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ORIGINAL ARTICLE

Neurofilament heavy subunit in cerebrospinal fluid: A biomarker of amyotrophic lateral sclerosis?

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Abstract

The objectives of this study were to investigate the presence of the three neurofilament subunits, ubiquitin, proteasome and 3-nitrotyrosine, in CSF samples of ALS patients. CSF samples were obtained by lumbar puncture from 10 ALS patients and six controls. All samples were analysed by Western blotting. Results revealed that neurofilament heavy subunit was identified in 70% of ALS cases and we conclude that this subunit may be a promising biomarker for clinical diagnosis of ALS.

Key words: *Amyotrophic Lateral Sclerosis, cerebrospinal fluid, proteinopathy, protein aggregation and neurofilament*

Introduction

Amyotrophic Lateral Sclerosis (ALS) is a progressive neurodegenerative disorder that results in the loss of motor neurons and leads to painless weakness, muscle atrophy, paralysis and death (1,2). Diagnosis is made by physical examination, with supportive electrophysiological findings and following exclusion of other conditions. There is no single biomarker that can corroborate the diagnosis. A histopathological hallmark of ALS is the accumulation of neurofilaments (NFs) and ubiquitinated inclusions inside motor neurons (1,3). NFs are major structural elements of the neuronal cytoskeleton and are composed of three different polypeptides: NF-L, NF-M, and NF-H subunits.

In a previous study, we performed a morphological and quantitative immunohistochemical analysis to evaluate the presence of abnormal accumulation of neurofilaments in control and ALS cases. Although we observed intense immunoreactivity in focal regions of motor neuron perikarya for all subunits, only NF-H showed a statistically significant difference (3).

The presence of ubiquitinated inclusions indicates that the ubiquitin/proteasome pathway may be involved in the pathogenesis of ALS. Ubiquitin is a small protein that associates with many regulatory and misfolded proteins. Ubiquitination is a signal for proteolysis by the proteasome 26S, a multicatalytic complex that has a regulatory component, the 19S particle, and a proteolytic component, the 20S proteasome (4,5). The 20S proteasome is composed of α and β subunits. In previous studies by our group, we observed the classic ubiquitin-positive aggregates and intense immunoreactivity for proteasome $\alpha\beta$ subunits in spinal cord motor neurons of patients who died of ALS (6).

There is considerable evidence implicating oxidative stress as a central mechanism by which motor neuron death occurs in ALS (7). Increased levels of 3-nitrotyrosine in the central nervous system have been found in patients and mouse models of familial

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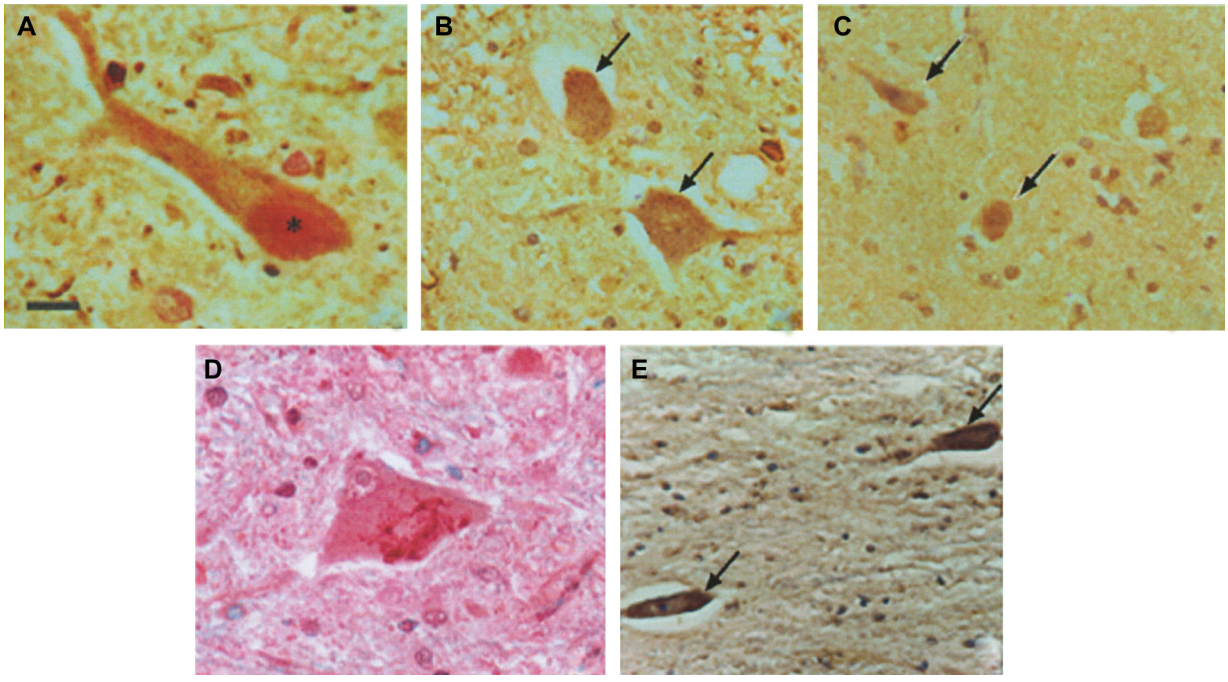

Figure 1. (A–E) Immunohistochemistry of ventral horn motor neurons in ALS tissue samples. (A) Immunohistochemistry for NF-H showing intense aggregation of the tested subunit in the neuron cell body (asterisk); (B) Immunohistochemistry for NF-M showing a more intense reaction in some cell body regions (arrows); (C) ALS atrophic and ballooned neurons showing strong immunostaining by immunohistochemistry for NF-L (arrows); (D) ALS motor neuron with intra-cytoplasmic inclusions strongly positive for ubiquitin; (E) ALS motor neuron displaying strong immunostaining for proteasome subunits (arrows). Scale bar: (A) 10 µm; (B–E) 35 µm.

ALS, suggesting a possible use of nitrated proteins as biomarkers (8,9).

This study was based on the underlying hypothesis that after the motor neuron cell membrane ruptures, the accumulated proteins leak into cerebrospinal fluid (CSF) circulation. We sought to identify neurofilament subunits, ubiquitin and proteasome and to measure the levels of 3-nitrotyrosine in CSF from ALS patients and controls.

Materials and methods

CSF samples were obtained by lumbar puncture from 10 ALS patients at the Instituto de Neurologia Deolindo Couto (INDC), Rio de Janeiro, and from six control patients at the CSF Laboratory of the Hospital Universitário Clementino Fraga Filho, Rio de Janeiro, Brazil. Collection and storage procedures were the same for the CSF samples obtained at both hospitals. The ALS patients included eight males and two females whose ages ranged from 30 to 67 years. Diagnoses of all ALS patients fulfilled the revised El Escorial World Federation of Neurology Research Group on Motor Neuron Disease Criteria (10) for definite ALS and all of them were in the intermediate stage of the disease. The disease presented as classic ALS in all patients. Control patients presented clinical signs or diagnoses of cephalalgia (n = 6). Informed consent was obtained from all participants and the study was approved by the ethics committee of INDC, Rio de Janeiro, Brazil.

CSF samples were labelled and stored at –80°C until analysis. Western blotting was undertaken using anti-NF-L (clone NR4/SIGMA), anti-NF-M (clone NN18/SIGMA), anti-NF-H (clone NE14/SIGMA), free ubiquitin and polyubiquitin chains (Dako), proteasome αβ subunits (Biomol), and 3-nitrotyrosine (Upstate) antibodies. Whole rat brain extracts were used as positive controls.

In brief, samples were previously treated with sample buffer (DTT, SDS, 1 *M* Tris, pH 6.8, glycerol, Bromophenol blue, and distilled water): 50–80 µg of proteins per sample was electrophoresed on a 7.5–12% SDS-polyacrylamide gel, at 100 V. Proteins from the gel were transferred for 6 h at 4°C onto immuno-blot PVDF membrane (Bio-Rad).

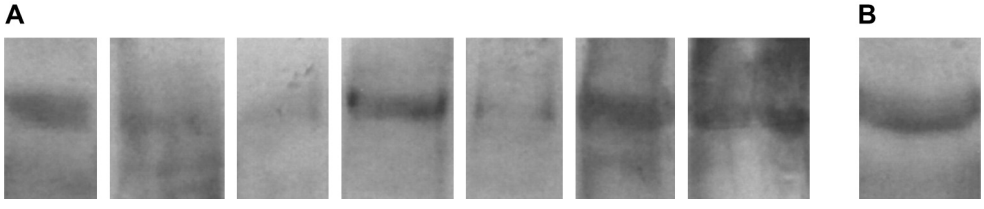

Figure 2. Western blotting for NF-H. (A) ALS CSF samples; (B) Whole rat brain extract as the positive control of the experiment, intensely stained.

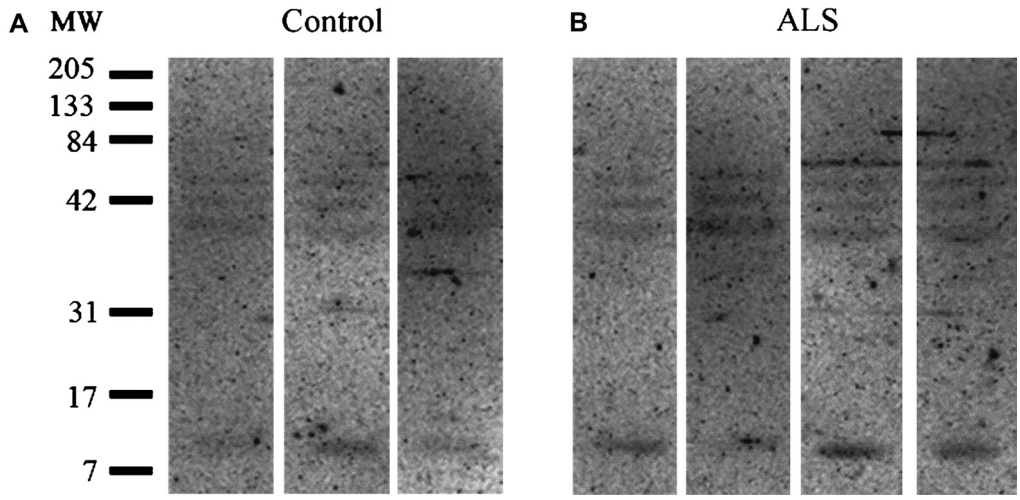

Figure 3. Western blotting for 3-nitrotyrosine. (A) Control CSF samples; (B) ALS CSF samples. Similar expression of 3-nitrotyrosine can be observed in both groups.

Membranes were incubated in blocking solution (0.05% Tween 20 in Tris-buffered saline) for 1 h at room temperature, followed by overnight antibodies incubation at 4°C with constant shaking. On the next day, membranes were washed 3 × 10 min in TBS-T buffer, then incubated with secondary antibodies. All antibodies were diluted in blocking buffer. For immunodetection, membranes were washed three times in TBS-T buffer, incubated with ECL solutions (Amersham Biosciences) and exposed to X-ray film (Kodak).

Results

As observed in Figure 1A–C, there was intense immunoreactivity in focal regions of neuronal perikarya for NF-H, NF-M, and NF-L subunits. Figure 1D shows ubiquitin-positive inclusions and in Figure 1E can be observed intense immunoreactivity for proteasome αβ subunits.

We investigated the expression of all these proteins in CSF samples from ALS and control cases. NF-H subunit was identified by Western blotting in seven of the 10 ALS cases analysed and not in the control cases (Figure 2A, B). Although NF-L and NF-M positive aggregates were also observed in ALS motor neurons, these subunits were not found in the CSF of ALS or control cases.

Ubiquitin and proteasome αβ subunits were not found in the CSF of ALS or control cases. Whole rat brain extract, used as the positive control of the experiment, was positive for all antibodies tested.

With regard to the oxidative stress marker, 3-nitrotyrosine was expressed in both ALS and control samples, with no significant difference between them (Figure 3).

Discussion

We have shown that NF-H subunit was present in seven of the 10 ALS cases analysed and in none of the control cases. These results are in agreement with our morphological and statistical analysis of NF subunits in the ALS spinal cord samples, where quantitative results were only significant for the heavy subunit (3). NF-L and NF-H have previously been identified in CSF of ALS patients, but all of them with ELISA technique (11–14). To our knowledge, this is the first report that demonstrates the presence of NF heavy chain subunit in ALS CSF by Western blotting, which is a more specific technique than ELISA. Further advantages of Western blotting include a lower level of non-specific immunological reaction and additional information regarding protein size.

We found no difference in levels of 3-nitrotyrosine in CSF from ALS and controls. This finding is similar to that of Ryberg et al. (2004), who analysed CSF from patients with Alzheimer's disease and ALS, and found no differences between patients and controls (15).

Similarly, ubiquitin and proteasome αβ subunits were not identified in ALS CSF. A possible explanation for the negative finding could be that ubiquitin is a small protein and is not as stable as NFs (4). Proteasome, on the other hand, is a large multisubunit protease and is therefore less likely to enter CSF circulation. NF-L or NF-M subunits were not identified in CSF from ALS patients, although its aggregates have been observed in ALS motor neurons (3).

Our findings support previous observations that NF heavy changes may be useful in the development of a panel of biomarkers for the clinical diagnosis of ALS.

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