Case Report

Value of Different Markers in Cerebrospinal Fluid for Diagnostics of Creutzfeldt-Jakob Disease: Evaluation in Three Cases*

Wertigkeit verschiedener Marker im Liquor cerebrospinalis bei der Diagnostik der Creutzfeldt-Jakob Krankheit: Evaluation an drei Fällen

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Abstract: Creutzfeldt-Jakob disease (CJD) is a very rare fatal neuronal disease with an incidence of about $1:10^6$. Diagnosis of CJD is often very complicated and requires a great number of clinical and laboratory methods. Although prior studies have shown increased values of neuron-specific enolase (NSE), protein S-100, protein tau, creatine kinase (CK-BB) and 14-3-3 protein in cerebrospinal fluid (CSF) of CJD patients, these markers have not been comparatively evaluated and repeated determinations over a longer observation time are often lacking. This study therefore compares the diagnostic value of these parameters in CSF of three CJD patients. Conventional parameters like cell number and morphology as well as CSF/serum-ratios of immunoglobulins were also determined. Concentrations of NSE, protein S-100 and protein tau were strongly increased, whereas CK-BB showed only slight variations and the results of the conventional CSF parameters were within the reference limits. In one of the three patients CSF analyses were performed four times within an observation time of three months. Concentrations of protein tau in CSF continuously increased, whereas concentrations of NSE and protein S-100 did not. Although the number of investigated patients is very small the data obtained suggests that the determination of NSE, protein tau, protein S-100 and CK-BB can be a useful tool for supporting the diagnosis of Creutzfeldt-Jakob disease.

Keywords: Creutzfeldt Jakob Syndrome/cerebrospinal fluid; Cerebrospinal Fluid Proteins; tau Proteins/cerebrospinal fluid; Phosphopyruvate Hydratase/cerebrospinal fluid.

Zusammenfassung: Die Creutzfeldt-Jakob Erkrankung (CJD) stellt mit einer Inzidenz von etwa $1:10^6$ eine seltene und bisher nicht behandelbare neurologische Erkrankung dar. Die Diagnose der Erkrankung ist oft kompliziert und erfolgt daher unter Anwendung zahlreicher Untersuchungsmethoden. In vorangegangenen Untersuchungen konnten im Liquor cerebrospinalis von Patienten mit Creutzfeldt-Jakob Erkrankung erhöhte Konzentrationen der neuronenspezifischen Enolase (NSE), Protein S-100, Protein Tau, Creatinkinase (CK-BB) und Protein 14-3-3 nachgewiesen werden. Vergleichende Untersuchungen der diagnostischen Wertigkeit dieser Parameter sowie Verlaufsbeobachtungen fehlen jedoch meist. Im Rahmen der vorliegenden Untersuchung wurden daher diese sowie konventionelle Parameter der Liquordiagnostik wie Zellzahl, Zellmorphologie und Liquor/Serum-Quotienten der Immunglobuline bei insgesamt drei Patienten mit Creutzfeldt-Jakob Erkrankung bestimmt. Dabei zeigten sich im Liquor cerebrospinalis stark erhöhte Konzentrationen von NSE, Protein S-100 und Protein Tau, während die CK-BB nur eine geringe Zunahme der Aktivität aufwies und die konventionellen Parameter innerhalb der jeweiligen Referenzbereiche lagen. Bei einem der drei Patienten konnten während der Beobachtungszeit von drei Monaten insgesamt vier Liquoruntersuchungen durchgeführt werden. Dabei zeigte die Konzentration von Protein Tau eine ständige Zunahme, während für die Konzentrationen von NSE und Protein S-100 kein Anstieg nachweisbar war. Die erhaltenen Daten zeigen, daß eine Bestimmung der Konzentrationen von NSE, Protein Tau, Protein S-100 und CK-BB im Liquor cerebrospinalis geeignet ist, zur Diagnostik der Creutzfeldt-Jakob Erkrankung beizutragen.

Schlüsselwörter: Creutzfeldt-Jakob Erkrankung/Liquor cerebrospinalis; Liquorproteine; tau-Proteine/Liquor cerebrospinalis; Phosphopyruvathydratase/Liquor cerebrospinalis.

Creutzfeldt-Jakob disease (CJD) is a very rare and incurable disease which mostly affects individuals after the fifth life decade and rapidly leads to death within a few months after symptom onset [1-3]. Clinically the disease is characterized by an uncharacteristic prodromal phase with various non-specific neurological

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and psychiatric symptoms followed by rapidly progressive dementia. Within the clinical course of the disease a combination of serious neurological symptoms like myoclonus, pyramidal or extrapyramidal signs and symptoms, visual or cerebellar signs or symptoms and akinetic mutism can be observed which is considered typical for or highly suggestive of CJD [3-7]. More than 60% of the patients show typical periodic sharp wave complexes in the electroencephalogram (EEG), but these findings are not specific for CJD [5, 8]. Other methods like computer tomogram or nuclear magnetic resonance imaging (NMR) also fail to prove the diagnosis of CJD, but are necessary to exclude other neurological diseases like ischemic stroke, hemorrhages and cerebral tumors or metastases [5, 6]. Therefore up to now the definite diagnosis of CJD can only be made by brain biopsy or autopsy [1, 5, 4]. Although the diagnosis is hampered by the lack of specific biochemical markers several attempts have been made for analysis of different proteins in cerebrospinal fluid. The detection of 14-3-3 proteins by a western blot procedure has been shown to be highly specific for CJD if other neurological diseases like acute viral encephalitis, acute stroke, subarachnoid hemorrhage or cerebral metastases are excluded [4, 9, 10]. However, this qualitative assay is technically demanding and lengthy and therefore cannot be used for routine analysis. In CSF of patients with CJD other studies have found increased concentrations of (1.) brain-type creatine kinase (CK-BB), the phosphate transporting enzyme in ATP metabolism [11], (2.) neuron-specific enolase (NSE), a glycolytic enzyme [6, 11, 12], (3.) protein S-100, an acidic calcium-binding protein located in glial cells and Schwann cells [5, 11] and (4.) protein tau, a neuronal axonal structure protein [13]. However, up to now only few studies have compared the diagnostic value of these markers at different times of the clinical course [11].

Materials and methods

Determination of protein tau

Concentration of protein-tau was measured by a commercially available sandwich-type ELISA (Innogenetics, Belgium). In the test 25 µl cerebrospinal fluid (CSF) and 75 µl conjugate solution 1 (contains two different biotinylated monoclonal antibodies against protein tau, HT7 and BT2) were added to each well. After incubation for 24 hours and washing of the wells 100 µl/well conjugate solution 2 (contains peroxidase-conjugated streptavidine) were added and incubated for 30 minutes. After washing once more 100 µl/well color substrate were added. The color reaction was stopped by adding 100 µl/well 2 mol/l H_2SO_4 and absorbances were measured at 450 nm.

Non standard abbreviations: CJD, Creutzfeldt-Jakob disease; CK-BB, creatine kinase BB band; CSF, cerebrospinal fluid; ECG, electrocardiogram; EEG, electroencephalogram; ELISA, enzyme-linked immunosorbent assay; Mr, relative molecular mass; NMR, nuclear magnetic resonance; NSE, neuron-specific enolase; Pt, particle.

Determination of neuron-specific enolase (NSE)

Neuron-specific enolase was determined by a immunoradiometric method (NSE IRMA, Sangtec Medical). In principle, the assay uses two monoclonal antibodies against different epitopes of the γ-subunit of NSE. 25 µl sample, control or standard solutions were added to a plastic tube. In a next step 200 µl tracer solution (contains a ^{125}J labelled antibody against NSE) and a plastic bead (coated with another antibody against NSE) were added which allows the indirect binding of the tracer antibody to the bead. After an incubation time of 3 hours at room temperature unreacted tracer antibody was removed by washing three times with demineralized water. Finally radioactivity was measured by means of a gamma counter and sample NSE concentrations were calculated by using the standard curve.

Determination of protein S-100

Protein S-100 was measured by a commercial monoclonal two-site immunoluminometric assay (LIA-mat Sangtec 100, Sangtec Medical). In a first step 100 µl diluent and 100 µl sample, control or standard solution were added to antibody coated polystyrene tubes which served for solid phase. After 1 hour incubation at room temperature the tubes were washed three times with 2 ml wash fluid. Then 200 µl tracer antibody (with covalently bound isoluminol derivative) were added to each tube and incubated for two hours at room temperature. Excess of the tracer antibody was removed by washing once more three times with 2 ml wash fluid. Finally the oxidation of isoluminol was started by addition of alkaline peroxide solution and catalyst and luminescence was immediately measured at 425 nm by means of a luminometer. Concentrations of protein S-100 were then calculated by use of the standard curve.

Determination of creatine kinase (CK-BB)

Activity of creatine kinase was determined automatically by means of a Beckman CX7 analyser (Beckman Instruments, Fullerton) under routine conditions. Due to the weak stability of the enzyme CK-BB was not measured in samples after freezing. Therefore CK-BB was only measured in 3 of 7 samples.

Determination of other parameters: Concentrations of albumin, IgG, IgA and IgM were measured nephelometrically in serum and CSF (Behring Nephelometer, Germany). Corresponding CSF-serum ratios of albumin, IgG, IgA and IgM were calculated according to *Reiber* [14]. CSF concentrations of total protein and glucose were determined on a Beckman CX7 analyser (Beckman Instruments, Fullerton). Cell concentration, cell morphology and activated B-lymphocytes were determined by means of a microscope. Analyses for oligoclonal IgG were performed by isoelectric focussing and western blot (Helena, Hartheim, Germany).

Case reports

Case 1

A 59-year-old woman has been hospitalized after anxiety and fear over the last three years and approximately six months of non-specific neurological symptoms increasing from slight ataxia and vertigo to severe neurological symptoms including dysarthria, nausea and dizziness, tremor, paralysis and myoclonus as well as psychopathological deficits (confusion in time, visual and acoustic hallucinations, affective symptoms and fear). A first stay in another hospital two months previously could not clear the etiology of the disorder. At this time clinical signs were more severe compared with the beginning but still limited to vertigo and ataxia as well as initial disturbances of cognitive and mnemonic functions.

Electrocardiogram (ECG) and the X-ray examination of the chest showed a small cardiovascular risk and the EEG minimal general disturbances. In summary, microangiopathic changes and slight stenosis of extracranial arteries were regarded as the main reason of the disorders. The rapid change for the worse of neurological states to dysarthria, aphasic and apractic dysfunctions, tremor of both hands and the trunk at rest and intentionally, rapid myoclonus, dysmetria, hypo- and akinesia, or cramps of the muscles of the lower extremities and increasing paralysis as well as an incontinence led to death in few months.

Other medical, social or occupational risks were not found, the patient only suffered from a dysfunction of the thyroid gland. In the family her mother died after a lymphoma. The diagnosis of Creutzfeldt-Jakob disease was confirmed by the clinical signs, including myoclonus and the EEG of rhyrthmic biphasic waves (3 to 4/sec). Analyses of the CSF sample taken three months after symptom onset at the beginning of the hospital stay showed a cell concentration of 1 MPt/l (normal range: <5 MPt/l) and no variations of cell morphology. CSF-serum ratios of albumin and immunoglobulins IgG, IgM and IgA were also within the reference limits and showed no alterations of the blood brain barrier and no local synthesis of immunoglobulins. Activated B-lymphocytes as well as oligoclonal IgG were also not detectable. On the other hand strong increases were observed in the concentrations of neuron-specific enolase (NSE) and protein tau (see table 1). The result of the analysis for protein 14-3-3 in CSF was also positive.

Case 2

A 54-year-old woman was initially hospitalized because of vertigo and paresthesia of both hands as well as mental problems over a few months. After the first diagnosis of a primary hyperparathyroidism the latter was surgically treated and serum concentrations of calcium and parathormone decreased to reference values. In contrast to the normalization of these laboratory findings the patient showed a rapid increase of psychological and neurological symptoms to somnolence, confusion, hemiparalysis of the left side with an increased reflexive state of the muscles and Babinski sign, hyperextension of the head and a tremor on both sides. After only a few weeks we found provoked and spontaneous myoclonus, cramps and rigidity of the muscles, primitive reflexes (e.g. sucking, licking) and the patient was not able to cooperate. In the EEG we found severe general dysfunctions (3 to 4 waves/sec) and rhythmic waves from the theta-spectrum and triphasic sharp waves all over the fields as well as small spikes. The patient died four months after the onset of the first clinical symptoms and two weeks after hospitalization in a nursing home. The clinical diagnosis of Creutzfeldt-Jakob disease was confirmed by neuropathological examination after death. Cerebrospinal fluid was analyzed twice within an observation period of one month. Total cell concentrations at both time points were 1 MPt/l and showed normal cell morphology. CSF-serum ratios of albumin and immunoglobulins IgG, IgM and IgA were within normal limits. Activated B-lymphocytes as well as oligoclonal IgG were not detectable. CSF concentrations of CK-BB, NSE, protein S-100 and protein tau were increased at both observations (see table 1). Protein 14-3-3 was determined in only one of the two samples and showed a positive result.

Case 3

The 61-year-old woman was hospitalized about two months after the first symptoms of progredient ataxia.

Table 1 Marker concentrations in three cases of Creutzfeldt-Jakob disease

Case number	Measurement number	CK [µmol/l*s]	NSE [µg/l]	protein S-100 [µg/l]	protein tau [pg/ml]
1	1	–	141.8	–	19215
2	1	–	35.0	7.9	9233
2	2	0.18	94.0	12.4	9328
3	1	0.07	20.1	7.6	5387
3	2	–	38.0	6.6	7706
3	3	0.11	18.8	6.4	7913
3	4	–	26.7	7.9	8898
Reference values		0.01-0.04[a]	<6.5	<0.5[a]	66.2-276[a]

[a]) as described by the manufacturer of the test kit

According to her husband she had fallen over while walking. During the first stay in a hospital these symptoms were seen as sensible ataxia, but also cerebellar and spinal disorders and an infectious process were supposed. In the first examination in our clinic the patient showed a very slight dysarthria, disturbances of eye movements, differences of muscular reflexes in favor of the left hand side and single muscular convulsions of the left arm as well as dysdiadochokinesia. Regarding the ataxia she was unable to stand and walk and fell down without help. Romberg´s sign was positive and a pallhypesthesia of the lower limbs was found. Only five to six weeks later the neurological symptoms and deficits had strongly increased and an unchangeable orientation and consciousness, a severe dysarthria and a severe dementia were observed. Generalised myoclonus and an increased muscular tension were seen. Psychological tests confirmed the clinical impression of a rapidly pronounced dementia, particularly concerning orientation, memory and visuoconstructive functions. The diagnosis of Creutzfeldt-Jakob disease was confirmed by EEG and clinical signs. In addition, the NMR showed a special feature with a higher signal in the T2 in the caput nuclei and putamen. The patient died a few months after the first clinical symptoms. Cerebrospinal fluid was analyzed four times within the observation period of three months. Total cell concentrations were found between 1 MPt/l and 2 MPt/l showing normal cell morphology. CSF-serum ratios of albumin and immunoglobulins IgG, IgM and IgA were within normal limits, activated B-lymphocytes and oligoclonal IgG were not detectable whereas concentrations of CK-BB, NSE, protein S-100 and protein tau were increased at all times. In contrast to the concentrations of CK-BB, NSE and protein S-100 the concentration of protein tau further increased during CJD progression (see table 1). Analysis for protein 14-3-3 was performed in only one of the samples and showed a positive result.

Discussion

This study describes the laboratory findings and clinical course of three patients with clinically suspected Creutzfeldt-Jakob disease (CJD). Based on the clinical findings and the results of the EEG all three patients were classified as "probable CJD" in accordance with the criteria published by *Masters* et al. [3, 4]. However, an autopsy was performed in only one of these patients. Therefore the diagnosis "definite CJD" resulted in only one of the observed patients. The age of all three patients as well as the observed clinical course stand in good agreement with prior studies which showed that most CJD patients are aged more than 50 years and die within 12 months after disease onset [1, 2, 3].

Aim of this study was the comparative evaluation of cerebrospinal fluid (CSF) parameters in CJD patients. Prior studies have shown that brain protein 14-3-3, a protein which plays a role in the conformational stabilization of other proteins [9], can be detected in CSF samples of patients with CJD. Although brain protein 14-3-3 can be detected in CSF of patients with other neurological diseases like viral encephalitis, acute stroke, subarachnoid hemorrhage and cerebral metastases, detection of this protein has been shown to be highly sensitive and specific for CJD after exclusion of these other diseases [4, 9, 10]. Immunological analyses for protein 14-3-3 were performed in an external laboratory and showed positive results in CSF samples of all three patients. However, the detection of protein 14-3-3 is tedious and therefore not available under routine conditions and in addition cannot provide information on the clinical stage, severity or source of the disease [9, 10].

In our study we determined "conventional" parameters of CSF (total cell number, cell morphology, CSF-serum ratio of albumin and immunoglobulins (IgA, IgM and IgG) and oligoclonal IgG) as well as creatine kinase (CK-BB), protein S-100, neuron-specific enolase (NSE) and protein tau. The results of our three cases demonstrate that "conventional" analyses of CSF fail to give any useful information for diagnosis and progression of CJD. These results stand in ample agreement to prior observations which also found normal or only slightly increased values of these parameters in CJD patients [6, 11]. On the other hand parameters of neuronal cell destruction (CK-BB, NSE, protein S-100 and protein tau) are increased in all three cases. These results confirm the observations of *Jimi* et al., who found increases of CK-BB, NSE and protein S-100 [11], *Otto* et al., who observed increased values of protein S-100 [5], as well as *Zerr* et al. [6], who described increased concentrations of NSE to be a useful marker for diagnosis of CJD by means of CSF analysis. Protein tau, an axonal structure protein, is found in various isoforms differing in their phosphorylation which are detectable by means of the used ELISA irrespective to their phosphorylation [15]. Increased concentrations of protein tau in CSF have been reported in patients with Alzheimer's disease when compared to controls [16, 17, 18]. However, increased concentrations of protein tau in CSF have also been found in demential diseases other than Alzheimer's disease [16] and non-demential neurodegenerative diseases like multiple sclerosis, acute encephalitis and acute vascular disorders [*Siekmeier*, unpublished]. Recently strong increases of protein tau in CSF of CJD patients in comparison to other demential diseases and controls were reported by *Otto* et al. [13]. However, *Otto* et al. have not evaluated the diagnostic value of protein tau in CSF in comparison to other proteins in respect to its time dependent behavior at different stages of CJD.

In our cases we observed a strong variability of CSF concentrations of CK-BB, NSE, protein S-100 and protein tau which might be caused by several factors.

In principle, secretion of cellular proteins into CSF is affected by their molecular weight, their cellular function and localization (structure proteins or en-

zymes, cytoplasmatic or mitochondrial localization), their distribution within the different cells of the central nervous system (i.e. neuronal or glial cells) and in general by the stage of disease activity and cell destruction. Neuron-specific enolase (NSE; Mr: 78 000 Dalton) is a glycolytic enzyme, which is localized in cytoplasm and axons of neuronal cells, extracerebral neuroendocrine cells and numerous extracerebral malignant tumors. Protein tau (Mr: 69 000 Dalton) has been characterized as a structure protein localized in neuronal axons. The brain specific isoform of creatine kinase (CK-BB; Mr: 80 000 Dalton) has been found in neurons, astrocytes and outside of the central nervous system (CNS) in smooth muscle cells of the gastrointestinal tract, bladder and uterus. Protein S-100 an acidic calcium-binding protein (Mr: 21 000 Dalton) has been predominantly found in glial cells and Schwann cells but also in heart, skeletal muscles and kidney. The abnormally high concentrations of these proteins in our samples are obviously caused by leaking from damaged cells into CSF. The increased concentrations of "neural" and "glial" proteins in CSF suggest that CJD affects not only neural cells but also glial tissues.

In one of our patients we took CSF samples at four different time points which allows the time dependent analysis of the concentration profiles of these proteins at different stages of CJD. We observed a continuous increase of the concentration of protein tau in CSF whereas the other proteins showed only minor variations. This observation confirms the results of *Skoog* et al. who found a relation between CSF concentration of protein tau and severity of dementia and brain atrophy [16]. However, the intensity of the observed increases might by affected by the stage of CJD and it can be speculated that in terminal stages of CJD when most neural cells are lost, concentrations of these proteins decrease [11].

Numerous investigations have shown increases of CK-BB, NSE, protein S-100 and protein tau also in other neurological degenerative, vascular and inflammatory diseases like Alzheimer's disease, Parkinson's disease, multiple sclerosis, cerebral infarction or bleeding, meningitis and encephalitis. The results of these studies imply that increased concentrations of the measured proteins are not specific for CJD. However, chronic degenerative demential neurological diseases commonly cause no or only mild increases of these proteins in CSF and therefore the differences were striking when compared to CJD. On the other hand acute cerebral disorders have other manifestations and are clinically distinguishable from CJD. The data therefore suggest that the determination of NSE, protein tau, protein S-100 and CK-BB can be useful for supporting the diagnosis of CJD.

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