Desmoplakin as a Potential Candidate for Cerebrospinal Fluid Marker to Rule Out 14-3-3 False Positive Rates in Sporadic Creutzfeldt-Jakob Disease Differential Diagnosis

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CSF marker · Creutzfeldt-Jakob disease · Desmoplakin · 14-3-3 · Tau

Abstract
Background: The detection of a 14-3-3 elevated level in cerebrospinal fluid (CSF) is a part of the diagnostic criteria for probable sporadic Creutzfeldt-Jakob disease (sCJD), as defined by the WHO. However, some pathological conditions associated with acute neuronal damage may result in a positive 14-3-3 test and thereby reduce test specificity in sCJD.

Objective: Desmoplakin has been previously identified as up-regulated CSF protein in sCJD and these studies aimed to investigate its diagnostic utility and compare it with two known CSF markers, 14-3-3 and tau.

Methods and Results: We tested CSF levels of 14-3-3, tau and desmoplakin in 58 sCJD patients and 81 control patients including 45 cases with an elevated 14-3-3 level due to other disease than sCJD. We detected an elevated CSF level of desmoplakin in 78% of the sCJD patients, while 14-3-3 (88%) and tau (91%) showed a higher positive rate. However, the false positive rate of newly tested desmoplakin was significantly lower in comparison to 14-3-3 and tau, and it accounted for only 11% versus 56% and 35%, respectively. Further reduction of false positive rates was achieved by combination of elevated tau level with a positive desmoplakin test. Moreover, in the non-sCJD group, desmoplakin level did not correlate with the level of both above-mentioned CSF markers, whereas a clear correlation was observed in the sCJD group.

Conclusion: Desmoplakin showed a low positive rate accompanied by a very low false positive rate. Thus, we conclude that desmoplakin is a promising candidate for supportive CSF marker to rule out 14-3-3 false positive cases in sCJD differential diagnosis.

Introduction

Creutzfeldt-Jakob disease (CJD) is a rare, fatal and rapidly progressing neurodegenerative disorder that belongs to the family of transmissible spongiform encephalopathies (TSEs). Sporadic CJD (sCJD) remains the most common TSE form worldwide as it accounts for approximately 85% of all human TSE cases.

TSEs are characterized by the accumulation of a scrapie form of prion protein (PrPSc) in the central nervous tissue. PrPSc differs from the cellular isoform (PrPc) by the higher β-sheet content, partial resistance to protease digestion and tendency to aggregation in the central nervous tissue [1].
sCJD has unusual degree of phenotypic heterogeneity, which is mainly influenced by the methionine/valine (M/V) polymorphism at codon 129 in the gene encoding PrP and by the presence of two major types of the protease-resistant form of the PrP (type 1 and type 2) [2]. This large spectrum of phenotypic variability has made the diagnosis of human prion diseases a demanding task.

Although neuropathological examination of the brain tissue remains the only way to obtain a definitive sCJD confirmation, the initial diagnosis still bases on the clinical phenotype defined by WHO diagnostic criteria [3]. The detection of 14-3-3 protein in cerebrospinal fluid (CSF) is a supportive tool with the highest diagnostic utility in sCJD diagnosis. However, the specificity and sensitivity of 14-3-3 protein test strongly depends on appropriate clinical context. Many pathological conditions associated with acute neuronal damage may result in positive 14-3-3 and thereby reduce 14-3-3 protein test specificity in sCJD [4, 5]. Therefore, other brain-derived proteins in CSF may have also diagnostic utility and support sCJD diagnosis. These additional CSF markers include S100b, NSE or tau protein. In particular, tau elevated more, we have analyzed efficiency of two tests combination (14-3-3 protein + desmoplakin, 14-3-3 protein + tau protein or tau protein + desmoplakin) in clinical diagnosis of sCJD [6, 7]. Moreover, the combination of several markers could be used to make sCJD diagnosis more accurate and precise [6, 8].

In our previous studies investigating CSF proteome alterations in sCJD, we could identify 33 differentially regulated proteins [9]. Subsequently, a truncated form of desmoplakin, which showed the highest up-regulation in sCJD, was selected for further examination with respect of its application in sCJD differential diagnosis.

In this study, we have aimed to investigate the diagnostic utility of desmoplakin in sCJD diagnosis. Furthermore, we have analyzed efficiency of two tests combination (14-3-3 protein + desmoplakin, 14-3-3 protein + tau protein or tau protein + desmoplakin) in clinical diagnosis of sCJD with emphasis to exclude false positive rates.

**Material and Methods**

**Patient Cohorts**

All patients were referred to German National Reference Center for Surveillance of Transmissible Spongiform Encephalopathy during the process of clinical diagnosis and surveillance of human TSEs. Among 58 sCJD patients with a median age of 68.5 years (range 38–79), 30 patients were classified as clinically probable sCJD and 28 patients had pathologically confirmed sCJD. For 56 sCJD patients (29 probable and 27 pathologically confirmed cases) polymorphism of PRNP codon 129 was determined.

The control non-sCJD population comprised the following two groups: the ‘14-3-3 false positive’ group (45 patients; selected on the basis of a positive 14-3-3 test and non-CJD diagnosis) and the ‘alternative clinical diagnosis’ group (36 patients; selected on the basis of initial referral as suspected CJD case, but an alternative clinical diagnosis was made at follow-up) with the median age of 70 years (range 37–84) and 68 years (range 37–87), respectively. A comparison of age by Mann-Whitney test did not reveal any statistically significant difference between patient populations. The calculated p value between the sCJD group and the ‘14-3-3 false positive’ group was 0.78; between the sCJD group and the ‘alternative clinical diagnosis’ group it was 0.36. The individual diagnoses of all patients are given in table 1.

This project was approved by the Ethics Committee of the University Medical Center Göttingen (11/11/93 supplemented in 18.09.1996 and 12.09.2002).

**CSF Samples**

CSF was taken by lumbar puncture during the diagnostic procedure and transferred to the German National Reference Center for Surveillance of Transmissible Spongiform Encephalopathy in order to carry out diagnosis towards CJD. Blood-contaminated CSF samples were excluded from the studies. The CSF samples were stored at −80 °C prior to analysis.

**Western Blot for 14-3-3 Protein**

14-3-3 protein in CSF was detected by Western blot after SDS-PAGE with chemiluminescent visualization [10, 11]. As a primary detection antibody, either rabbit polyclonal anti-14-3-3-β (1:2,000, Santa Cruz Biotechnology) or rabbit polyclonal anti-14-3-3-β (1:3,000, Abcam) was used; variations in primary antibody had no influence on the test results. Positive and negative controls were included in each run. The positive controls consisted of recombinant 14-3-3-β protein (Abfrontier) and 14-3-3-β positive CSF from sCJD patients, and the negative control consisted of CSF from patients with clinical diagnosis of an alternative disease. The quality control of performed tests is proven by a yearly ring trial between worldwide TSE diagnostic centers.

**Western Blot for Desmplakin**

Sixty microliters CSF were separated on 12% SDS-PAGE gels and transferred to PVDF membranes. Membranes were blocked with 5% skimmed milk in phosphate buffer saline with 0.2% Tween-20 (PBST) for 1 h at room temperature. Subsequently, membranes were incubated overnight at 4 °C with rabbit polyclonal anti-desmoplakin I + II antibody (1:750, Abcam). Thereafter, membranes were washed with PBST and incubated for 1 h at RT with corresponding horseradish peroxidase-conjugated secondary antibody: goat anti-rabbit (1:7,500, Jackson Research). The immunoreactivity was detected after immersing the membranes into enhanced chemiluminescence solution and exposing to chemiluminescence Hyperfilm (Amersham Biosciences). A positive control consisting of a 14-3-3-positive CSF from a sCJD patient was included in each run.

**ELISA for Tau Protein**

Tau protein in CSF was measured using commercially available ELISA (Innotest hTau-Ag; Inogenetics) according to the manufacturer’s protocol. As the standard detection range of this kit is 75–1,200 pg/ml and a concentration >1,300 pg/ml is considered to be relevant in CJD diagnosis [12], CSF samples from sCJD patients have to be diluted in sample diluent prior measurement.
Ruling Out 14-3-3 False Positive Rates in sCJD Differential Diagnosis

**Statistical Analysis**

Descriptive statistics were calculated for every patient population. The comparison of age between patient populations was carried out by a non-parametric Mann-Whitney test. The correlation coefficient (p) was computed using Spearman’s rank order correlation coefficient test.

**Results**

**Single CSF Markers**

In the sCJD group, 88, 91 and 78% of the patients were positive for 14-3-3, tau and desmoplakin, respectively (table 2). In the control groups, 28 patients showed an elevated tau level and only 9 had a positive desmoplakin test. The rate of false positive results differed among the control groups selected for this analysis. In the ‘14-3-3 false positive’ group (selected on the basis of an elevated 14-3-3 level which was not CJD-related), 23 were positive for tau and 7 for desmoplakin. In the ‘alternative clinical diagnosis’ group (selected on the basis of initially suspected CJD and subsequent differential diagnosis), none of the patients were positive for 14-3-3, 5 patients were positive for tau, and only 2 were positive for desmoplakin (table 2; fig. 1).

Interestingly, 3 out of 4 patients with meningiosis carcinomatosa, belonging to the ‘14-3-3 false positive’ subgroup, were positive for desmoplakin and all of them showed a physiological CSF tau level. On the other hand, many patients with different kinds of encephalitis showed an elevated tau level, but they were negative for desmop-
plakin (table 1). Furthermore, the rate of positive tests differed across sCJD patients with various codon 129 genotypes. All VV-sCJD patients were positive for three CSF markers. Out of 26 MM-sCJD patients, 22, 23 and 18 were positive for 14-3-3, tau and desmoplakin, respectively. The MV-sCJD-positive rate pattern rate looked similar to the MM1-sCJD one.

**Combination of Two CSF Markers**

In order to analyze whether a combination of two markers may improve CSF testing utility in the clinical diagnosis of sCJD, the analysis of three possible two CSF marker combinations was performed. The combination of a positive 14-3-3 test and an elevated tau level showed an 88% positive rate and 28% false positive rate, while the combination of both positive 14-3-3 and desmoplakin tests showed a lower 78% positive rate and also a lower 9% false positive rate. When the elevated tau level was accompanied by a positive desmoplakin test, the false positive rate accounted for only 4% (table 3).

**CSF Marker Correlation**

Subsequently, an analysis of biological factors hypothetically influencing CSF level of the investigated markers was computed. In the sCJD group, all CSF markers showed a positive correlation: 14-3-3 versus tau (rho = 0.829; p < 0.001), 14-3-3 versus desmoplakin (rho = 0.595; p < 0.001) and tau versus desmoplakin (rho = 0.45; p < 0.001). Interestingly, there was no correlation between desmoplakin and 14-3-3 (rho = 0.156; p = 0.164) or tau (rho = −0.006; p = 0.957) in the non-sCJD group, while within this group there was a positive correlation between 14-3-3 protein and desmoplakin.

**Table 2.** Overall positive and false negative as well as false positive and negative rate for single CSF markers (14-3-3 protein, tau protein and desmoplakin)

| CSF marker       | Rate                  |
|------------------|-----------------------|
|                  | positive | false negative | false positive | negative |
| 14-3-3 protein   | 51/58 (88%) | 7/58 (12%) | 45/81 (56%) | 36/81 (44%) |
| Tau protein      | 53/58 (91%) | 5/58 (9%) | 28/81 (35%) | 53/81 (65%) |
| Desmoplakin      | 45/58 (78%) | 13/58 (22%) | 9/81 (11%) | 72/81 (89%) |

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**Fig. 1.** Application and efficiency of CSF markers in sCJD differential diagnosis. Among sCJD patients with both positive 14-3-3 and tau tests, 71% of the patients also showed an elevated desmoplakin level in CSF. Among non-sCJD patients with both false positive 14-3-3 and tau tests, only 9% (2 out of 23) of the patients showed an elevated desmoplakin level in CSF.

**Fig. 2.** Correlation between tau level (pg/ml) and 14-3-3 protein (a) as well as between tau level (pg/ml) and desmoplakin (b) in the sCJD group. The lower tau level corresponded with the negative 14-3-3 or negative desmoplakin test.
and tau protein (p = 0.4; p = 0.004) (table 4). Moreover, the lower tau level corresponded with the negative 14-3-3 or negative desmoplakin test (fig. 2).

**Discussion**

In the present study, we aimed to determine the diagnostic utility of desmoplakin which is a potential candidate for sCJD marker supporting to rule out 14-3-3 false positive rates, and to make a comparison between desmoplakin with two known CSF markers: 14-3-3 and tau. Moreover, we performed a correlation analysis between CSF markers in the sCJD and non-sCJD control group.

In our previous studies investigating CSF proteome alterations in sCJD, we could identify 33 differentially regulated proteins [9]. Subsequently, a truncated form of desmoplakin, which showed the highest up-regulation in sCJD, was selected for further examination with respect to its application in sCJD diagnosis.

Desmoplakin is a major high molecular weight protein of desmosomes involved in the cell junctions. It has been previously shown that PrP<sup>C</sup> interacts with desmosomal proteins, including desmoplakin, in epithelial cells where it regulates cellular distribution of junction-associated proteins [13]. Subsequently, a truncated form of desmoplakin, which showed the lowest down-regulation in sCJD, was selected for further examination with respect to its application in sCJD diagnosis.

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A newly tested desmoplakin showed a lower positive rate than the already used 14-3-3 and tau biomarker, but its high specificity (low false positive rate) could be utilized to rule out false positive cases in sCJD differential diagnosis.

It has been claimed that the elevated CSF level of 14-3-3 protein is associated with its release from an area of massive brain damage that is not sCJD-specific. Indeed, many acute neuronal damages such as cerebral hypoxia, different encephalitis, brain tumors or complex focal seizure can give a positive 14-3-3 test. In our studies, we included 45 patients classified as 14-3-3 false positive cases; among them, 23 showed an elevated level of tau and only 7 cases had a positive desmoplakin test. For instance, all encephalitis, complex focal seizure, hypoxic brain damage or brain tumors cases were negative for desmoplakin. The exceptions for negative desmoplakin test were meningiosis carcinomatosa cases. Interestingly, these cases had a physiological tau level in CSF. A potential explanation might be that these proteins are selectively up-regulated and/or involved in specific pathological processes.

In general, the computed overall false positive rates for 14-3-3, tau and desmoplakin were 56%, 35%, and 11%, respectively.

A further decrease of false positive rates could be achieved by the combination of a positive 14-3-3 test with positive desmoplakin test or by the combination of an elevated tau level with a positive desmoplakin test. For these combinations only 9% or 4% cases were rated as false positive, respectively. Subsequently, an analysis of

| Table 3. Overall positive and false negative as well as false positive and negative rates for combination of two CSF markers (14-3-3 protein + tau protein, 14-3-3 protein + desmoplakin, tau protein + desmoplakin) |
|---|---|---|---|---|
| CSF marker | Rate | positive | false negative | false positive | negative |
| 14-3-3 protein + tau protein | 51/58 (88%) | 7/58 (12%) | 23/81 (28%) | 58/81 (72%) |
| 14-3-3 protein + desmoplakin | 45/58 (78%) | 13/58 (22%) | 7/81 (9%) | 74/81 (91%) |
| Tau protein + desmoplakin | 45/58 (78%) | 13/58 (22%) | 3/81 (4%) | 78/81 (96%) |

| Table 4. Correlation between CSF markers (14-3-3 protein vs. tau protein, 14-3-3 protein vs. desmoplakin, tau protein vs. desmoplakin) in sCJD and differential diagnosis (non-sCJD) (p value) |
|---|---|---|
| CSF markers | sCJD | Non-sCJD |
| 14-3-3 protein vs. tau protein | 0.829; <0.001 | 0.4; 0.004 |
| 14-3-3 protein vs. desmoplakin | 0.595; <0.001 | 0.156; 0.164 |
| Tau protein vs. desmoplakin | 0.45; <0.001 | –0.006; 0.957 |
biological factors hypothetically influencing the CSF level of the investigated markers was computed. In sCJD, all three CSF markers were positively correlated; an increased tau level corresponded with a positive 14-3-3 or desmoplakin test. Interestingly, there was no correlation between desmoplakin and 14-3-3 or tau in the non-sCJD group. This observation can further favor our concept that desmoplakin may be supporting a CSF marker to rule out 14-3-3 false positive cases.

Similarly to 14-3-3, we found that the desmoplakin level, thus sensitivity of the desmoplakin-based test, was influenced by PRNP codon 129 polymorphism [5, 6, 15]. All VV were positive, whereas the sensitivity in the MM group was the lowest, once again suggesting a potential involvement of specific pathways in single disease subtypes. In general, among sCJD patients with both positive 14-3-3 and tau tests, 71% of the patients also showed an elevated desmoplakin level in CSF. However, among non-sCJD patients with both false positive 14-3-3 and tau tests, only 8% (2 out of 23) of the patients showed an elevated desmoplakin level in CSF.

In summary, desmoplakin is a promising candidate for CSF markers which support ruling out 14-3-3 false positive cases and it should be considered to include desmoplakin in the diagnostic workup in suspected CJD cases.

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Disclosure Statement

All authors disclose any actual and potential conflicts of interest including any financial, personal or other relationships with other people or organizations.

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