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.

**Main Outcome Measures:** The thymosin β4 test results in CJD and other 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.


**CREUTZFELDT-JAKOB DISEASE (CJD)** is a rare, fatal neurodegenerative disease belonging to the group of transmissible spongiform encephalopathies or prion diseases. A progressive cognitive dysfunction resulting in dementia represents the main clinical sign and most patients show a spectrum of additional neurological disturbances, including symptoms and signs of motor system dysfunction. Considerable progress in understanding prion disease has improved the knowledge on CJD, and several neuropathological and biochemical findings have been reported to support the clinical diagnosis. In particular, in recent years cerebrospinal fluid (CSF) analysis has become increasingly important in the diagnosis of sporadic CJD (sCJD). Currently, the altered levels of some brain-derived proteins in CSF, like the 14-3-3 protein, tau protein, neuron-specific enolase, and S100B protein, accompanied by characteristic clinical manifestations and typical changes on electroencephalogram and magnetic resonance imaging scans, are highly suggestive of the disease. However, the specificity of each diagnostic criterion does not reach 100%. For these reasons, the clinical diagnosis of CJD may sometimes be difficult in routine practice, as well as remaining problematic for differentiating sCJD from other neurological conditions leading to dementia that may resemble the clinical features. Recently, by applying a proteomic gel-free mass-based approach using matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) protein profiling, increased levels of thymosin β4 protein were observed for the first time in the CSF of patients with CJD. The use of an independent immunoassay technique provided validation of these results. To evaluate thymosin β4 specificity as relevant to the diagnosis of CJD, and
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 hypointensities without atrophy whose diagnosis of limbic encephalitis was made on the features of limbic signs and symptoms (ie, seizures of temporal semiology, disturbance of epicerebral memory, affective disturbances with prominent mood lability, or dis-inhibition), 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 × g 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.

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 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 = .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/true negatives/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.
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.

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