

CSF 14-3-3 protein and its ζ isoform are prognostic markers in HIV positive patients with CNS disease

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ABSTRACT:

- **Background:** 14-3-3 proteins have been detected in the cerebrospinal fluid (CSF) of patients with different neurological disorders as markers of neuronal damage. Since a previous study showed the prognostic value of this test in patients with bacterial meningitis, we wanted to evaluate its value in HIV-positive patients presenting with central nervous system (CNS) diseases.
- **Patients and Methods:** We studied the trend of CSF level of 14-3-3 protein and its ζ isoform in 15 consecutive HIV patients presenting with various CNS diseases (cerebral lymphoma and cryptococcal meningitis were the most common). Lumbar Punctures were performed only according to clinical necessities; levels of 14-3-3 proteins were measured according to published methods and their total amount was quantified against control cases with hydrocephalus.
- **Results:** Fifteen patients were enrolled: 10 male, mean age 39 and mean CD4 counts of 66 (13-226) cells $\times 10^6$ /litre. Cerebral lymphoma and cryptococcal meningitis were the most common diseases, respectively 40% and 33%. All patients showed positivity for 14-3-3 protein and its zeta isoform in CSF at admission. All who had a favourable outcome cleared the protein from their CSF in advance to the normalization of standard laboratories values (8/8). Seven non-survivors did not show clearance of the 14-3-3 protein or its zeta isoform. No difference emerged between total 14-3-3 protein and its zeta isoform in terms of quantity, association with a specific disease and prognostic value.
- **Conclusions:** Detection of 14-3-3 protein and its ζ isoform in the CSF warrant further evaluation as a possible prognostic marker in HIV-positive subjects with CNS disorders and as a tool to guide treatment strategies.
- **Key words:** CSF, HIV, Neurological disorder, 14-3-3 protein.

INTRODUCTION

Patients with human immunodeficiency virus (HIV) infection frequently present with central nervous system (CNS) symptoms related to HIV infection itself, drug toxicity, opportunistic infections or neoplastic disorders. Even if some of these can be successfully treated, differ-

ential diagnosis and management are complicated; emerging diagnostic and prognostic markers may prove to be clinically beneficial in this complex group of patients.

14-3-3s are a family of ubiquitous proteins acting as signalling protein-kinases in eukaryotic cells. This family is made up of seven isoforms in mammals (beta, gamma,

epsilon, zeta, eta, sigma, tau)¹. They are abundantly expressed in brain tissue with some degree of anatomical specificity² and have been detected in the cerebrospinal fluid (CSF) of patients with different neurological disorders, probably as neuronal damage markers for cell disruption and leakage of brain proteins into this compartment³.

Data about the measurement of CSF 14-3-3 protein level in patients infected with HIV are contrasting. Two studies found no correlation between the presence of these proteins and AIDS dementia complex^{4,5} whilst a separate study⁶ detected specific 14-3-3 isoforms in certain HIV-related neuropathologies (including HIV dementia). In addition, a recent work conducted on SIV-infected macaques not only implicated CSF levels of 14-3-3 proteins as worthy markers of early neuronal damage but also viral replication in CNS and progression of CNS pathology⁷.

Furthermore, the prognostic value of CSF 14-3-3s as markers of neuronal damage is supported by a study published from our research group in HIV-negative patients with bacterial meningitis⁸. Supported by these elements, we evaluated the presence and the prospective trend of global and zeta-isoform 14-3-3 proteins in CSF samples from HIV-positive patients presenting with neurological symptoms, comparing these observations with the conventional diagnostic and prognostic parameters.

PATIENTS AND METHODS

All HIV-positive patients presenting with CNS disease who were admitted to our department from 2001 until February 2004 were enrolled in the study if at least two CSF samples were available. CSF aliquots of 1 ml were collected and stored at -20°C. Analysis of CSF samples included cell count, biochemistry determination, total protein level and microbiological investigations (CSF PCR HIV performed using Roche Amplicor 1.5, Roche Diagnostics Corporation, Indianapolis, USA). Lumbar Punctures were performed on admission and accordingly to clinical necessity.

Analyses of CSF samples, for 14-3-3 protein (and ζ isoform), were carried out with no prior knowledge of patient's diagnosis. These results were only available after termination of clinical care, so they did not influence clinical practice. Patients were observed until death or discharge with a follow-up of at least six months. CSF 14-3-3 detection was performed by the Department of Neurology, University of Verona. CSF aliquots of 100 μ l were mixed with 7 volumes of ice-cold methanol, kept at -20 °C for 2 hours, and then centrifuged at 20,800 g for 30 minutes. The pellet was dissolved in 40 μ l of sample buffer (3% sodium dodecyl sulphate, 3% beta-mercaptoethanol, 2 mM ethylenediaminetetraacetic acid, 10% glycerol, and 62.5 mM Tris [pH, 6.8]) and boiled for 5 minutes. For each sample, 10 μ l (the equivalent of 25 μ l of CSF), 5 μ l, and 1.25 μ l of sample buffer/well were loaded onto a 13% polyacrylamide gel and transferred to polyvinylidene difluoride membranes (Immobilon P; Millipore). Membranes were incubated with

anti-14-3-3 beta polyclonal rabbit IgG and anti-zeta-14-3-3 IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a 1:500 dilution and revealed with anti-rabbit horseradish peroxidase IgG (Amersham) at a 1:3000 dilution. The blots were developed using an enhanced chemiluminescent system (Amersham). Densitometric values for each sample were obtained with a computer-assisted laser scanner (GS-710 Calibrated Imaging Densitometry; BioRad), after correction for background. The total amount of 14-3-3 protein as quantified from each diluted and undiluted CSF sample was expressed in arbitrary units. Control CSF specimens included samples obtained from 3 patients with benign intracranial hypertension and from 2 patients with normotensive hydrocephalus.

Objective of this study was to evaluate the trend of CSF 14-3-3 total protein and its zeta isoform in a setting of HIV-positive patients with neurological involvement and to investigate any association of this marker to the prognosis and diagnosis of these diseases.

STATISTICAL ANALYSIS

Categorical variables are expressed as numbers and proportions, continuous variables are expressed as mean \pm standard deviation (SD) or median (interquartile range (IQR)). CSF parameters in the CSF of survivors and non-survivors were compared using Mann Whitney test or Student *t*-test as appropriate.

RESULTS

Fifteen patients were enrolled. Their characteristics are as follows: 10 male, mean age 39 (26-60) years, mean CD4 counts of 66 (13-226) cells $\times 10^6$ /litre and viral load of 280,000 (480-650,000) copies $\times 10^6$ /litre. Their range of diagnoses included Lymphoma (n=6), Cryptococcus neoformans meningitis (n=5), Mycobacterium tuberculosis meningitis (n=2), Toxoplasma gondii encephalitis (n=1), Neisseria meningitidis meningitis (n=1), AIDS dementia complex (n=1) and aseptic meningitis associated with HIV seroconversion (n=1). Two patients had comorbidities. Seven patients out of fifteen were receiving HAART on admission.

For treating cerebral lymphomas different chemotherapy combinations were used accordingly to our haematologist's advice (r-CHOP, ABVD; all were treated); standard therapies for opportunistic infections were administered. Five patients with lymphoma (83%) and two with cryptococcal meningitis died (40%), thus showing an overall mortality of 46.7%; all other patients recovered but one showed major neurological sequelae at six months of follow-up.

Results of the conventional CSF analysis on admission showed no significant difference between patients who later died and those who had a favourable outcome. Median values for surviving and deceased patients were respectively: median leukocyte count, 238 (10-1930) and 20 (0-90) cells $\times 10^6$ /litre (p: not significant (ns)); median

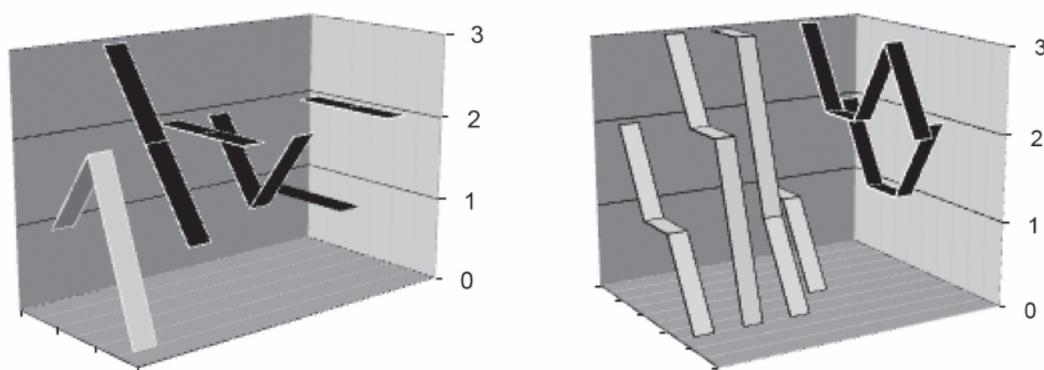


Figure 1. Results of the consecutive determinations of total 14-3-3 protein in CSF in patients with at least 3 samples collected (each ribbon is a patient). Left: patients with cerebral lymphoma. Right: patients with other CNS disorders. The dark lines represent the patients who died. The y axis represents the arbitrary unit scale (3=+++, 2=++, 1=+, 0=-) reflecting 14-3-3 concentrations.

total protein concentration, 103.25 (54-237) and 65 (46-199) mg/dl (*p*: ns); and median glucose concentration, 35 (15-52) and 42 (32-50) mg/dl (*p*: ns).

In the group presenting with neoplastic diseases, only one patient survived and showed a complete remission of lymphoma. The results concerning the determination of total and zeta-isoform 14-3-3 protein levels are showed in Figure 1.

Baseline samples from all study subjects were positive for both markers; the degree to which the samples tested positive, according to our arbitrary scale, showed no consistent difference between survivors and non-survivors (the median value was 2 in both groups). Furthermore, no correlation was found between 14-3-3 concentration and specific neuropathologies. 14-3-3 protein levels were demonstrated as undetectable in all survivors (8/15, 53.3%); however those with persistent levels, invariably, did not survive.

Conventional CSF parameter values recorded at the time of test normalization (for survivors in the two groups, respectively patient 1 and patients 7-13) or of the last determination before death (patients 2-6 and 14-15) are reported in table 1. CSF leukocyte counts, total protein and glucose levels in survivors were still respectively outside normal limits in 75, 100, and 50% of cases at the time of the first negative test.

DISCUSSION

14-3-3 protein and ζ isoform concentrations were measured in CSF samples serially taken from 15 patients infected with HIV infection presenting with CNS disease. All patients at admission tested positive and those who remained positive died. Those who demonstrated clearance of 14-3-3 protein from the CSF survived.

Table 1. CSF analysis (14-3-3 and ζ isoform levels, conventional parameters) at the time of the first negative 14-3-3 test in patients who had a positive outcome and at the time of the last determination in the non-survivors. A: neoplastic B: non-neoplastic

| Pt | Outcome | Disease | CSF 14-3-3 (ζ isoform), AU ^a | CSF WBC (cells $\times 10^6$ /litre ^b) | CSF protein (mg/dl ^c) | CSF glucose (mg/dl ^d) | |
|----|---------|----------|--|--|-----------------------------------|-----------------------------------|-----|
| A | 1 | Survived | Burkitt's Lymphoma | - (+) | 5 | 45* | 66 |
| | 2 | Deceased | Burkitt's Lymphoma | + (+) | 4 | 51 | 59 |
| | 3 | Deceased | Burkitt's Lymphoma | ++ (++) | 0 | 57 | 49 |
| | 4 | Deceased | Cryptococcus meningitis, Primary Cerebral Lymphoma | ++ (+++) | 4 | 65 | 73 |
| | 5 | Deceased | Non-Hodgkin's Lymphoma | + (++) | 0 | 50 | 51 |
| | 6 | Deceased | Non-Hodgkin's Lymphoma | ++(+++) | 50 | 262 | 3 |
| B | 7 | Survived | Neurotoxoplasmosis, AIDS dementia complex | - (-) | 0 | 48* | 58 |
| | 8 | Survived | Cryptococcus meningitis | - (-) | 20* | 56* | 47* |
| | 9 | Survived | Cryptococcus meningitis | - (-) | 80* | 73* | 50 |
| | 10 | Survived | Cryptococcus meningitis | - (-) | 33* | 48* | 36* |
| | 11 | Survived | Tubercular meningitis | - (-) | 84* | 98* | 32* |
| | 12 | Survived | HIV primary infection | - (-) | 24* | 60* | 42* |
| | 13 | Survived | Tubercular meningitis | - (-) | 30* | 49* | 64 |
| | 14 | Deceased | Cryptococcus meningitis | ++ (++) | 10 | 95 | 32 |
| | 15 | Deceased | Cryptococcus meningitis | ++ (++) | 98 | 81 | 36 |

^a 14-3-3 proteins are normally absent in CSF. AU, arbitrary units. ^b Normal values, 0-10 cells $\times 10^6$ /litre.

^c Normal values, <45 mg/dl. ^d Normal values, 50-80 mg/dl. * Altered values (in survivors).

CNS: central nervous system; CSF: cerebrospinal fluid.

In all the survivors, the 14-3-3 concentration in the CSF was negative, thus suggesting that in HIV patients without CNS pathologies this marker is likely to be normal. This was evident in a patient who presented with meningism (headache, stiff neck and fever) due to acute HIV infection: his rapid recovery was mirrored in 14-3-3 concentrations, as well as CSF PCR for HIV (extremely high on admission, falling to undetectable levels towards recovery). These observations seem to suggest a role for this marker in unambiguously distinguishing the presence, or absence, of neuropathology causing neuronal damage.

It is interesting to highlight that our only survivor in the neoplastic group, who demonstrated complete remission of non-Hodgkin lymphoma, had normalization of CSF 14-3-3 levels. In a disease with such an unfavourable outcome, this could be a promising prognostic tool.

Our data seem to suggest that 14-3-3 protein and its ζ isoform are cleared from the CSF earlier than the conventional parameters (even if they showed a trend towards normalization and were only slightly altered at the time of their last determination). Should this be confirmed, such a test may become relevant in clinical practice by allowing objective evaluation of treatment as well as prognosis before normalization of conventional tests. In complicated long-lasting meningitis, normalization of CSF 14-3-3 could be anticipatory of healing and a positive outcome.

As far as it emerged from this study, there is no significant difference in either the concentration or the trend of the ζ isoform compared to the global 14-3-3. Future studies may now look closely at how isoforms of CSF 14-3-3 protein may be used as a diagnostic tool. As it has been suggested by some previous reports, different isoforms may be associated with specific cerebral localizations.

Although this marker was studied in a small and very heterogeneous group of patients and no control group was analysed (since we considered unethical to perform

lumbar puncture on healthy HIV-positive patients) with severe neuropathologies, measurement of the 14-3-3 proteins deserves further clinical investigation as a possible addition to the conventional CSF parameters used in diagnostic and prognostic evaluation of HIV-positive patients presenting with CNS diseases.

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