ORIGINAL ARTICLE

A new marker for ischemic cerebrovascular stroke: Phosphorylated Neurofilament H

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Abstract *Background and purpose:* A main problem in patients with ischemic CVS is the absence of a blood marker that can be collected to diagnose and predict prognosis. The objective of our study was to assess if Phosphorylated Neurofilament H (*pNF-H*) might provide useful diagnostic and prognostic information in such patients.

Methods: Thirty patients presenting to the critical care department with ischemic CVS were studied. Blood samples for Phosphorylated Neurofilament H were assayed on admission and after 7 days. Neurofilament levels were correlated with Glasgow coma scale, CT findings and NIHSS on admission and after 7 days.

Results: Neurofilament H levels showed a negative correlation with GCS on admission and after 7 days in, ischemic stroke (0.37, 0.56); hence higher neuromarker levels were associated with lower GCS. There was a negative correlation between neurofilament levels and ASPECTS CT scores ($r = 0.64, 0.89$). NIHSS showed positive correlations with neurofilament levels.

Conclusion: Phosphorylated Neurofilament H can be used as a useful tool to assess patients with acute ischemic CVS. Levels of the neurofilament correlated with the degree of conscious level in such patients and with CT findings hence can be used to assess short term prognosis.

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Introduction

Following CNS injury, certain proteins are released from neurons. A test that can quantify the levels of these released proteins might provide useful information about the level of injury, and would be particularly useful if such protein could be detected in blood. There has been a growing appreciation that many kinds

of CNS injury and disease states are the result of axonal injury and degeneration [1,2]. Accordingly, a convenient method of detecting ongoing axonal loss might be particularly useful experimentally and clinically. The perfect marker to detect axonal injury should have several properties; it should be specific to axons, it should be profuse enough so that it can be readily detectable after the significant dilution that occurs following the release into blood, and it should be resistant to proteases so that it is not broken down prior to or following release. Numerous studies have tried to identify and characterize candidate biochemical surrogate markers for brain damage including S-100 protein, Glial Fibrillary Acidic Protein (GFAP) and Neuron Specific Enolase (NSE). These studies establish that these proteins are expressed predominantly in neurons, astroglia, oligodendroglia and other brain cells. They are released into the brain parenchyma and transit to cerebrospinal fluid (CSF) and blood in association with acute brain damage and barrier disruption. Unfortunately, owing to limitations in sensitivity, specificity, and standardized quantification across multiple laboratories and studies, none of these proteins has emerged as a widely used diagnostic or prognostic clinical tool or a validated surrogate measure for brain damage. Studies that correlate such protein levels with various clinical outcomes are lacking.

Over the last 20 years, many steps have been taken in the classification of Neurofilament proteins and their functions. Early studies by Hsieh et al have shown that phosphorylation of neurofilaments is required for the proper functioning of axons by regulating axons' diameter.[10] The Neurofilament H protein sequence contains unusual tandemly repeated 6–8-amino-acid sequences centered on the sequence lysine–serine–proline. The serine residues of the KSP are phosphorylated [3] and are axon specific [4]. This phosphorylated form of NF-H (here referred to as pNF-H) is known to be more resistant to calpain and other proteases [5,6] Also, pNF-H is highly immunogenic, and the multiple repeated phosphorylated sites are an excellent target for antibody-based assays. Taken together, these facts suggest that pNF-H might be a good candidate for a biomarker of axonal injury.

Patients and methods

Study design

This randomized study was prospectively conducted on 30 patients presenting to the Critical Care Department Cairo University Hospitals during the period from January 2010 to January 2011. Informed written consents had been obtained from the relatives and the study was approved by the Hospital's Ethics Committee.

Inclusion criteria

Thirty patients presenting to the Critical Care Department of the University of Cairo Hospitals with ischemic Cerebrovascular stroke were enrolled in the study. Ischemic Cerebrovascular Stroke was defined as the presence of a neurological deficit with a clearly defined time of onset, and a baseline CT scan of the brain that shows no evidence of intracranial hemorrhage.

Exclusion criteria

The following patients were excluded:-

- Patients with chronic neurological disease.
- Patients with seizure activity.
- Patients with renal impairment.
- Patients with Multi-organ Failure Syndrome.
- Patients with multiple trauma.
- Patients younger than 18 years and older than 65 years.
- Patients who were receiving or candidates for thrombolytic therapy.

Five patients were excluded, because they had one or more reason for exclusion.

Methods

- Full history taking from the patients, relatives or witnesses with stress on the onset of neurological symptoms.
- Complete general and focused neurological examination.
- Blood samples were taken for routine labs and for pNF-H levels. Samples were taken within the first 24 h (pNF-H1) and after 7 days (pNF-H2).
- Non-contrast CT brain (NCCT) on admission and after 7 days. Image review was independently performed on a workstation by radiologists or neurologists. ASPECTS CT score was used to assess CT findings.
- Glasgow coma scale was calculated on admission (GCS1) and repeated after 7 days (GCS2).
- The National Institute of Health Stroke Scale (NIHSS) was used on admission (NIHSS1) and after 7 days (NIHSS2). The level of stroke severity was measured by the NIH stroke scale scoring system: $0 = i$, $1–4 = minor\ stroke$, $5–15 = moderate\ stroke$, $15–20 = moderate/severe\ stroke$, $21–42 = severe\ stroke$.

The ASPECTS score

The Alberta stroke program early CT score (ASPECTS) is a 10-point quantitative topographic CT scan score used in patients with ischemic stroke. Segmental assessment is made and 1 point is removed from the initial score of 10 if there is evidence of infarction in the following regions [7]:

- Caudate.
- Putamen.
- Internal capsule.
- Insular cortex.
- M1: "anterior MCA cortex," corresponding to frontal operculum.
- M2: "MCA cortex lateral to insular ribbon" corresponding to anterior temporal lobe.
- M3: "posterior MCA cortex" corresponding to posterior temporal lobe.
- M4: "anterior MCA territory immediately superior to M1".
- M5: "lateral MCA territory immediately superior to M2".
- M6: "posterior MCA territory immediately superior to M3".

The ASPECTS score was calculated on each of the CTs done to every patient in Group B on admission and after 7 days.

Neurofilament H assay method

Blood samples were drawn from each patient on admission and after 7 days. The BioVendor Human Phosphorylated

Neurofilament H ELISA, standards, quality controls and samples were placed and left in microplate wells that contained chicken polyclonal anti-pNF-H antibody. One hour later, detection rabbit polyclonal anti-pNF-H antibody was added and incubated with captured pNF-H for 60 min. After another wash, horseradish peroxidase (HRP) conjugated antibody against rabbit antibody was added. After 60 min of incubation and the last washing step, the remaining conjugate was allowed to react with the substrate solution. The reaction was stopped by the addition of acidic solution and absorbance of the resulting yellow product was measured.

Data analysis

The Pearson correlation analysis of data was performed using Statistical Package for the Social Sciences (SPSS) software. The association of subject characteristics to pNF-H levels was studied with multiple regressions. Neurofilament H data were square root transformed to effect normality of distribution of residuals. Relationships between the square root of pNF-H were investigated within groups with the Pearson correlation and the two-sample t-test. Analysis of correlation was used to assess the different relationships between pNF-H and other variables.

Results

General characteristics of patients

The study of demographic characteristics of the patients showed a mean age of 49 years with a range of 49–51 years. Males represented 60% of the population. Out of the thirty patients studied, 22 patients were diabetics (73%), 21 were dyslipidemics (70%), 16 were smokers (53%) and 22 were hypertensive (73%). The mean GCS on admission (GCS1) was 7.1 and was 8.1 after 7 days (GCS2).

Mean Neurofilament H levels

The mean Neurofilament level (pNF-H1) was 35.4 ± 21 pg/ml on admission and was 89.3 ± 54.7 after 7 days (pNF-H2). Hence the Neurofilament level was significantly higher after 7 days ($p < 0.005$).

Correlation between GCS and p-NFH levels

As shown by the scatter diagram there was a negative correlation between the level of Neurofilament H and the Glasgow
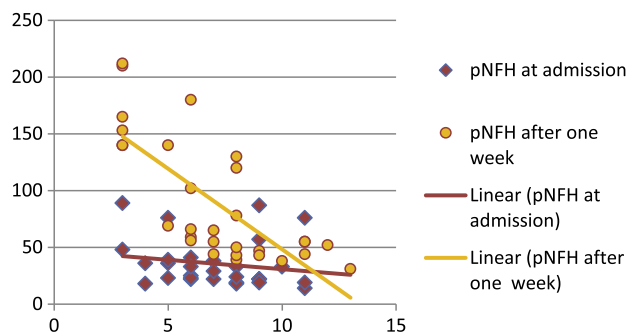

Figure 1 Correlation of Phosphorylated Neurofilament H with Glasgow Coma.
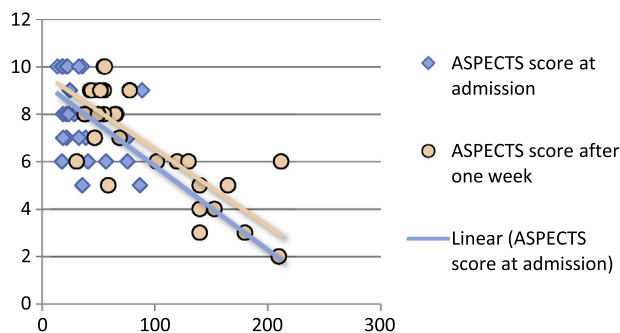

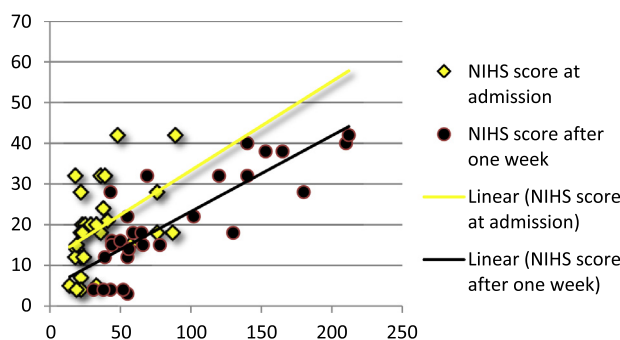**Figure 2** Correlation between Neurofilament H and ASPECTS score.

Figure 3 Correlation of Neurofilament H with NIHSS.

Coma Scale on admission and after 7 days in patients with ischemic CVS (−0.374, −0.51 with $P < 0.005$) (Fig. 1).

Mean ASPECTS score

CT assessment showed the mean ASPECTS score was 8.1 on admission and 6.7 after 7 days. The difference was statistically significant, lower after 7 days hence most patients with ischemic CVS showed an increase in infarct size after 7 days $p < 0.005$).

Correlation between ASPECTS score and mean Neurofilament levels

There was a negative correlation between the level of Neurofilament detected and the ASPECTS scores on admission and after 7 days (−0.64 and −0.89, respectively, $p < 0.005$). In other words, the higher the level of pNF-H the lower the ASPECTS score (Fig. 2).

Correlation between Neurofilament H levels and NIHSS

There was a positive correlation between the Neurofilament level and the NIHSS patients (0.55 on admission and 0.8 after 7 days $p < 0.005$) (Fig. 3).

Discussion

Our results show that pNF-H is quantifiable in the blood of patients with ischemic CVS and hence the appearance of this form of neurofilaments in the serum of humans might indicate

a neuronal injury. Blood collection is more practical and safer than CSF sampling, this suggests that analysis of blood for pNF-H could be a useful clinical tool to conveniently assess, diagnose and follow up patients with ischemic CVS. Other studies have shown that vertebrate neurons are sensitive to mechanical and metabolic damage and a major portion of the pathophysiology following ischemic CVS is due to axonal damage [1]. Therefore, a convenient blood assay of axonal loss could be of great utility.

Our results showed that there was a significant increase in the mean pNF-H levels in patients with ischemic CVS after 7 days (from 35 to 89 pg/ml). This indicated that the process of neuronal degeneration in ischemic CVS is an ongoing process that does not stop with the primary ischemic insult but continues on for days and weeks.

We showed a significant correlation between the GCS and hence the degree of consciousness and the mean pNF-H value i.e. those with higher pNF-H levels had lower GCS. This is of great importance as the GCS is a frequently utilized score that assess changes in consciousness, and the presence of a significant correlation with the GCS is a point that gives strength to our marker studied.

ASPECTS CT score showed a negative correlation with pNF-H, which again means that higher levels of the marker were found in those with a lower ASPECTS score and hence worse CT findings. The mean ASPECTS score was lower after 7 days indicating an increase in infarct size, and this also correlated with a higher pNF-H levels. This denotes that our marker might be able to predict an increase in the infarct size in patients with ischemic CVS.

Our data showed a positive correlation between pNF-H and NIHSS scale i.e. those with higher NIHSS and hence greater disability had higher mean pNF-H levels both on admission and after 7 days. Again this shows that our marker is able to detect levels of disability in patients with ischemic CVS.

Sellner et al. [8] studied serum biomarkers in patients presenting with acute stroke. They studied 18 patients (15 ischemic, 3 hemorrhagic) and analyzed 3 biomarkers: neurofilaments, S-100 proteins and Glial Fibrillary Acidic Protein (GFAP). Serial blood samples were collected, starting within the first 6 h and daily up to 6 days. Their data showed a significant increase in neurofilament levels within the first day and at all other times compared to the S-100B, and GFAP was not detected at any time in all patients. Although their study showed an increase in the level of neurofilament in ischemic stroke patients, they did not correlate their findings with the clinical parameters – as GCS or NIHSS – or with radiological findings.

Sing et al. [9] measured levels of pNF-H in stroke and correlated these levels with measures of stroke severity. Blood samples were collected from 54 ischemic stroke patients at day 1, week 1 (days 7–10) and weeks 3–6, and an ELISA was used to measure pNF-H levels in each patient at each time-point. Serum pNF-H levels were significantly elevated in stroke patients compared to healthy controls. Blood pNF-H levels that reflect the severity of ischemic stroke correlated with outcome and rise during the weeks after stroke. Similarly in our study, the pNF-H levels correlated with the severity of the stroke as reflected by GCS, ASCPECTS and NIHSS.

An important issue to mention is, if Neurofilaments are located in axons, axonal loss is a major problem in many kinds of human neurological damage and disease states, such as Traumatic brain injury, multiple sclerosis, and amyotrophic lateral sclerosis. This can be problematic in patients with chronic CNS disease and those with multiple co-morbidities. Although we excluded those patients with any chronic CNS diseases, in real clinical practice this will be hard.

Conclusion

Neurofilament H is detected in the plasma of patients with acute ischemic CVS. Levels of Neurofilament H correspond to the severity of injury as shown by the presence of significant correlations between Neurofilament levels and GCS, NIHSS and the radiological findings. Thus Neurofilament H seems to be a promising marker for the diagnosis and prognosis of patients with ischemic CVS and for the short term follow up of such patients. However, further studies will be needed to complement our results in larger groups of patients. A more specific study of the pattern of rise, fall and peak of the marker will be very helpful in this group of patients.

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