis using the optimized cutoff value (1.2% area) revealed a sensitivity of 100% and specificity of 98.5% for differentiating CJD from the other patient groups. The area under the curve was calculated to be 0.998 (Figure 2A). The positive predictive value and negative predictive value were 95% and 100%, respectively. The
efficiency (defined as true positives/total sample size tested) was 99%. We also estimated the specificity excluding the NCIC group from the receiver operating characteristic analysis; in so doing, we observed a negligible difference in the specificity (98%) and an area under the curve of 0.998 (Figure 2B). In Table 2, a full comparison of specificity, sensitivity, positive predictive value, negative predictive value, and efficiency of thymosin β4 assay against 14-3-3 and tau proteins is reported. Moreover, we assessed whether any correlation between thymosin β4 and tau levels was present among CJD cases, and a good correlation was found (r = 0.56; P = .008).

There is great interest in developing new specific biomarkers to support antemortem diagnosis of CJD because the 14-3-3 protein, which is the unique protein currently included in the diagnostic criteria, does not always have a high sensitivity.11-13 Furthermore, the specificity of this biomarker is poor and many conditions associated with acute neuronal damage may result in a positive CSF 14-3-3 finding, raising questions about the differentiation of sCJD from other neurodegenerative diseases.

Table 1. Baseline Clinical Features and Investigation Findings of All Patients

<table>
<thead>
<tr>
<th></th>
<th>NCIC</th>
<th>RPD</th>
<th>AD</th>
<th>FTD</th>
<th>CJD</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of patients</td>
<td>25</td>
<td>9</td>
<td>18</td>
<td>15</td>
<td>21</td>
</tr>
<tr>
<td>Sex, No.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M</td>
<td>15</td>
<td>2</td>
<td>7</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>F</td>
<td>10</td>
<td>7</td>
<td>11</td>
<td>8</td>
<td>14</td>
</tr>
<tr>
<td>Age, y, range</td>
<td>27-80</td>
<td>25-82</td>
<td>48-85</td>
<td>50-81</td>
<td>40-69</td>
</tr>
<tr>
<td>Tau level, pg/mL, mean (range)</td>
<td>140.33 (48-358)</td>
<td>468.8 (39-1607)</td>
<td>276.85 (137-525)</td>
<td>366.83 (111-1137)</td>
<td>5881.25 (741-17150)</td>
</tr>
<tr>
<td>14-3-3 Protein level, No.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>25</td>
<td>5</td>
<td>14</td>
<td>12</td>
<td>5</td>
</tr>
<tr>
<td>Weak-positived</td>
<td>0</td>
<td>1</td>
<td>4</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Positive</td>
<td>0</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>16</td>
</tr>
<tr>
<td>Thymosin β4, % of area, mean (SD)</td>
<td>0.45 (0.25)</td>
<td>0.87 (0.31)</td>
<td>0.61 (0.22)</td>
<td>0.32 (0.19)</td>
<td>2.93 (1.52)</td>
</tr>
</tbody>
</table>

Abbreviations: AD, Alzheimer disease; CJD, Creutzfeldt-Jakob disease; FTD, frontotemporal dementia; NCIC, non–cognitively impaired control; RPD, rapid-onset progressive dementia.

This does not include 4 patients with RPD who were not examined for tau protein level.

Results were assessed with Western blot assay.

These included 2 patients with sporadic CJD and 3 patients with genetic CJD.

All weak-positive samples were considered negative.

Figure 1. Comparison of thymosin β4 relative peak area (percentage of area) in the examined groups. A, Mean (SD) data statistically analyzed by t test. B, Individual values for each group. The line represents the optimal cutoff (1.2% area). AD indicates Alzheimer disease; CJD, Creutzfeldt-Jakob disease; FTD, frontotemporal dementia; NCIC, non–cognitively impaired control; and RPD, rapid-onset progressive dementia.

Figure 2. Receiver operating characteristic analysis at different cutoff points of cerebrospinal fluid thymosin β4 levels. A, Receiver operating characteristic analysis for diagnosis of patients with Creutzfeldt-Jakob disease (n = 21) vs all other patients (n = 67). The area under the curve was 0.998. B, Diagram for patients with Creutzfeldt-Jakob disease vs patients with dementia (n = 42). For this curve, the area under the curve was 0.998.
characterized by a rapidly progressive dementia. Comparable sensitivity was revealed for tau protein and S100B especially in the early stage of sCJD, in contrast with the findings of other studies in which tau has been suggested to be the most sensitive marker in the early stage of sCJD. However, the specificity of these proteins in the complex is still poor, and although their association may improve the sensitivity, the specificity often remains suboptimal. The aim of this study was to investigate whether a proper differential diagnosis of CJD could be made using the CSF levels of thymosin β4 that were previously reported by our team to be consistently and specifically expressed in the CSF of patients with CJD. Thus, we compared the thymosin β4 levels (MS relative peak area) in the CSF of patients with CJD with those of patients with other forms of dementia to assess the differences among groups. The results showed a marked increase of thymosin β4 level in all 21 patients with CJD compared with all the other patients, supporting our initial hypothesis. Only 1 patient with limbic encephalitis demonstrated a thymosin β4 level higher than the optimized cutoff value (1.2% area). Therefore, this biomarker might reveal a sensitivity of 100% with a specificity of 98.5% for differentiating CJD from the other patient groups. Moreover, a full comparison on the examined samples between the 14-3-3, tau, and thymosin β4 biomarkers showed higher value of sensitivity and efficiency for this last protein. The reason for the increased CSF thymosin β4 level in CJD, as well as the pathogenic relevance of this protein, is still uncertain. The release of this brain-derived protein into the CSF is likely correlated, like 14-3-3 protein, tau, and neuron-specific enolase, with its leakage following rapid neuronal damage. However, it may have arisen principally from the glial hyperactivation (gliosis) given that thymosin β4 has been detected mainly in human glial cells and demonstrated as being upregulated in hyperactivated microglial cells. Although overall function of thymosin β4 is known, the effect on the central nervous system has not been completely clarified. There is evidence that thymosin β4 is expressed in the developing brain where it regulates outgrowth of growing neuritis on neurons, likely playing a role in neuroprotection, synaptogenesis, axon growth, cell migration, and plastic changes. Insufficient data have been reported on the protein levels of thymosin β4 in central nervous system tissue in healthy and pathological conditions. The expression of thymosin β4 increases in focal rat brain ischemia and in rat brain after global transient hypoxia, whereas a downregulation of thymosin β4 gene expression occurs in prion-infected mouse neuronal cells. Interestingly, an overexpression of thymosin β4 has been demonstrated in the glomeruli of rats used as models for segmental glomerulosclerosis, which plays a role in sclerosis interacting with complement proteins. Because there are indications that complement components may be involved in the prion disease, the understanding of the interaction between thymosin β4 and complement proteins in the central nervous system might reveal new molecular mechanisms that could be responsible for the neuronal alterations. In summary, based on these findings, despite the fact that the relatively small number of patients might limit the conclusion taken from this analysis, we propose that CSF levels of thymosin β4 protein could be effectively used to discriminate CJD from other forms of dementias.

Table 2. Sensitivity, Specificity, PPV, NPV, and Efficiency for Each Marker

<table>
<thead>
<tr>
<th>14-3-3 Protein</th>
<th>Tau Protein</th>
<th>Thymosin β4 Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>CJD</td>
<td>16/21</td>
<td>21/21</td>
</tr>
<tr>
<td>Other dementia</td>
<td>3/67</td>
<td>1/63</td>
</tr>
<tr>
<td>Sensitivity, %</td>
<td>71</td>
<td>99</td>
</tr>
<tr>
<td>Specificity, %</td>
<td>95</td>
<td>98.4</td>
</tr>
<tr>
<td>PPV, %</td>
<td>84</td>
<td>95</td>
</tr>
<tr>
<td>NPV, %</td>
<td>93</td>
<td>97</td>
</tr>
<tr>
<td>Efficiency, %</td>
<td>91</td>
<td>96</td>
</tr>
</tbody>
</table>

Abbreviations: CJD, Creutzfeldt-Jakob disease; NPV, negative predictive value; PPV, positive predictive value.

The numbers indicate the positive samples divided by the total samples investigated.

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REFERENCES